AN ABSTRACT OF THE THESIS

Ishan A. Patel for the degree of Honors Baccalaureate of Science in Bioengineering presented on May 15, 2013. Title: Rational design of ex vivo model of thrombosis.

Abstract approved:

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Dr. Owen McCarty

When vessel walls within the vasculature are damaged, exposed extracellular matrix (ECM) proteins trigger a series of events that lead to the formation of a hemostatic plug (Andrews *et al.* 2004, Furie *et al.* 2008, Watson 2009). Thrombus formation requires an orchestrated series of receptor-mediated events facilitating platelet recruitment, platelet activation, and initiation of coagulation. This process ultimately leads to thrombin generation and thrombus formation. In diseased vessels, this process leads to the propagation of intravascular thrombus formation and vessel occlusion, which is the underlying cause of cardiovascular disease.

We have developed a model of occlusive thrombus formation that relies on gravity to drive blood flow through a capillary tube under a constant pressure gradient. We have used this model to identify a role for laminin in the formation of occlusive thrombi in a FXII-dependent manner. We have also used this model to characterize the procoagulant phenotype of breast cancer epithelial cell lines in the presence of physiologically relevant, pressure-driven blood flow. The use of this model may be expanded to characterize the mechanisms of thrombosis and to determine the efficacy of pharmacological agents designed to prevent occlusive thrombus formation.

Key Words: thrombosis, coagulation, *ex vivo* model

DEDICATION

To my family, friends, mentors, and supporters
Without you, this would not be possible.

Rational design of *ex vivo* model of thrombosis

by

Ishan A. Patel

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Ishan A. Patel, Author

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CHAPTER TWO: COMMON METHODS AND MATERIALS

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ANOVA	analysis of variance
APC	activated protein C
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CTI	corn trypsin inhibitor
ECM	extracellular matrix
FG	fibrinogen
FII	factor II (prothrombin)
FIIa	activated factor II (thrombin)
FIX	factor IX
FIXa	activated factor IX
FV	factor V
FVa	activated factor V
FVII	factor VII
FVIIa	activated factor VII
FVIII	factor VIII
FVIIIa	activated factor VIII
FX	factor X
FXa	activated factor X
FXI	factor XI
FXIa	activated factor XI
FXII	factor XII
FXIIa	activated factor XII
FXIII	factor XIII
GP	glycoprotein
HK	high molecular weight kininogen
LDL	low-density lipoprotein
PAR	protease-activated receptor
PBS	phosphate buffered saline
PGI_2	prostaglandin I_2
PS	phosphatidylserine
RBC	red blood cell
RNA	ribonucleic acid
RT	room temperature
SEM	standard error of the mean
TF	tissue factor
tPA	tissue-type plasminogen activator
Tx	thromboxane
vWF	von Willebrand factor

CHAPTER ONE:

INTRODUCTION AND BACKGROUND

1.1 BLOOD: AN OVERVIEW

Blood is a highly specialized fluid tissue that performs many intricate functions in the human body. One of the key functions blood plays is the transport of nutrients, such as oxygen, to the cells of the body as well as transport of appropriate wastes for disposal. The composition of blood is also complex - to match the intricacy of function that it performs. Blood cells are fluidized in blood plasma. Plasma is composed of 92% water by volume and also contains dissolved ions, proteins, hormones, dissolved oxygen, and carbon dioxide . Blood is a mixture of cells and plasma - 55% of blood is plasma, while the remaining approximate 45% are red blood cells (RBC), leukocytes (or white blood cells, WBC), or platelets (termed thrombocytes in non-mammalian vertebrates). These have the functions of nutrient transport, immunity, and hemostasis, respectively. The most abundant of these cells are the RBC's - which includes an iron-containing protein called hemoglobin designed specifically for the transport of oxygen to the site of metabolic cells. The next most abundant cells are the platelets whose responsibilities include blood clotting (coagulation). Finally, the third most abundant type of cells are the leukocytes which scour the vascular piping of the body to identify and destroy old cells as well as attacking pathogens and other foreign substances. (Aarts *et al.* 1988, Woldhuis *et al.* 1992)

1.2 PLATELETS

Platelets average between 2-3 μm in diameter and are anucleate- by the far the smallest of blood cell constituents. In normal, healthy adults, platelets circulate in

numbers between 150,000 - 400,000 per µL of whole blood. The main function of platelets is to rapidly respond to sites of vascular injury and initiate coagulative processes. They have a critical role in thrombosis and haemostasis. (Najean *et al.* 1969, Italiano *et al.* 1999)

1.2.1 Platelet Production

Platelets are derived from precursor cells in the bone marrow called megakaryocytes. Large megakaryocytes localize near the sinusoidal walls of the lumen-bone marrow interface which facilitate the protrusion of large segments of megakaryocytic cytoplasm to circulation. Megakaryocytes develop systems of many organelles representing budding platelets. The shear force exerted on the invaginated plasma membrane of the megakaroycyte leads to fragmentation and the formation of immature platelets, or pro-platelets. Maturation of these pro-platelets leads to mature thrombocytes through the blood circulation. The dominant hormone regulating the production and development of megakaryoctyes is thrombopoietin. (George 2000)

1.2.2 Platelet Circulation

Platelets have an average lifespan of about 10 days in circulation. Older platelets have reduced functionality compared to younger ones. The spleen is a transient storage of platelets - reserving about one-third of platelets in circulation. Splenic contractions during an activated, coagulative state may increase platelet numbers in the blood. (Najean *et al.* 1969, George 2000)

1.2.3 Platelet Structure and Function

Platelets most importantly contain granules that are secreted during platelet activation, classified as either dense granules or α-granules. The secretions from the granules contribute to platelet activation and cohesion. Dense granules contain

adenosine diphosphate (ADP), serotonin, zinc, and calcium which potentiate activation and sustain blood coagulation. α-granules house cohesion proteins, chemokines, and components essential for coagulation as well as negative regulators to blood coagulation. (Andrews *et al.* 1997)

Platelets also express several integrins formed by non-covalent associations of two integral membrane subunits. One of the most important integrins found on the surface of platelets is the GPIIb/IIIa platelet-specific integrin (also known as $\alpha_{IIb}\beta_3$). GPIIb/IIIa specifically binds plasma proteins such as fibrinogen (FG) and von Willebrand Factor (vWF) and is also important for platelet-to-platelet interactions. (Vicente *et al.* 1990, Doggett *et al.* 2002, Liddington *et al.* 2002, Andrews *et al.* 2004)

Protease-activated receptors (PARs), belonging to the G-protein coupled receptor family, can also be found on the surface of platelets. Human platelets express two variants: PAR-1 and PAR-4. The main function of the PAR-family of receptor is presumed to perform signaling functions that lead to various structural changes, granule secretion, and platelet aggregation. (Zucker *et al.* 1985)

Finally, there are about 25,000 copies of normal glycoprotein (GP)IB/IX/V receptor complexes on the surface of platelets. This family of receptors has a variety of functions, including binding sites for coagulation-specific proteins involved in the injury response as well as serves as the transmitter of intercellular signals with interactions with other platelet receptors. (Marguerie *et al.* 1979, Ruggeri *et al.* 1982, Wagner *et al.* 1996)

Within platelets, a concert of signaling events is orchestrated to activate platelets at the site of a vascular injury. **Figure 1.1** shows some of the platelet surface receptors and the resulting intercellular signal transduction that drive activation.(Offermanns 2006)

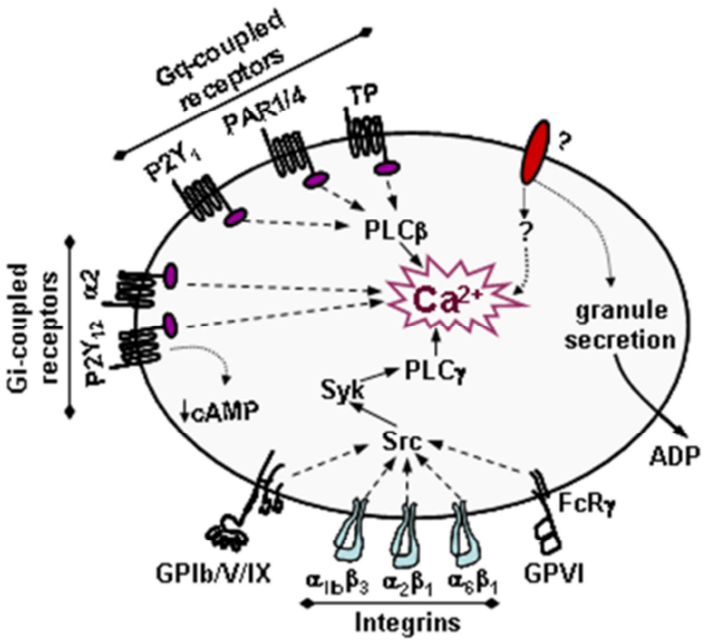

Figure 1.1 - *Platelet receptors and signaling pathways*. Intracellular signaling transduction is initiated through receptor-ligand interactions resulting in platelet activation and granule secretion.

1.2 THE VESSEL WALL

1.2.1 Endothelial Cells

A monolayer of endothelial cells encompasses the lumen of all blood vessels, known as the endothelium. This monolayer compartmentalizes the flowing blood and the important vasculature of the blood vessel wall. The function of the endothelium is crucial, performing a variety of different roles including maintenance of the vasculature, hemostasis, and resistance to leukocyte adhesion. Unbroken, healthy endothelial cells (**Figure 1.2**) secrete many substances into the free-flowing bloodstream that downregulate platelet activation and coagulation - such as nitric oxide (NO), prostaglandin (PGI_2), and tissue-type plasminogen activator (tPA). (de Groot *et al.* 1988, Hansen *et al.* 2011)

1.2.2 Extracellular Matrix

Underneath the endothelium there is a vast meshwork of proteins that behaves as both a scaffold and filter, called the extracellular matrix (ECM). The composition of the ECM can vary greatly and can also influence many auxiliary cellular processes including survival, proliferation, and permeability (Auger *et al.* 2005). This concept becomes especially important during the development of atherosclerotic plaques which can cause vessel damage and rupture leading to an adverse occlusive clotting event. The two most abundant ECM proteins include collage IV and laminin - both of which are known to bind platelet surface receptors leading to signaling events culminating in platelet activation (Inoue *et al.* 2006, White-Adams *et al.* 2010).

1.2.2-1 Collagen

Collagen IV is a self-polymerizing, heterotrimetic molecule that can assemble into dimers or tetramers via their non-collagenous 1 or 7S domains, respectively (Kratzer *et al.* 1985, Vandenberg *et al.* 1991). An intricate 3-dimensional fibrous network is created when these multimers are interwoven with each other (**Figure 1.2**). One of the most important features of the multimeric chains are the Gly-Xaa-Yaa triple repeats - a ~1400 residue domain that interacts with the Apple 3 domain of plasma von Willebrand Factor (Khoshnoodi *et al.* 2008, White-Adams *et al.* 2010). This interaction is a critical step in the response of platelets to injured blood vessels.

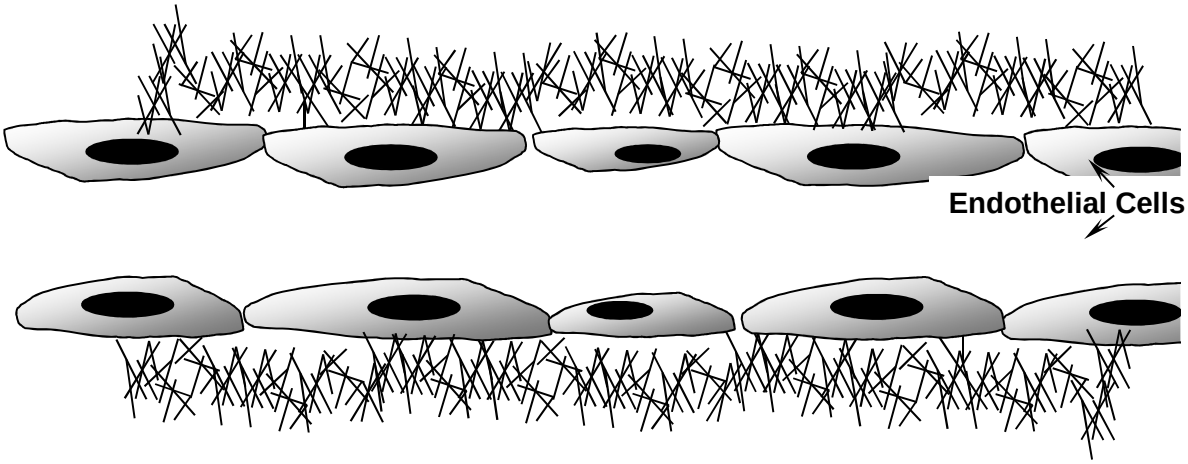

Figure 1.2 - *Prototypical blood vessel components and structure*. Blood flow is localized in the lumen of a vessel that is lined with an endothelial cell layer supported by a meshwork of extracellular matrix proteins including polymerized laminin and a network of collagen fibers. The cell membranes of the endothelial cells are bound to the matrix by integrin receptors.

1.2.2-2 Laminin

Another important component of the ECM includes the proteins from the laminin family. They can be found as heterotrimers composed of α, β, and γ chains. The structure of laminin is cross-shaped - the subunits merge in a coiled-coil formation. The short arms are critical for reversible, divalent cation-dependent laminin polymerization while the long arm can interact with integrins on cell surfaces. Networks of laminin in the ECM have been shown to associate themselves with Type IV collagen both directly and through linkage by fellow ECM protein, nidogen. (McKee *et al.* 2007)

1.3 HEMOSTASIS

1.3.1 Platelet Response

Sub-endothelial ECM contact subsequent to vessel wall injury exposes flowing blood to the platelet agonists such as collagen and laminin. Solubilized vWF quickly binds to exposed collagen and laminin. A subunit of the GPIB/IX/V platelet receptor then interacts with the bound vWF tethering the platelet to the vWF-collagen (or -laminin)

matrix (**Figure 1.3**). Interaction of various other platelet integrins results in the activation of several signaling cascades. Many of these signaling pathways releases intracellular Ca^{2+} and sustained levels of this ion accelerates and intensifies platelet activation (**Figure 1.1**). The cellular contents of the dense and α-granules are released during platelet activation, which activate nearby platelets to which are in turn recruited to the growing hemostatic plug (**Figure 1.3**). Finally, the platelets provide a negatively-charged surface by exposing phosphatidylserine (PS). This negative-charged backdrop supports the assembly of procoagulant complexes (**Figure 1.4**). (Marcus *et al.* 1965, Bevers *et al.* 1983, Kratzer *et al.* 1985, Hindriks *et al.* 1992)

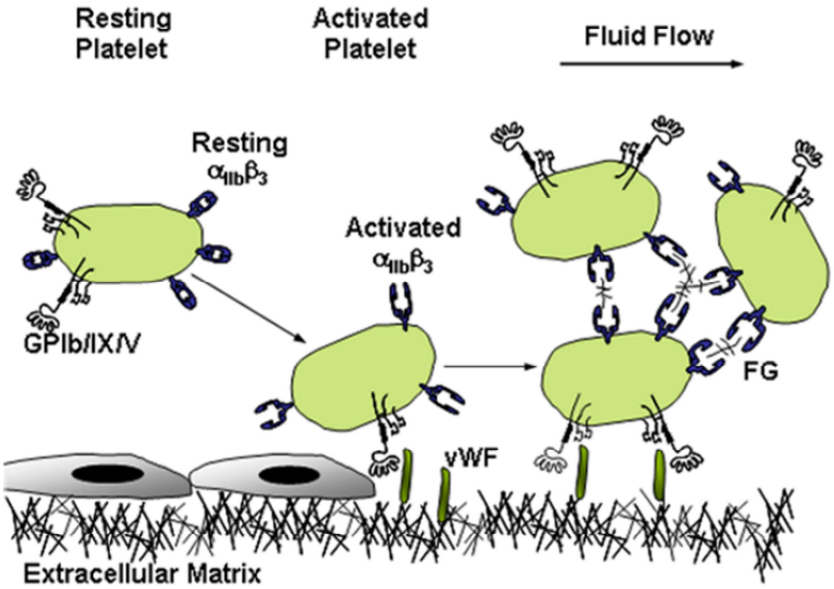

Figure 1.3 - *Platelet response to injury*. Exposure of flowing blood to the sub-endothelial matrix components initiates interaction of vWF and platelet GPIb eventually leading to the further recruitment of platelets. This aggregate becomes crosslinked by fibrinogen and vWF within the hemostatic plug.

1.3.2 Coagulation Response

1.3.2-1 Vitamin K-Dependent Coagulation Factors

Coagulation factors that are solubilized in the blood plasma freely float in the bloodstream in an inactivated zymogen form. They are sequentially activated to their serine protease form by limited proteolysis. Factors II (prothrombin), VII, IX, X, and the anticoagulant precursor Protein C, are all vitamin-K dependent proteins (Huntington 2009). Post-translational modifications to these proteins to their fully active form depend on vitamin-K which results in carboxylation of glutamic acid residues in the Gla domains of the coagulation factors (Sunnerhagen *et al.* 1995). The Gla domains of the coagulation factors associate with exposed phosphatidylserine surfaces in the presence of Ca^{2+} leading to the assembly of coagulation factor-activating complexes (**Figure 1.4**). This feature facilitates the rapid progression of coagulation at the injury site. (Nemerson 1968, Stenflo 1972, Nemerson 1975)

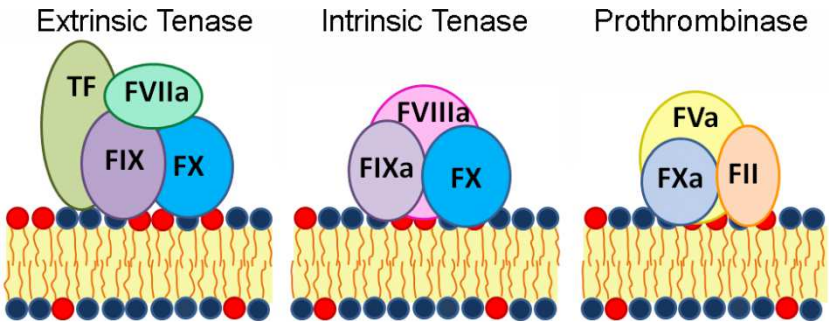

Figure 1.4 - *Coagulation factor activation complexes on the surface of platelets*. Calcium-mediated interactions between PS (red) and coagulation factors lead to factor binding on the platelet membrane surface. The TF-FVIIa complex (left) promotes the activation of FIX and FX in the extrinsic tenase complex. FIXa and cofactor FVIIIa assemble to activate FX (middle) in the intrinsic tenase complex. Prothrombin (Mohle *et al.*) is activated to thrombin (FIIa) by FXa and FVa in the prothrombinase complex (right).

1.3.2-2 Extrinsic Coagulation

Damage to the endothelium exposes tissue factor (TF) to flowing blood, initiating coagulation through the extrinsic pathway (**Figure 1.5**). Exposed TF serves as a cofactor for activated factor VII (FVIIa). As a complex, the TF-FVIIa activates factor IX (FIX) and factor X (FX) to their protease forms, FIXa and FXa, respectively. (Nemerson 1975, Osterud *et al.* 1977, Gailani *et al.* 1991, Mann 2003)

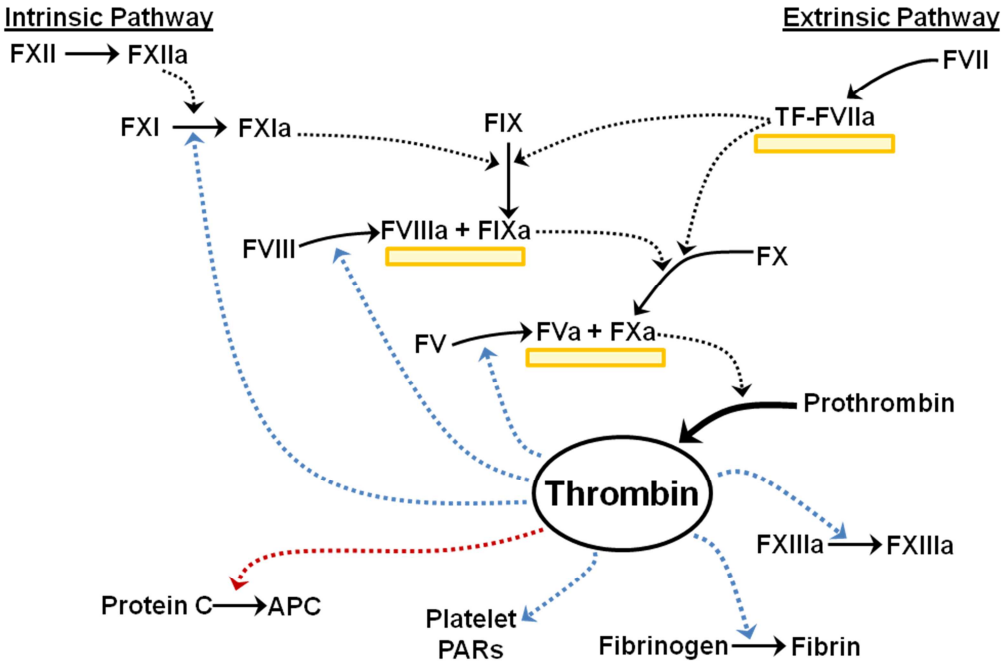

Figure 1.5 - *The coagulation cascade.* Fibrin formation is dependent upon the activation of thrombin either by the extrinsic (TF-mediated/injury) or the intrinsic pathways. Thrombin has numerous enzymatic functions including generation, amplification, and regulation of thrombin production at the site of vascular injury.

As illustrated in the **Figure 1.5**, FIXa associates with activated Factor VIII (FVIIIa) to activate FX in the tenase complex. Following, FXa joins its cofactor, activated factor V (FVa), in the prothrombinase complex to convert prothrombin (a zymogen, FII) to its activated serine protease form, thrombin (FIIa). Thrombin cleaves solubilized fibrinogen that is freely floating in flowing blood to its insoluble form, fibrin. Thrombin also activates factor XIII (FXIII) to its active form, FXIIIa, which crosslinks the fibrin to a mesh that

stabilizes the platelet plug at the site of injury. Thrombin also participates in a positive feedback systems leading to explosions in the concentration of thrombin by way of activation of upstream coagulation components such as factor XI and cofactors V and VIII. This generates thrombin at ever-increasing rates in order to ensure fibrin deposition to the site of vascular injury. (Bevers *et al.* 1982, Kakkar *et al.* 1995, Mann *et al.* 2003, Lu *et al.* 2004, Mackman 2009)

1.3.2-3 Intrinsic Coagulation: FXI Contributions

Activation of the intrinsic pathway is a second mechanism for the initiation of coagulation. Intrinsic, or contact activation, has been traditionally associated with the presence of foreign substances exposed to flowing blood, such as polyphosphates released by dense granules. These molecules initiate the cleavage of factor XII (FXII) into activated FXII (FXIIa). Tethered to a surface, FXIIa is a potent activator of factor XI (FXI). Studies suggest that a significant number of patients with FXI deficiencies experience bleeding in response to coagulation challenges, such as surgery. This would suggest that FXI may be crucial to sustained thrombin generation during vascular injury. (White-Adams *et al.* 2009)

FXI is a 160 kDa homodimer that circulates in the bloodstream complexed with high molecular weight kinnogen (HMWK). Activated FXI (FXIa) is generated by FXIIa, auto-activation, or by thrombin. Further, FXIa activation of FIX leads to the convergence of the two arms of the coagulation cascade pinpointed at FX activation. (Gruber *et al.* 2003, White-Adams *et al.* 2009, White-Adams *et al.* 2010)

1.3.2-4 Regulation of Coagulation

Thrombin, along with its ability to generate additional thrombin via feedback amplification, also participates in several events that downregulate thrombin generation in order to confine coagulation to the site of vascular injury. This is important because

without these mechanisms, the explosive nature of coagulation would generate large clots without regulation. In particular, thrombin activates the coagulation factor, protein C. Protein C is a vitamin K-dependent protein and is the zymogen precursor to activated protein C (APC). APC is responsible for inactivating cofactors VIIIa and Va, thereby negatively regulating thrombin generation. (Crawley *et al.* 2008)

1.4 COAGULATIVE DISORDERS: THROMBOSIS

Activation of platelets, formation of the platelet plug, coagulation, and the creation of a tight, fibrin mesh are essential for normal haemostasis. However, these events can be triggered in a disease state, such as rupture of an atherosclerotic plaque. Pathological stimuli or imbalances in coagulative processes may lead to thrombus formation. This event can be a precursor to many diseases such as heart attack, pulmonary embolism, or stroke. All of these diseases represent a significant portion of morbidity and mortality in advanced nations. (Lloyd-Jones *et al.* 2010)

Combating pathological thrombus formation requires anti-platelet or anticoagulation agents, such as aspirin, warfarin, or heparin. Anticoagulants or thrombolytic agents (such as tissue-type plasminogen activator, tPA) are used to break up thrombi in diseases such as stroke. (Chuang *et al.* 2001, Gray *et al.* 2008)

Antithrombotic agents are effective in preventing thrombus formation by either blocking the recruitment of platelets (i.e. preventing platelet aggregation) or removing thrombi from circulation by using fibrinolytic agents. However, usage of these powerful anti-thrombotics may carry severe hemorrhagic risk. For example, tPA treatment for stroke is currently the only approved treatment in the US but it has been shown to increase the instances of brain hemorrhage, has only 3-hour time window for efficacy, and can directly damage neurons. Similarly, aspirin exhibits the ability to inhibit activation and aggregation of platelet but can also predispose patients to a bleeding

diathesis. Oral platelet receptor $\alpha_{IIb}\beta_3$-blockers can cause an increase in cardiovascular disease mortality. Therefore, it is imperative to find treatments that can address the issue of thrombosis while still maintaining hemostasis. (Ansell *et al.* 2008, Gray *et al.* 2008, Lovelock *et al.* 2010, Wallentin *et al.* 2010, Akl *et al.* 2011, Donati *et al.* 2012)

Several novel factor inhibitors have been developed to remedy the problems of current anticoagulation therapies. Of particular interest is the role of factor XI (FXI) and ways to inactivate this factor. Studies have shown, as mentioned previously, that FXIa is required for sustained thrombin generation at the site of vascular injury. However, inactivation of this factor using novel monoclonal antibodies may retain the body's ability for hemostasis while preventing thrombosis. Two anti-FXI monoclonal antibodies have been created - anti-human FXI mAb, 1A6, which blocks the ability to activate FIX, and the anti-murine FXI mAb, 14E11, which blocks the ability of FXIIa to activate FXI (**Figure 1.6**). (Gruber *et al.* 2003, Tucker *et al.* 2009, White-Adams *et al.* 2009, Berny *et al.* 2010)

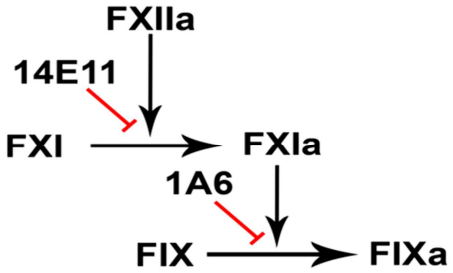

Figure 1.6 - *Novel FXI inhibitors.* Neutralizing anti-human FXI antibody (1A6) inhibits FXIa from activating FIX. Antibody 14E11 binds FXI and interferes with FXI activation by FXIIa *in vitro*.

Several studies have pointed to the correlation between thrombosis and cancer (Meyer *et al.* 1973, Baron *et al.* 1998, Sorensen *et al.* 1998, Sorensen *et al.* 2000, Rickles *et al.* 2001). Cancer metastasis, a process whereby cancer cells detach from the primary tumor, migrate in the vasculature, survive the circulating environment, exit into new tissue, and colonize in the invaded microenvironment, represents a major source of morbidity and mortality in cancer patients. Epithelial cancers, such as certain types of

breast cancer, are not well understood in terms of behavior of cancer cells in the fluid phase during transit in the vasculature (Berny-Lang *et al.* 2011). There is mounting evidence that supports the idea of coagulation activation in cancer. Therefore, the need arises to understand cancer in terms of coagulation in order to elucidate novel treatment therapies. (Berny-Lang *et al.* 2011)

1.5 MODELS OF THROMBOSIS

1.5.2 In-vitro models

Over the course of new drug evaluation in the field of thrombosis, rational drug design and high throughput screening are performed to select suitable candidates for possible *in vivo* studies. Potency assays are performed *in vitro* to further evaluate potential compounds for specified potency and selectivity. They are evaluated further in human plasma using *in vitro* clotting assays such as Activated Partial Thromboplastin Time (aPTT) or Prothrombin Time (PT), or in the case of antiplatelet agents, by platelet aggregation tests. (Gruber *et al.* 2003, Pickering *et al.* 2004)

A commonly used *in vitro* flow model is the parallel plate which mimics the *in vivo* environment of the vasculature. Shear rates, though vary widely in the vasculature, can be precisely controlled and manipulated to mimic those observed in both venous and arterial environments. The mechanism of action (**Figure 1.7**) to create the shearing environment relies on a syringe pump that either pulls blood through a thrombogenic capillary tube at a constant volumetric flow rate. Using a specialized microscope, the developing clot can be visualized with the aid of fluorescence or contrast microscopy. (Berny *et al.* 2008, White-Adams *et al.* 2009, Berny *et al.* 2010)

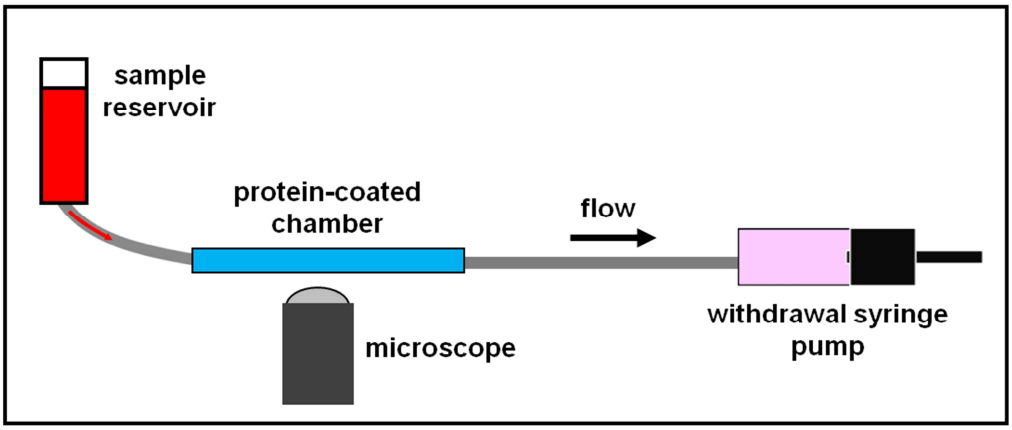

Figure 1.7 - *Schematic of the parallel-plate in vitro flow system*. Anticoagulated whole blood or reconstituted blood (washed platelets and RBCs) are added to a sample reservoir. The blood sample is pulled through a protein-coated chamber at desired shear conditions with a syringe pump. The flow chamber is mounted above a microscope for imaging of platelet interactions and aggregate formation during and after perfusion

1.5.1 In-vivo models

Currently, *in vivo* models use rodents to test efficacy of novel antithrombotic drugs. Typically, researchers can measure thrombus formation using thrombotic injury models that employ the use of either electrolytic or ferrous chloride-induced carotid artery injuries. For compounds that are meant for the venous side, several methods cause stasis in the inferior vena cava which results in thrombus formation. Formation of thrombus in the stasis region can be accelerated by addition of FXa or TF directly.

Other animals may be used to confirm and extend results from rodents. Rabbits are used in a variety of models, as the blood biochemistry of rabbits is closer to human blood biochemistry than rodent blood biochemistry. Additional mammalian models of thrombosis include canine, porcine, and non-human primate models. One such model is the baboon arteriovenous shunt model, wherein a thrombogenic segment is placed in an exteriorized chronic arteriovenous shunt. Blood flow is regulated through the segment to

venous levels of shear, and thrombus formation is quantified by measuring the deposition of radiolabeled platelets and fibrin. (Gruber *et al.* 2002, Gruber *et al.* 2003)

The vast majority of *in vivo* models require controlled incisions in anesthetized animals. These produce relatively constant experimental environments as the injuries can be experimentally controlled and standardized. Currently, genetic models of thrombosis include genetic knock-out mice. These mice have been created with the deletion of one specific, coagulation protein. The effects of the deletion can be studied and novel therapeutics could be used to mimic the deletion (Ni *et al.* 2000). Conversely, prothrombotic genetic models have been created through the deletion of fibrinolytic pathways. These mice tend to have vascular occlusive diathesis. Interestingly, these mice may be acceptable models for myocardial infarction caused by thrombosis. These models, though useful for genetic studies and for the basis for finding new treatments for diseases can be extremely variable and hard to standardize. Ideally, researchers want a model for intravascular thrombus formation that is spontaneously triggered in a diseased blood vessel. The inherent difficulty of working with spontaneous models has forced researchers to employ the use of external injury models or additions of thrombogenic surfaces to generate a thrombotic phenotype under well-defined experimental conditions *in vivo*. (Tabrizi *et al.* 1999, Welsh *et al.* 2012)

1.6 THESIS OVERVIEW

Vascular injury perpetuates a carefully planned and neatly orchestrated sequence of events that result in the deposition of platelets and activation of coagulation pathways to stop aberrant blood loss. Development of a model to recapitulate a venous blood flow system is essential to understanding the molecular pathways of coagulation. This information could be used to generate new and efficacious drugs that would address the need for anti-thrombotics that retain the overall hemostatic functions of the

body. In order to evaluate these potential mechanisms, a validated ex vivo model of thrombus formation is needed. This thesis centers on the development of an *ex vivo* model of thrombosis and reveals new insights to the molecular pathways that contribute to thrombosis.

Chapter Three describes the development and validation of the ex vivo model as well as two studies that were used in conjunction with the *ex vivo* flow model to provide new insight into the process of thrombus formation in a variety of settings. In Chapter Four, the findings from my thesis research are summarized and areas of interest for future work are considered and outlined.

CHAPTER TWO:

COMMON MATERIALS AND METHODS

2. 1 ETHICAL CONSIDERATIONS

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

2.2 COLLECTION OF HUMAN BLOOD

Human venous blood was drawn by venipuncture from healthy male or female volunteers, age 18 or order, who had been aspirin-free for at least two weeks prior or ibuprofen-free for at least 6 days prior. Blood was collected into a syringe containing 0.38% (final concentration, v/v) sodium citrate (a metal chelator) at room temperature. The first 1 mL of the venipuncture draw was generally discarded to prevent inclusion of tissue factor (TF).

2.3 FLOW ASSAY

The events of hemostasis occur in a dynamically changing environment of flowing blood. Wall shear stresses on the blood vessel wall range from 10-70 dynes/cm^2 in the arteries and about 1-6 dynes/cm^2 in veins. These correspond to shear rates of about 260-1800 s^{-1} and 30-160 s^{-1}, respectively. Connecting these effects of flow and stress to flow assays allows for characterization of hemostatic function *in vitro*. Rectangular glass capillary tubes were used to develop a ex vivo thrombosis model. This model used gravity to drive recalcified whole blood through the glass capillary tube

under a constant pressure gradient (**Figure 2.1**). The height of the blood required to drive this system was calculated using the Navier-Stokes equation within the capillary tube, which in the *z*-direction in Cartesian coordinates is:

$$\rho_b\left(\frac{\partial u_z}{\partial t}+u_x\frac{\partial u_z}{\partial x}+u_y\frac{\partial u_z}{\partial y}+u_z\frac{\partial u_z}{\partial z}\right)=-\frac{\partial P}{\partial z}+\mu_b\left(\frac{\partial^2 u_z}{\partial x^2}+\frac{\partial^2 u_z}{\partial y^2}+\frac{\partial^2 u_z}{\partial z^2}\right) \qquad [2.1]$$

where ρ_b is blood density, *t* is time, u is velocity, *P* is pressure (including effects due to gravitational forces), and μ_b is blood viscosity. Under the assumptions that the flow is laminar and does not change with time (steady-state); velocity is only in one direction (unidirectional) in the *z*-direction; the flow is fully developed and does not change as a function of the *z*-coordinate; the width of the capillary (*x*-direction) is much larger than the depth (*y*-direction) and thus the velocity is only a function of *y*; and that the blood behaves a Newtonian, incompressible, and isothermal fluid in this model, the Navier-Stokes Equation (2.1) simplifies to Equation 2.2:

$$\frac{\partial P}{\partial z}=\mu\frac{\partial^2 u_z}{\partial y^2} \qquad [2.2]$$

Upon double integration of Equation 2.2, we employed the following boundary conditions for pressure: at the entrance of the capillary ($z = 0$), the pressure term was defined as the atmospheric pressure (P_a) plus the pressure resulting from the height of blood ($z = h_b$) in the reservoir ($\rho_b g h_b$). At the exit of the capillary (z = -hc), the pressure was defined as the atmospheric pressure minus the resulting pressure from the height of the capillary ($\rho_b g h_c$) plus the pressure term from the depth that the capillary was submerged in the phosphate buffered saline (PBS; $\rho_{pbs} g h_{pbs}$). The boundary conditions for velocity were: (1) at the wall, velocity was zero due to the assumption of a no slip boundary condition; (Karnicki *et al.*) at the center of the tube (defined in the *y*-direction as 2*a*), the velocity was maximum and the gradient of velocity in the *y*-direction (the shear rate) was zero. Solving the Equation 2.2 employing the above boundary conditions and assumptions

resulted in Equation 2.3 where the shear rate at the wall (γ_w) can be calculated given a width of capillary (a) and height of the reservoir of blood (Plow *et al.*):

$$\gamma_w = a\left[\frac{\rho_b g\left(h_c + h_b\right) - \rho_{pbs} g h_{pbs}}{h_c \mu_b}\right] \qquad [2.3]$$

where γ_w is the wall shear rate, ρ_b is the density of blood, ρ_{pbs} is the density of PBS, h_c is the height of the capillary tube, h_b is the height of the blood in the reservoir, h_{pbs} is the depth that the capillary tube is submerged in PBS, g is the magnitude of acceleration due to gravity, μ_b is the viscosity of blood, and a is half the width of the capillary along the x-direction. An initial capillary wall shear rate of 350 s^{-1} can be achieved in a 0.2 x 2.0 x 50 mm glass capillary tube (Vitrotube™ Catalog # 5002, Vitrocom, Mountain Lakes, NJ) by maintaining a height of blood (Plow *et al.*) in the reservoir at 2.2 cm.

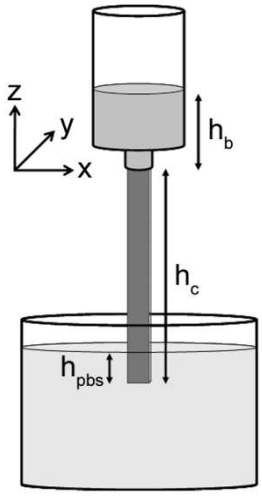

Figure 2.1 - *Pressure driven occlusive thrombus formation model*. Diagram of *ex vivo* model of thrombus formation. Recalcified blood flows through height of the capillary (h_c) into a bath of PBS by action of the pressure generated by the constant height of blood.

Glass capillary tubes were coated with variety of thrombogenic proteins at varying concentrations according to the experiment (Berny *et al.* 2010, White-Adams *et al.* 2010, Berny-Lang *et al.* 2011). The tubes were then washed with PBS and blocked with BSA (5 mg/mL) for 1 hour. Coated tubes were vertically mounted below a reservoir

(**Figure 2.1**). Citrated whole blood samples were recalcified with $CaCl_2$ and $MgCl_2$ (final concentrations at 7.5 and 3.75 mM, respectively) and serially added to the syringe reservoir in order to maintain constant height of blood. Recalcified blood was allowed to drain from the reservoir, through the coated capillary, into a PBS bath. Time to occlusion of the capillary was recorded as the time blood first exited the capillary into the bath until the time the blood ceased to flow from the capillary (**Figure 2.2**).

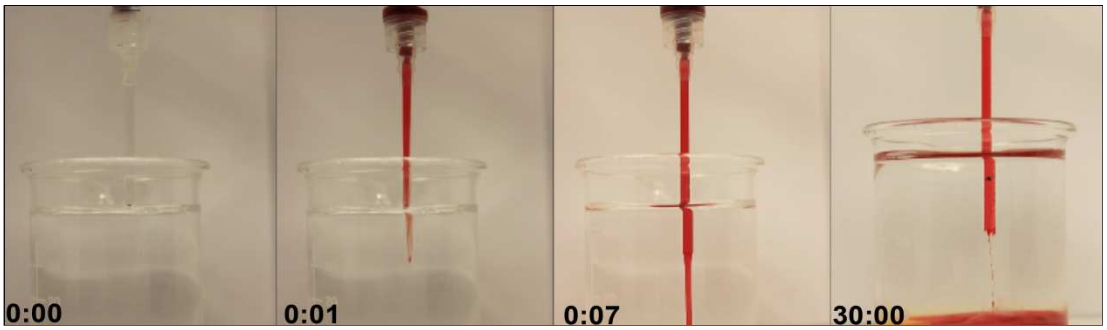

Figure 2.2 - *Time to occlusion for whole blood in the model of thrombosis.* Over a time period of 30 minutes shown above, whole blood flows through the coated capillary into the PBS bath. Time to occlusion is recorded from the time blood exits the capillary until the time blood ceases to flow from the capillary.

2.4 COMMON REAGENTS

Unless other specified, reagents used for blood collection, preparation of blood components, or for flow assays were from Sigma-Aldrich (St. Louis, MO). Recombinant tissue factor (TF, Innovin) was purchased from Dade Behrine (Marburg, Germany or Deerfield, IL) and fibrillar collagen was from Chrono-Log (Havertown, PA).

CHAPTER THREE:

RESULTS AND DISCUSSION

The following three sections chronicle the use of the *ex vivo* model of thrombosis in three different scenarios: developing the model, using the model in to characterize the role of laminin in thrombus formation, and using the model for to evaluate the prothrombotic phenotype of epithelial breast cancer cells.

3.1 MODEL DEVELOPMENT

3.1.1 Materials and Methods

Glass capillary tubes were coated with fibrillar collagen (100 µg/mL) for 1 hour at room temperature, washed with PBS, and blocked with denatured bovine serum albumin (BSA, 5 mg/mL) for 1 hour. These coated capillary tubes were vertically mounted as previously described in section 2.3. Whole blood samples (either treated with inhibitors or not) were recalcified with $CaCl_2$ and $MgCl_2$ (7.5 and 3.75 mM, respectively) and serially added to the syringe in order to maintain the height of blood at 2.2 cm. Time to occlusion was recorded as the time the blood first exited the capillary into the PBS bath until the blood ceased to flow from the capillary tube. Experiments were observed over a 40 minute time period. If occlusion did not occur after 40 minutes, experiments were terminated and a time point of 40 minutes was recorded.

3.1.2 Results and Discussion

3.1.2-1 Results

Recalcified blood occluded the collagen-coated capillary tub after a mean time of 17.4 ± 1.7 minutes (**Figure 3.1**). Occlusion time was significantly increased in BSA-only coated capillary tubes (30.6 ± 1.7 minutes, $P < 0.05$). No occlusion was seen in the

absence of the recalcification step. With the addition of platelet receptor $\alpha_{IIb}\beta_3$ antagonist, epitifibatide, was added (20 µg/mL; Cor Therapeutics Inc South San Francisco, CA), occlusion times in the collagen-coated capillary tube were extended to 30.0 ± 3.3 minutes (**Figure 3.1**). Thrombus formation was dependent on the action of thrombin. Direct thrombin inhibitor, hirudin, was added (20 µg/mL; CIBA-Geigy Pharmaceuticals, Horsham, UK) and failed to occlude the collagen-capillary tube over the 40 minutes of blood flow (**Figure 3.1**). Factor X inhibitor, rivaroxaban, failed to occlude the capillary tube when added to the blood (10 µmol/L; Bayer Healthcare, Leverkusen, Germany) (**Figure 3.1**). Pretreatment of blood with activated protein C (APC, 5 µg/mL; Haematologic Technologies Inc, Essex Junction, VT), a natural anticoagulant that inhibits activated factors V and VIII, prolonged occlusion time to 39.4 ± 0.6 minutes (**Figure 3.1**).

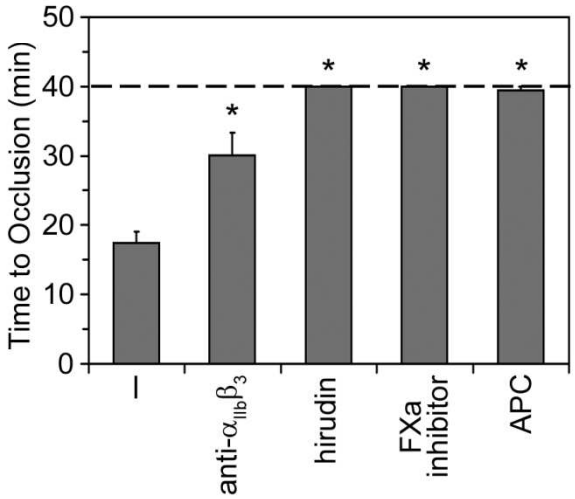

Figure 3.1 - *Pressure driven occlusive thrombus formation on a collagen matrix.* Sodium citrate anticoagulated whole blood was recalcified with $CaCl_2$ and $MgCl_2$ (final concentrations 7.5 and 3.75 mM, respectively), serially added to the reservoir (1 mL aliquots), and allowed to drain through collagen-coated (100 µg/mL) capillary tubes into PBS bath. Experiments were performed in the presence of PBS (-), the integrin $\alpha_{IIb}\beta_3$ antagonist eptifibatide (20 µg/mL, anti-$\alpha_{IIb}\beta_3$), the thrombin inhibitor hirudin (20 µg/mL), the activated factor X (FXa) inhibitor rivaroxaban (10 µM), or activated protein C (5 µg/mL, APC). Time to occlusion is reported as mean ± SEM from at least three experiments. Statistical significance of differences between means was determined by ANOVA. **P* < 0.05 with respect to PBS-treatment (−).

3.1.2-2 Discussion

The aim of this study was to successfully recreate the process of intravascular thrombosis in a gravity driven, *ex vivo* model. The times to occlusion (17 minutes) that were observed on collagen were within the range of times of occlusion reported for mouse vessels exposed to $FeCl_3$. Prolongation of time to occlusion with the addition of known antithrombotic reagents (e.g. hirudin, rivaroxaban, epitifibatide) is in agreement with established mechanisms of thrombus formation. This model can be used and expanded upon to evaluate occlusion times on a variety of surfaces to determine mechanisms that regulate coagulation under a physiologically relevant constant pressure gradient, to characterize efficacy of novel pharmaceutical agents designed to prevent occlusive thrombi, and/or to investigate the role of tortuosity in intravascular thrombosis.

This work was originally published by Springer; copyright 2010 in *Cellular and Molecular Bioengineering 2010; Volume, Number 2, Pages: 187-189*.

3.2 THE LAMININ STUDY

3.2.1 Rationale

Initiation of coagulation and thrombus formation following exposure of blood to the extracellular matrix protein, collagen, has been extensively investigated. The roles of the minor components of the extracellular matrix, such as laminin, remain ill-defined. The aim of our study was to define the role of laminin in initiating coagulation and supporting thrombus formation under physiologically-relevant levels of shear.

3.2.2 Materials and Methods

Glass capillary tubes were coated in the manner described above using laminin (50 µg/mL), collagen (100 µg/mL), or tissue factor. Surfaces were blocked with denatured BSA (5 mg/mL) and washed with PBS. Sodium citrate anticoagulant, donor whole blood was pretreated with inhibitors in selected experiments for at least 10 minutes at room temperature before being serially added (1 mL aliquots) after recalcification with $CaCl_2$ (7.5 mM) and $MgCl_2$ (3.75 mM) (**Figure 2.1**). In select experiments, blood was pretreated with corn trypsin inhibitor (CTI, an FXIIa inhibitor; 40 µg/mL), 14E11 (20 µg/mL), or 1A6 (20 µg/mL). Flow through the capillary was driven by the force of gravity at a height that would produce an initial shear rate of 300 s^{-1} according to Equation 2.3. The time to capillary occlusion was recorded with a maximum observation time of 40 minutes (**Figure 3.2**). Data is shown as means ± SEM. Statistical significance of differences between means was determined by ANOVA. Probability values of $P < 0.05$ were selected to be statistically significant.

3.2.3 Results and Discussion

3.2.3-1 Results

The data demonstrates that capillary occlusion occurred after 22.0 ± 3.2 minutes in laminin-coated tubes (**Figure 3.2**). Similar occlusion times were observed in collagen- or tissue factor-coated capillary tubes. BSA coated tubes had occlusion times after 30.6 ± 1.6 minutes. Occlusive thrombus formation on any surface was thrombin dependent, as the direct inactivation of thrombin through the action of hirudin (20 µg/mL) abrogated occlusive thrombus formation in the capillary under the time limit of 40 minute (**Figure 3.2**). Likewise, pretreatment of blood with CTI, 1A6, or 14E11 abrogated occlusion in laminin- and collagen-coated capillary tubes (**Figure 3.2**). In contrast, occlusion times in

the tissue factor coated capillaries were equivalent in the presence of vehicle, CTI, 14E11, or 1A6 (**Figure 3.2**).

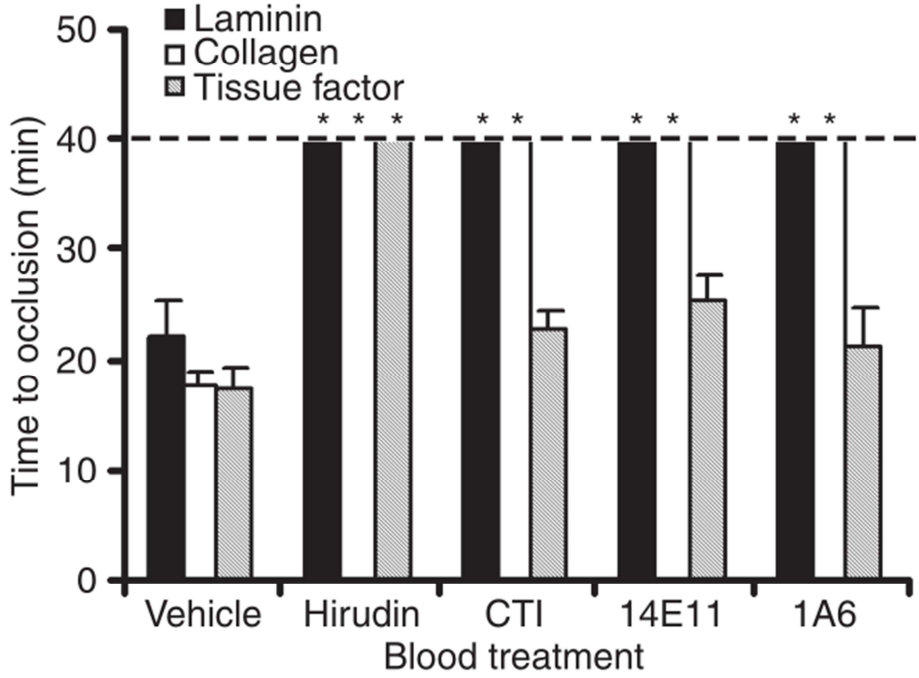

Figure 3.2 - *Laminin supports occlusive thrombus formation under a constant pressure gradient*. Sodium citrate anticoagulated whole blood (0.38%) was recalcified with $CaCl_2$ and $MgCl_2$ (final concentrations 7.5 and 3.75 mM, respectively), serially added to the reservoir (1 mL aliquots), and allowed to drain through collagen- (100 µg/mL), laminin- (50 µg/mL), or TF-coated capillary tubes into PBS bath. Experiments were performed in the presence of PBS (vehicle), the direct thrombin inhibitor hirudin (20 µg/mL), the activated factor XIIa (FXIIa) inhibitor CTI (40 µg/mL), or factor XI inhibitors 14E11 or 1A6 (20 µg/mL each). Time to occlusion is reported as mean ± SEM from at least three experiments. Statistical significance of differences between means was determined by ANOVA. *P < 0.05 compared to occlusion time in the presence of vehicle on each representative surface.

3.2.3-2 Discussion

This study demonstrates that immobilized laminin on glass capillary surfaces is able to initiate and support thrombus formation and occlusion in the presence of gravity driven flow of whole human blood (**Figure 3.2**). Laminin joins the registry of ECM proteins, including collagen, which contributes to FXII activation during exposure of

circulating blood in the event of injury. Further studies are required to define the mechanisms by which laminin triggers blood coagulation and platelet recruitment and activation. The current study suggests that laminin supports platelet recruitment, adhesion, and activation under flow conditions analogous to collagen.

This work was originally published in the *Journal of Thrombosis and Haemostasis* in 2010. *J Throm. Haemost.* 2010 March. **8**: 1295-301.

3.3 THE EPITHELIAL BREAST CANCER STUDY

3.3.1 Rationale

Studies have demonstrated a correlation between elevated levels of circulating TF and thrombosis in cancer patients. While TF is a key initiator of blood coagulation, cancer cells seem to have several procoagulant functions. In the present study, the aim was to characterize occlusive thrombus formation under physiologically relevant shear conditions with epithelial cells originating from breast tumors.

3.3.2 Materials and Methods

An anti-TF antibody (clone D3H44) was from Genentech (South San Francisco, CA). Rivaroxaban, a FXa inhibitor, was obtained from Bayer Healthcare (Leverkusen, Germany). Hirudin, a direct thrombin inhibitor was obtained from CIBA-Geigy Pharmaceuticals (Horsham, UK). Dulbecco's Modified Eagle Medium (DMEM) for MDA-MB-231 and MCF-10A cells, fetal bovine serum (FBS), horse serum, and recominant trypsin (TrypLE) were from Invitrogen (Carlsbad, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

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

cells were cultured and prepared with assistance from Joseph Aslan and Garth Tormoen of the McCarty laboratory.

As described in Section 2.3, glass capillary tubes were incubated with fibrillar collagen (100 µg/mL) for 1 hour at room temperature, washed with PBS, and blocked with denatured BSA (5 mg/mL) for 1 hour at room temperature, and washed with PBS. These were vertically mounted below a syringe reservoir and submerged in a PBS bath (**Figure 2.1**) Sodium citrated whole blood was incubated with vehicle, MDA-MB-231, or MCF-10A cells for 5 minutes. Aliquots (500 µL) of treated blood were recalcified with the addition of 7.5 mM $CaCl_2$ and 3.75 mM $MgCl_2$ and serially added to the reservoir to maintain a prescribed height that would yield an initial wall shear rate of 285 s^{-1} through the capillary according to Equation 2.3. The time to occlusion of the capillary was recorded over an observation window of 60 minutes (**Figure 3.3**).

Data is presented as mean ± SEM. One-way ANOVA with Tukey post-hoc test was employed to determine statistical significance between means. Significance differences for all tests required $P < 0.05$ (**Figure 3.3**).

3.3.3 Results and Discussion

3.3.3-1 Results

Time to capillary occlusion was significantly decreased in the presence of either MDA-MB-231 or MCF-10A cells (**Figure 3.3**). This reduction in time to occlusion was abrogated by the presence of anti-TF antibody or the thrombin inhibitor, hirudin. The time to occlusion for the vehicle was 36.0 ± 2.0 minutes (**Figure 3.3**). With the addition of MDA-MB-231 cells at a concentration of 1×10^3 cells/mL, a significant reduction of time to occlusion was observed (21.0 ± 0.5 minutes; **Figure 3.3**). At a concentration of 4×10^4 cells/mL of MDA-MB-231 cells, there was an even greater reduction of time to occlusion (10.0 ± 0.5 minutes; **Figure 3.3**). Addition of anti-TF antibody to the higher concentration

(4×10^4 cells/mL) of MDA-MB-231 cells increased the time to occlusion to the levels similar to vehicle without cells (37.0 ± 1.0 minutes; **Figure 3.3**). Finally, addition of hirudin to the higher concentration (4×10^4 cells/mL) of MDA-MB-231 cells completely prevented capillary occlusion during the 60 minute observation period (**Figure 3.3**). Similar results were obtained for the MCF-10A cell line although the reduction in clotting time between vehicle and 1×10^3 cells/mL of MCF-10A cells was not significant (**Figure 3.3**).

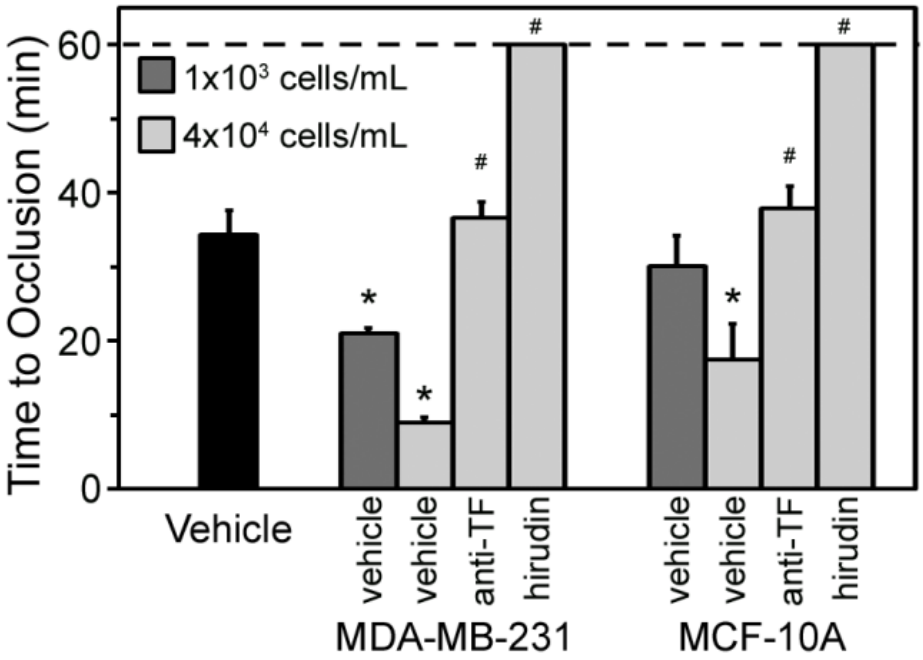

Figure 3.3 - *Cultured breast epithelial cells promote TF-dependent occlusive thrombus formation in flowing blood, ex vivo*. Human sodium citrate-anticoagulated whole blood was mixed with vehicle, MDA-MB-231 or MCF-10A cells (4×10^4 or 1×10^3/mL) for 5 minutes at room temperature. In selected experiments, blood was treated with a neutralizing antibody to TF (anti-TF, 20 µg/mL) or the thrombin inhibitor, hirudin (20 µg/mL), in the presence of MDA-MD-231 or MCF-10A cells. Treated blood was recalcified with $CaCl_2$ and $MgCl_2$ (final concentration 7.5 and 3.75 mM, respectively), added to a reservoir to a set height, and allowed to drain through collagen-coated capillaries into a PBS bath. The time to thrombotic occlusion (time until blood ceased to flow from the capillary) was recorded. Data are mean ± SEM from three or more experiments. *P<0.05 versus vehicle treatment in the absence of cells. #P<0.05 versus vehicle treatment of corresponding cell type at 4×10^4 cells/mL.

3.3.3-2 Discussion

This data suggests that epithelial cells from breast cancers that enter circulation under pathologic conditions may contribute to thrombus formation in the presence of physiologically relevant shear forces (**Figure 3.3**). This data also suggests that coagulation may be inhibited in the presence of either anti-TF or hirudin. It has been shown that the coagulation potential exists in these cell lines upon incubation in whole blood however, it has been yet to be determined if and how these circulating tumor cells utilize mechanisms of coagulation during transit in the vasculature. What can be concluded from these studies is that the *in vitro* cultured MDA-MB-231 and MCF-10A cells lines have procoagulant tendencies that promote occlusive thrombus formation during pressure-driven flow.

This work was originally published by IOP Science in February 2011. *Phys Biol*. 2011 Feb; 8(**1**):015014

CHAPTER FOUR:

FUTURE DIRECTIONS AND CONCLUSIONS

4.1 Expand *ex vivo* model to a quantitative model of thrombosis

Chapter 3 describes the development and characterization of an *ex vivo* model of occlusive thrombosis formation on collagen or laminin matrices. In agreement with standardized animal models of occlusive thrombus formation, the time to occlusion was increased with the addition of inhibitors of coagulation. This model, therefore, is an ideal platform for future work to evaluate mechanisms of thrombus formation and to test efficacy of novel antithrombotics.

Currently, the occlusive model relies on an operator to qualitatively assess the time to occlusion. Imaging modalities added to the model would greatly enhance the quantitative functionality of the assay. Laser dopler flow (LDF) probe coupled with optical coherence microscopy could provide additional data during the experimental observation time. Blood velocity exiting the capillary during an experiment could be continuously monitored. Standards for occlusion (e.g. 5% of max blood velocity through the capillary) would provide a means by which to standardize the *ex vivo* model.

4.2 Determine the role of donor characteristics thrombus formation

We have used ex vivo model of thrombus formation to determine mechanism of thrombosis in many physiological settings. The outcomes of these experiments have yielded noteworthy insights to the nature of thrombosis in an *in vitro* setting. The problem with this model, however, is that it does not take into account the inherent variability in donor blood. Whole blood differentials, from donor to donor, would unsurprisingly yield diversified blood cell counts: red blood cells, white blood cells, platelets, and other

proteins necessary for coagulation are present in fluctuating amounts. The reason behind these differences lies in the native variability within a donor. Even with the same donor, blood counts may significantly change due to the dynamic nature of the body. As a general rule of thumb, we have been assuming, for the purposes of our experiments, that donor blood is generally the same. This rule, however, can radically change the outcome of the experiment and therefore there is a need to quantify the extent by which these variables transform the end point of the experiment. The aim of this study would be to establish the extent of the variances between these donor qualities in the outcome of the diagnostic technique. It has become clearly evident, through several case-studies, that patients can have huge dissimilarities in their blood workup. This is natural – each person, clinically, may be healthy; however, their range of blood constituents may undergo significant changes from day to day and from person to person. Therefore, there is a need to assess the variance in the factors that contribute to occlusive thrombus formation. From this data, we can interpret the clinical efficacy of the model as well as establish a baseline for occlusive thrombus formation.

4.3 Characterize TF-dependent coagulation kinetics at zero, low, and high flow rates

Tissue factor is an essential transmembrane glycoprotein constitutively expressed by extravascular tissues that serves as the physiological initiator of coagulation. Tissue factor (TF) activity was thought to be regulated through its limited exposure to blood, as TF exposure only occurred in the event of blood vessel injury. This regulatory mechanism is unique from the other coagulation factors, which circulate in the blood as inactive zymogens or procofactors. Recently, the discovery that TF is persistent at low levels within the vasculature (circulating tissue factor, CTF) has challenged conventional views of TF regulation through limited exposure. CTF has been shown to contribute to experimental thrombus formation, suggesting a role for CTF in hemostasis

and demonstrating that CTF is capable of driving coagulation. However, CTF has also been linked to several disease states associated with thrombotic and hemorrhagic phenotypes. Therefore, the influence of CTF on coagulation is variable, and the conditions that drive CTF to induce coagulation are not well understood. It has not been postulated how CTF fits into the ethos of hemostasis, where coagulation factors are present but only activated in the event of blood vessel injury.

The occlusive model of thrombosis assay can be utilized to elucidate the coagulation kinetics for CTF in both closed and open systems, and under conditions of flow relevant to venous and arterial shear rates. Characterization of TF-dependent coagulation kinetics at zero, low and high flow rates would determine the role of shear rate and transport parameters on the coagulation kinetics of TF under physiologically relevant flow conditions. Using this data, it could be determined whether CTF coagulation in the circulation is transport-limited.

4.4 Conclusions

The work presented in this thesis has contributed to studies pertaining to the mechanisms further linking platelets, the ECM, or procoagulant cancer cells to coagulation. These mechanisms were elucidated using a validated model of occlusive thrombus formation under physiologically relevant flow conditions.

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APPENDIX A

BIOGRAPHICAL SKETCH

Ishan Ashwin Patel was born on 26 July 1990 and raised in Corvallis and Redmond, Oregon by his parents Ashwin and Bakula Patel and grandparents, Virji and Lilavati K. Madalia. He is the older brother to Janki A. Patel.

As a high school student, Ishan earned valedictorian in June of 2008 at the International School of the Cascades and Redmond High School. Ishan played varsity tennis his senior year and started attending Oregon State University immediately after graduating from high school during the summer of 2008.

Ishan studied honors bioengineering at Oregon State University while pursuing minors in Spanish and chemistry. As a freshman, Ishan actively participated in the OSU CBEE Club as a freshman representative, the Pre-Med society, and was able to win The Waldo-Cummings Outstanding Freshman Award as well as the opportunity to work in the lab of Dr. Owen McCarty at Oregon Health & Science University under the scholarship of Pete and Rosalie Johnson.

Upon completing his first research experience with Dr. McCarty, Ishan actively engaged in teaching the honors general chemistry sequence as a lab instructor. He also presented his research at the 2010 American Institute of Chemical Engineers (AIChE) Pacific Northwest Regional Conference at the University of Washington, winning 1st place among students from all over the PNW region and the chance to compete at the National AIChE Student Conference in Salt Lake City, UT.

Ishan was sparked by the implications of his research that he was doing during his very first experience with Dr. McCarty. This led to two additional summers of working at OHSU under Dr. Owen McCarty sponsored by two funded grants from the American

Heart Association. Ishan was able to take the culmination of his research experience to Japan to present a poster at the 2011 International Society of Thrombosis and Haemostasis conference in Kyoto. His final undergraduate research experience occurred during the summer of 2012 where he worked on a collaborative research project under the directions of Dr. McCarty and Dr. Ross Levine at Memorial Sloan-Kettering Cancer Center in New York City. Ishan's task was to advance some of the clinical applications of a new and novel cancer detection platform developed at OHSU.

After the culmination of his bioengineering studies, Ishan won Most Outstanding Bioengineering Student award from the Department of Chemical, Biological, and Environmental Engineering. Using his research topics in thrombosis and hemostasis, Ishan was also able to win the nation's highest math and engineering scholarship only given to the nation's top 300 students annually: The Barry M. Goldwater Scholarship. Finally, the University Honors College at Oregon State University recognized Ishan and his contributions to research and to the community through his active participation in teacher by awarding him the Joe Hendricks Scholarship for Academic Excellence.

Ishan will attend Oregon Health & Science University to pursue Doctor of Medicine in the Fall of 2013. He hopes to continue to do research at the academic level, actively participate in the Portland community, and travel.

A list of publications and presentations to date follows:

PUBLICATIONS

1. Berny MA**, Patel IA**, Simonson P, White-Adams TC, Gruber A, Rugonyi S, McCarty OJ, “Rational design of an ex vivo model of thrombosis” *Cell Mol Bioengineering*; 2010 Jun; 3:187-189.

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5. Larson MK, Tormoen GT, Weaver LJ, Luepke KJ, **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 Feb 1;304(3):C273-9.

PRESENTATIONS

Presentations at Meetings and Conferences

1. **Patel IA**, Berny-Lang MA, Gruber A, McCarty OJ "Rational Design of ex vivo Blood Thrombosis Model" *2010 AIChE Regional Conference Paper Competition at the University of Washington*, Seattle, Washington (Apr. 2010).
2. MA Berny, JE Aslan, J Pang, GW Tormoen, D Marrinucci, **IA Patel**, CP Loren, PE Bock, A Gruber, P Kuhn, OJ McCarty, "Characterization of the procoagulant activity of circulating tumor cells" *Gordon Research Conference: Hemostasis*, Waterville, NH (Jul, 2010).

3.A Itakura, JE Aslan, S Sinha, TC White-Adams, **IA Patel**, AA Vandenbark, GG Burrows, H Offner, OJ McCarty, “Characterization of human platelet binding of recombinant T cell receptor ligand” *XIth European Symposium on Platelet and Granulocyte Immunobiology*, Beaune, France (Oct, 2010).

4. **Patel IA**, Berny-Lang MA, Tormoen G, White-Adams TC, Tucker EI, Gruber A, McCarty OJ, “Rational Design of Ex Vivo Model of Thrombosis” *2010 Annual National Student Conference, American Institute of Chemical Engineers Paper Competition*, Salt Lake City, Utah (Nov 2010).

5. **Patel IA**, Berny-Lang MA, Tormoen G, White-Adams TC, Tucker EI, Gruber A, McCarty OJ, “Rational Design of Ex Vivo Model of Thrombosis” *Celebrating Undergraduate Excellence at Oregon State University*, Corvallis, Oregon (May 2011).

6. **Patel IA**, Berny-Lang MA, Tormoen G, White-Adams TC, Tucker EI, Baker SM, Gruber A, McCarty OJ, “Rational Design of Ex Vivo Model of Thrombosis” *XXIIIrd Congress of the International Society on Thrombosis and Haemostasis*, Kyoto, Japan (Jul 2011).

7. **Patel IA**, Berny-Lang MA, Tormoen G, White-Adams TC, Tucker EI, Baker SM, Gruber A, McCarty OJ, “Rational Design of Ex Vivo Model of Thrombosis” *2011 Annual National Student Conference, American Institute of Chemical Engineers*, Minneapolis, Minneasota (Oct 2011).

8. **Patel IA**, Berny-Lang MA, Tormoen G, White-Adams TC, Tucker EI, Baker SM, Gruber A, McCarty OJ, “Rational Design of Ex Vivo Model of Thrombosis” *School of Chemical, Biological, and Environmental Industrial Advisory Board at Oregon State University*, Corvallis, Oregon (Nov 2011).

APPENDIX B

RATIONAL DESIGN OF EX VIVO MODEL OF THROMBOSIS MANUSCRIPT

Cellular and Molecular Bioengineering (© 2010)
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Rational Design of an *Ex Vivo* Model of Thrombosis

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Abstract—The underlying pathogenesis of cardiovascular disease is the formation of occlusive thrombi. While many well-defined animal models recapitulate the process of intravascular thrombosis, there is a need for validated *ex vivo* models of occlusive thrombus formation. Using the force of gravity to provide a constant pressure gradient, we designed and validated an *ex vivo* model of thrombosis. Times to occlusion on a collagen matrix in our model were within the range of occlusion times observed in murine thrombosis models. Prolongation of time to occlusion in the presence of platelet $\alpha_{IIb}\beta_3$ antagonists or inhibitors to thrombin or activated factor X is in agreement with established mechanisms of thrombus formation. The use of this model may be expanded to characterize the mechanisms of thrombosis and to determine the efficacy of pharmacological agents designed to prevent occlusive thrombus formation.

Keywords—Thrombosis model, Coagulation, Platelets, Shear.

ABBREVIATIONS

ECM	Extracellular matrix
TF	Tissue factor
PBS	Phosphate buffered saline
BSA	Bovine serum albumin
APC	Activated protein C

Upon damage to vessel walls, exposed extracellular matrix (ECM) proteins and released tissue factor (TF) trigger a series of events that lead to the formation of a hemostatic plug in order to prevent blood from escaping a damaged blood vessel. A number of *in vitro* models have been established to simulate primary hemostasis under physiologically relevant shear and pressure conditions.[3,4,7,9] While the activation of platelets and the coagulation cascade are essential for normal hemostasis, intravascular formation of occlusive thrombi represents the underlying pathogenesis of cardiovascular diseases.[2,5,12,13] Although there currently exist a number of well-defined animal models of thrombosis, whereby the time to vessel occlusion is measured, there is a need for validated *ex vivo* models to determine the propensity of blood to form an occlusive thrombus. *In vitro* flow adhesion models have been widely utilized to study receptor-ligand mediated interactions under physiologically relevant levels of shear,[8] illuminating the initial steps of platelet recruitment and activation on ECM proteins under flow. However, these parallel-plate or capillary flow models are driven by a constant volumetric flow rate, in contrast to the pressure gradient that maintains blood circulation *in vivo*. The current study describes the design of an *ex vivo* model of intravascular occlusive thrombus formation in blood driven by a constant pressure gradient.

Our thrombosis model uses gravity to drive blood flow through a capillary tube under a constant pressure gradient (Fig. 1a). The height of the blood required to drive this system was calculated using the Navier–Stokes equation within the capillary tube, which for the z-direction in Cartesian coordinates, is:

$$\rho_b\left(\frac{\partial u_z}{\partial t}+u_x\frac{\partial u_z}{\partial x}+u_y\frac{\partial u_z}{\partial y}+u_z\frac{\partial u_z}{\partial z}\right) = -\frac{\partial P}{\partial z}+\mu_b\left(\frac{\partial^2 u_z}{\partial x^2}+\frac{\partial^2 u_z}{\partial y^2}+\frac{\partial^2 u_z}{\partial z^2}\right) \quad (1)$$

where ρ_b is blood density, t time, u velocity, P pressure (including effects due to gravitational forces), and μ_b blood viscosity. Under the assumptions that the flow is laminar and steady state (does not change with time); velocity is unidirectional, and only flows in z direction; the flow is fully developed and does not change as a function of z; the width (x-direction) of the capillary is much larger than the depth (y-direction) and thus velocity is only a function of y; blood behaves as a Newtonian, incompressible, and isothermal fluid in

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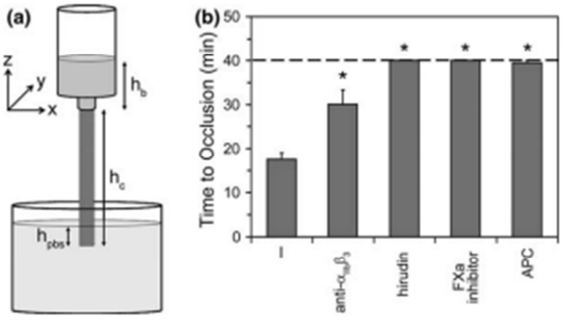

FIGURE 1. Pressure driven occlusive thrombus formation on a collagen matrix. (a) Diagram of *ex vivo* model of thrombus formation. (b) Sodium citrate anticoagulated whole blood was recalcified with $CaCl_2$ and $MgCl_2$ (7.5 and 3.75 mM final concentrations, respectively), added to the reservoir and allowed to drain through collagen-coated capillary tubes into a PBS bath. Experiments were performed in the presence of PBS (−), the integrin $\alpha_{IIb}\beta_3$ antagonist eptifibatide (anti-$\alpha_{IIb}\beta_3$), the thrombin inhibitor hirudin, the activated factor X (FXa) inhibitor rivaroxaban, or activated protein C (APC). Time to occlusion is reported as mean ± SEM from at least three experiments. Statistical significance of differences between means was determined by ANOVA. * $p < 0.05$ with respect to PBS-treatment (−).

this model, the Navier–Stokes equation simplifies to Eq. (2):

$$\frac{\partial P}{\partial z} = \mu \frac{\partial^2 u_z}{\partial y^2} \tag{2}$$

Upon integration of Eq. (2), we employed the following boundary conditions for pressure: at the entrance of the capillary ($z = 0$), the pressure term was defined as the atmospheric pressure (P_a) plus the pressure resulting from the height of the blood ($z = h_b$) in the reservoir ($\rho_b g h_b$). At the exit of the capillary ($z = -h_c$), the pressure term was defined as the atmospheric pressure (P_a) minus the pressure resulting from the height of the capillary ($\rho_b g h_c$) plus the pressure term from the depth that the capillary was submerged in phosphate buffered saline (PBS; $\rho_{pbs} g h_{pbs}$). In addition, boundary conditions for velocity were: at the wall, velocity was zero due to the assumption of a no slip boundary condition; at the center of the tube (width in the y-direction was defined as $2a$), the velocity was maximum and the gradient of velocity in the y-direction (the shear rate) was zero. Thus solving the equation resulted in the formula given in Eq. (3), whereby the shear rate at the wall (γ_{wall}) can be evaluated for a given width of the capillary tube (a) and height of the reservoir of blood (h_b).

$$\gamma_{wall} = a\left[\frac{\rho_b g(h_c + h_b) - \rho_{pbs} g h_{pbs}}{h_c \mu_b}\right] \tag{3}$$

where γ_{wall} is wall shear rate, ρ_b is the density of the blood, ρ_{pbs} is the density of the PBS, h_c is the height of the capillary tube, h_b is the height of the blood in the reservoir, h_{pbs} is the depth that the capillary is submerged in PBS, g is acceleration due to gravity, μ_b is viscosity of blood, a is 1/2 the width of the capillary along the short-axis. Thus, an initial capillary wall shear rate of 350 s^{-1} can be achieved in a 0.2 × 2.0 × 50 mm glass capillary tube (Vitrotube™ Catalog # 5002, VitroCom, Mountain Lakes, NJ) by maintaining the height of blood (h_b) in the reservoir at 2.2 cm.

Glass capillary tubes were coated with fibrillar collagen (100 μg/mL) for 1 h at room temperature and blocked with denatured BSA (5 mg/mL) for 1 h as previously described.[1] Collagen-coated capillaries were vertically mounted below a reservoir (Fig. 1a). Whole blood samples (1 mL) were recalcified with $CaCl_2$ and $MgCl_2$ (final concentrations 7.5 and 3.75 mM, respectively) and serially added to the reservoir in order to maintain a constant height of blood. Recalcified blood was allowed to drain from the reservoir, through the capillary, into a PBS bath. Time to occlusion of the capillary was recorded as the time blood first exited from the capillary into the PBS until the time blood ceased to flow from the capillary (occlusion). Experiments were observed over a 40 min period. If occlusion did not occur after 40 min, experiments were terminated and a time point of 40 min was recorded.

Our data show that recalcified blood occluded in the collagen-coated capillary tube after a mean time of 17.4 ± 1.7 min (Fig. 1b). Time to occlusion was significantly increased in BSA-coated capillary tubes (30.6 ± 1.7 min, $p < 0.05$). No occlusion was observed in the absence of recalcification (data not shown). When the platelet receptor $\alpha_{IIb}\beta_3$ was inhibited with

the $\alpha_{IIb}\beta_3$ antagonist, eptifibatide (20 μg/mL; Cor Therapeutics Inc, South San Francisco, CA), occlusion times in collagen-coated capillaries were extended to 30.0 ± 3.3 min (Fig. 1b). Occlusive thrombus formation was thrombin dependent, as recalcified blood pretreated with the direct thrombin inhibitor, hirudin (20 μg/mL; CIBA-Geigy Pharmaceuticals, Horsham, UK), failed to occlude in the capillary over 40 min of blood flow. In the presence of the activated factor X inhibitor, rivaroxaban (10 μM; Bayer Healthcare, Leverkusen, Germany), occlusion times were also greater than 40 min (Fig. 1b). Further, pretreatment of blood with activated protein C (APC, 5 μg/mL; Haematologic Technologies Inc, Essex Junction, VT), a natural anticoagulant which inhibits activated factors V and VIII,[14] prolonged occlusion times to 39.4 ± 0.6 min.

This aim of this study was to develop an *ex vivo* model of occlusive thrombus formation that successfully recapitulates the process of intravascular thrombosis. The times to occlusion (17 min) we observed on collagen were within the range of times to occlusion reported for mouse vessels exposed to $FeCl_3$.[11,12] Prolongation of time to occlusion in the presence of known antithrombotic reagents is in agreement with established mechanisms of thrombus formation.[6,10] The use of this model may be expanded to evaluate the occlusion times on a variety of surfaces to determine the mechanisms that regulate the coagulation cascade under a physiologically relevant constant pressure gradient, to characterize the efficacy of pharmacological agents designed to prevent occlusive thrombus formation, and to determine the role that vascular tortuosity plays in intravascular thrombosis.

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APPENDIX C

LAMININ PROMOTES COAGULATION AND THROMBUS FORMATION IN A FACTOR XII-DEPENDENT MANNER MANUSCRIPT

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ORIGINAL ARTICLE

Laminin promotes coagulation and thrombus formation in a factor XII-dependent manner

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Summary. *Background:* Laminin is the most abundant non-collagenous protein in the basement membrane. Recent studies have shown that laminin supports platelet adhesion, activation and aggregation under flow conditions, highlighting a possible role for laminin in hemostasis. *Objective:* To investigate the ability of laminin to promote coagulation and support thrombus formation under shear. *Results and methods:* Soluble laminin accelerated factor (F) XII activation in a purified system, and shortened the clotting time of recalcified plasma in a FXI- and FXII-dependent manner. Laminin promoted phosphatidylserine exposure on platelets and supported platelet adhesion and fibrin formation in recalcified blood under shear flow conditions. Fibrin formation in laminin-coated capillaries was abrogated by an antibody that interferes with FXI activation by activated FXII, or an antibody that blocks activated FXI activation of FIX. *Conclusion:* This study identifies a role for laminin in the initiation of coagulation and the formation of platelet-rich thrombi under shear conditions in a FXII-dependent manner.

Keywords: factor XI, factor XII, fibrin, laminin, platelets.

Introduction

When vessel walls are damaged, exposed extracellular matrix (ECM) proteins trigger a series of events that lead to the formation of a hemostatic plug [1,2]. Hemostasis requires an orchestrated series of receptor-mediated events to facilitate platelet recruitment to the ECM in the presence of shear, leading to platelet adhesion, rapid cellular activation, and accumulation of additional platelets (see review [3]). ECM-bound von Willebrand factor (vWF) plays a critical role in initial platelet deposition at high shear due to the rapid on-rate of binding between the platelet receptor glycoprotein Ibα (GPIbα) and vWF [4–6]. The platelet receptors GPVI and $\alpha_2\beta_1$ mediate platelet activation and subsequent adhesion to the exposed matrix proteins, while integrin $\alpha_{IIb}\beta_3$-mediated binding of additional platelets is required for the formation of a stable hemostatic plug [7].

Concomitant with platelet recruitment and activation are the first steps of blood coagulation (secondary hemostasis), namely the exposure of blood to tissue factor [8]. Initiation of the coagulation cascade leads to the sequential conversion of coagulation factors into their corresponding active serine protease forms, and culminates in the generation of thrombin. Thrombin not only activates platelets and cleaves fibrinogen, which leads to fibrin production and clot formation, but also mediates the activation of coagulation factors (F) XIII and XI and cofactors V and VIII, which in concert contribute to the rapid and efficient arrest of bleeding at sites of vascular injury.

Recent work has highlighted a dual role of exposed ECM proteins in contributing to thrombus formation. van der Meijden [9] and colleagues demonstrated that the ECM protein collagen triggers the activation of coagulation via FXII, and thus subsequent FXI activation, in addition to the well-defined role for collagen in mediating platelet recruitment and activation. It is currently unknown whether additional ECM proteins, such as laminin, can contribute to the initiation of coagulation and thrombus formation in a similar fashion.

The members of the laminin family of heterotrimers are major constituents of all basement membranes, the 50- to 100-nm thick layer of specialized ECM protein complexes found basolateral to all cell monolayers (epithelium and endothelium) in tissues. We recently demonstrated that laminin activates platelets through the collagen receptor GPVI, and it is established that laminin supports platelet adhesion through the integrin $\alpha_6\beta_1$ [10,11]. Further studies by Inoue and colleagues revealed that immobilized laminin supports platelet recruitment under shear flow in a GPIbα-vWF dependent

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manner [12]. Thus, the process of platelet recruitment, activation and adhesion on laminin under shear is mechanistically analogous to the interactions of platelets with collagen under shear, prompting the hypothesis that laminin and collagen may act together to promote hemostasis. In the present study, we aimed to determine if the similarity in function between laminin and collagen extends to the initiation of coagulation. Here, we present the first evidence that laminin can contribute to the activation of FXII and that surface-associated laminin alone can trigger the formation of fibrin- and platelet-rich clots under shear.

Experimental procedures

Reagents

Plasma-derived FXII was purchased from Haematologic Technologies, Inc. (Essex Junction, VT, USA). Plasmin and corn trypsin inhibitor (CTI) were from Enzyme Research Laboratories, Inc. (South Bend, IN, USA). Pefachrome FXIIa was obtained from Centerchem, Inc. (Norwalk, CT, USA). Tissue factor was purchased from Siemens Healthcare Diagnostics (Deerfield, IL, USA). Purified D-dimer was obtained from Cell Sciences (Canton, MA, USA). Equine Type I collagen was from Chrono-log Corporation (Havertown, PA, USA). Murine monoclonal anti-factor XI antibodies 1A6 and 14E11 were cloned, expressed and purified as described [13,14]. Laminin from human placenta was purchased from Sigma-Aldrich (St Louis, MO, USA). A silver stain of laminin subjected to SDS–PAGE under reducing conditions showed bands at molecular weights consistent with those listed in the Sigma product sheet (Supplemental Fig. S1). The predominant laminin isoform present in this preparation has been shown to be α5β1γ1, as characterized elsewhere [15]. All other reagents were from Sigma-Aldrich, Inc. (St Louis, MO, USA) or previously named sources [16–18].

Collection of human blood and preparation of plasmas and washed platelets

Human venous blood was drawn by venipuncture from healthy adult volunteers into sodium citrate. In order to reduce potential tissue factor contamination, the first 2 mL of each blood draw were taken into a separate syringe and discarded. Platelet-rich plasma (PRP) was prepared by centrifugation of citrated whole blood (in 0.38% w/v sodium citrate) at 200 × *g* for 20 min. Fresh PRP was prepared prior to each experiment. For washed platelet preparation, platelets were pelleted by centrifugation of PRP (in 0.38% sodium citrate and 1:10 v/v acid/citrate/dextrose) at 1000 × *g* in the presence of prostacyclin (0.1 μg mL^{-1}). The pellet was washed once by centrifugation and resuspended in modified Tyrode buffer (in mmol L^{-1}: 129 NaCl, 0.34 Na_2HPO_4, 2.9 KCl, 12 $NaHCO_3$, 20 HEPES, 5 glucose, 1 $MgCl_2$), pH 7.3 as previously described [18].

Platelet-poor plasma (PPP) was prepared by centrifugation of citrated whole blood (in 0.32% w/v sodium citrate) from three separate donors at 2150 × *g* for 10 min. Further centrifugation of the plasma fractions at 2150 × *g* for 10 min yielded PPP, which was then pooled and stored at −80 °C until use.

Clotting times and activation of FXII

Clotting times of human PRP or PPP were measured with a KC4 Coagulation Analyzer (Trinity Biotech, Bray, Co. Wicklow, Ireland). Samples were pretreated at room temperature (RT) for 3 min with 1A6, 14E11 or CTI, followed by incubation with vehicle, collagen or laminin in the absence or presence of tissue factor for 3 min at 37 °C. Coagulation was then initiated by the addition of Ca^{2+} (16.6 mmol L^{-1} final), and clotting time was recorded.

Cleavage of the activated factor XII (FXIIa) substrate, Pefachrome FXIIa, was monitored in the presence of vehicle or laminin. Reactions contained combinations of purified FXII (95 nmol L^{-1}), laminin (5–50 μg mL^{-1}), CTI (4 μmol L^{-1}), prekallikrein (30 nmol L^{-1}), and high-molecular-weight kininogen (30 nmol L^{-1}) in HEPES buffer (136 mmol L^{-1} NaCl, 5 mmol L^{-1} HEPES, 2.7 mmol L^{-1} KCl, 2 mmol L^{-1} $MgCl_2$, 0.42 mmol L^{-1} NaH_2PO_4, pH 7.45). After addition of 0.8 mmol L^{-1} Pefachrome FXIIa substrate, the rate of increase in absorption at 405 nm was determined for a period of 10 min.

Detection of phosphatidylserine exposure

Washed platelets were incubated over laminin- or collagen-coated coverslips for 30 min at 37 °C. Coverslips were gently washed with modified Tyrode buffer to remove non-adherent cells before incubation with Oregon Green® 488 conjugated Annexin V (Invitrogen Corp., Carlsbad, CA, USA) in a binding buffer (in mmol L^{-1}: 140 NaCl, 10 HEPES, 2 $CaCl_2$, pH 7.4). Coverslips were assembled onto a Quick change Chamber (Warner Instruments) and imaged using differential interference contrast (DIC) optics and fluorescence microscopy.

Recalcified blood flow

Glass capillary tubes (0.2 × 2 mm; VitroCom, Mountain Lakes, NJ, USA) were incubated for 1 h at RT with a solution of laminin (50 μg mL^{-1}), collagen (100 μg mL^{-1}), or denatured BSA (5 mg mL^{-1}). Surfaces were blocked with 5 mg mL^{-1} denatured BSA for 1 h prior to assembly into a flow system on the stage of a Zeiss Axiovert 200 M microscope (Carl Zeiss, Thornwood, NY, USA). Sodium citrate (0.38% w/v) anticoagulated whole blood was perfused through the chamber for 12 min at an initial wall shear rate of 250 s^{-1}. A solution of 37.5 mmol L^{-1} Ca^{2+} and 18.75 mmol L^{-1} Mg^{2+} was perfused at 1:5 vol/vol of the blood using a second, infusion syringe pump. The Ca^{2+}/Mg^{2+} solution entered the perfusion system via a Y-connection just prior to the capillary. Blood flow was followed by washing with modified Tyrode buffer and imaging the capillaries using DIC optics. Washed capillaries were treated for 5 min with 1x lysis buffer (10 mmol

L^{-1} Tris, 150 mmol L^{-1} NaCl, 1 mmol L^{-1} EGTA, 1 mmol L^{-1} EDTA, 1% NP-40 and 5 mmol L^{-1} PMSF), followed by treatment with 1 μmol L^{-1} plasmin for 40 min at RT, and the capillary eluate was collected for analysis.

Fibrin deposition during flow experiments was evaluated by separating the eluate samples by 6% SDS–PAGE under non-reducing conditions. Gels were evaluated by immunoblotting with anti-fibrinogen antiserum (MP Biomedicals, Irvine, CA, USA) to detect the 220 kDa fibrin degradation product, D-dimer. Sample D-dimer levels were compared with known amounts of purified D-dimer on the same blot (1–100 ng).

Capillary occlusion assay

Capillary tubes were prepared as described above, aligned vertically and connected to a reservoir. The capillary exit was immersed in PBS. Sodium citrate-(0.38% w/v) anticoagulated whole blood was sequentially supplemented with 7.5 mmol L^{-1} Ca^{2+} and 3.75 mmol L^{-1} Mg^{2+} in 1 mL aliquots to reduce the residence time of recalcified blood during each experiment. Flow through the capillary was driven by the force of gravity, and the height of the sample reservoir was regulated in order to produce an initial shear rate of 300 s^{-1} according to the following equation [19]:

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

where γ_{wall} is wall shear rate, ρ_b is the density of the blood, ρ_{pbs} is the density of the PBS, h_c is the height of the capillary tube, h_b is the height of the blood in the reservoir, h_{pbs} is the height that the capillary is submerged in PBS, g is acceleration due to gravity, μ is viscosity of blood, and $2a$ is the width of the capillary. The time to capillary occlusion was recorded, within a maximum observation period of 40 min.

Data analysis

Data are shown as means ± SEM. Statistical significance of differences between means was determined by ANOVA. If means were shown to be significantly different, multiple comparisons were performed by the Tukey test. Probability values of $P < 0.05$ were selected to be statistically significant.

Results

Laminin shortens clotting times in recalcified plasma in a FXII-dependent manner

The extracellular matrix protein laminin has recently been shown to support platelet recruitment and activation [10,12]. Our study was designed to determine the role of laminin in the initiation of coagulation. The presence of 40 μg mL^{-1} laminin shortened the clotting time of recalcified platelet-rich plasma (PRP) compared with vehicle (Fig. 1A). Pretreating PRP with the FXIIa inhibitor, CTI (4 μmol L^{-1}), 14E11 (20 μg mL^{-1}),

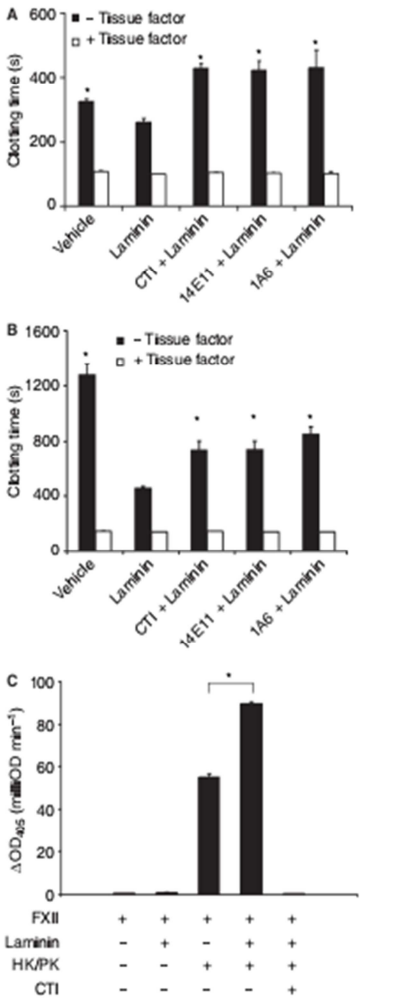

Fig. 1. Laminin enhances coagulation. Human sodium citrate-anticoagulated (A) platelet-rich plasma (PRP) or (B) platelet-poor plasma (PPP) was pretreated with 4 μmol L^{-1} CTI, 20 μg mL^{-1} 14E11 or 20 μg mL^{-1} 1A6. Laminin was then added to the plasma (40 μg mL^{-1} in PRP, 10 μg mL^{-1} in PPP) in the absence (black bars) or presence (white bars) of 1 pmol L^{-1} tissue factor. Following a 3-min incubation, coagulation was initiated by the addition of 16.6 mmol L^{-1} $CaCl_2$, and clotting times were recorded. Data reported are mean ± SEM of at least three experiments; *$P < 0.05$ compared with clotting times in the presence of laminin alone. (C) FXII activation was monitored in the presence of 50 μg mL^{-1} laminin. Experiments were performed in the presence of high molecular weight kininogen/prekallikrein (HK/PK, 30 nmol L^{-1} each) or CTI (4 μmol L^{-1}) as indicated. FXIIa was detected by measuring cleavage of its chromogenic substrate, Pefachrome FXIIa. Data are mean ± SEM of three experiments. (*$P < 0.05$).

which binds to the second apple (A2) domain of FXI and inhibits FXI activation by FXIIa [13], or the anti-FXI mAb 1A6 (20 μg mL^{-1}), which binds to the third apple (A3) domain of FXI and blocks FIX binding to FXIa [13], prior to incubation with laminin prolonged clotting times compared with the presence of laminin alone (Fig. 1A). The presence of CTI, 14E11 or 1A6 did not affect clotting times in the presence of laminin plus 1 pmol L^{-1} tissue factor (Fig. 1A). Similar trends were observed in platelet-poor plasma (PPP, Fig. 1B), suggesting that the presence of platelets plays a minimal role in the mechanism by which laminin shortens clotting times.

Collagen has been shown to activate FXII and promote coagulation in recalcified PRP [9]. In experiments performed here, PRP was incubated with either collagen (40 μg mL^{-1}) or vehicle, followed by the initiation of coagulation by adding $CaCl_2$. In accord with van der Meijden *et al.* [9], the presence of collagen significantly shortened clotting time compared with vehicle (Supplemental Fig. S2A). Clotting times in the presence of collagen, but not collagen plus 1 pmol L^{-1} tissue factor, were prolonged by the addition of 1A6, 14E11 or CTI (Supplemental Fig. S2A).

In order to address the possibility that reduced clotting times in the presence of laminin may be attributed to a residual amount of contaminating collagen, we determined the minimum concentration of collagen required to generate equivalent functional responses. Our results show that a collagen concentration of at least 0.625 μg mL^{-1} was required to reduce clotting times in PRP to a similar degree as observed for 40 μg mL^{-1} laminin (Supplemental Fig. S3). It is noteworthy that the addition of 0.3125 μg mL^{-1} collagen had no effect on PRP clotting times, yet concentrations of collagen as low as 0.3125 μg mL^{-1} retained the ability to induce platelet shape change in PRP aggregations (Supplemental Fig. S4A-B). In contrast, the addition of 40 μg mL^{-1} laminin to PRP failed to initiate either platelet shape change or aggregation (Supplemental Fig. S4B). A similar set of results was observed with washed human platelets (data not shown). These studies provide evidence against the suggestion that contamination by collagen plays a role in mediating the functional response we observed with laminin.

Activation of FXII in the presence of laminin

We used a plasma-free system to measure the effects of laminin on FXII activation. The incubation of laminin with FXII in the presence of high-molecular-weight kininogen (HK) and prekallikrein (PK) significantly accelerated the cleavage of a FXIIa chromogenic substrate compared with the presence of HK and PK alone (Fig. 1C). Chromogenic activity was negligible in the presence of CTI (Fig. 1C).

Laminin-bound platelets expose phosphatidylserine

A population of activated platelets expose procoagulant phosphatidylserine (PS) on their outer membrane surface, which serves as a site for assembly and activation of the tenase and prothrominase complexes [20,21]. To study the ability of laminin to support PS exposure on platelets, we layered purified platelets on laminin for 30 min before staining the adherent cells with fluorescently-labeled annexin V, which binds to PS with high affinity. Our data showed that approximately 23% of laminin-bound platelets bound annexin V (Fig. 2). The portion of collagen-bound platelets that supported annexin V binding was 49% (Supplemental Fig. S2B). A minimal degree of platelet adhesion and PS exposure was observed on BSA-coated surfaces (data not shown).

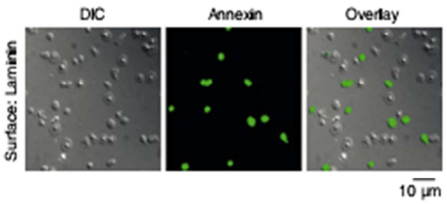

Fig. 2. Laminin-bound platelets expose phosphatidylserine (PS). Washed human platelets were exposed to a surface of immobilized laminin. Bound platelets were incubated with a solution of fluorescently-labeled annexin V to visualize PS exposure. Images were recorded using DIC and fluorescence microscopy. Corresponding brightfield and fluorescent images are shown alone and in the overlay.

Laminin supports thrombus formation in the presence of flow

In order to characterize the ability of laminin to support thrombus formation, recalcified blood was perfused over immobilized laminin at a shear rate of 250 s^{-1} for 12 min at 37 °C. Thrombus formation was visually recorded using DIC microscopy and the degree of fibrin formation was analyzed by Western blotting for the fibrin degradation product, D-dimer, following clot lysis with plasmin. Our results demonstrate that laminin supported platelet adhesion and aggregation and fibrin deposition in recalcified blood under shear (Fig. 3A). Fibrin strands were visible throughout the laminin-coated capillary (Fig. 3A), and D-dimer was readily detected in clot lysates (Fig. 3A). The degree of fibrin deposition, platelet aggregation and D-dimer detection was visibly higher for laminin as compared with BSA-coated surfaces (Fig. 3C). In order to rule out effects of collagen contamination in the laminin preparations, flow experiments were repeated on collagenase-treated laminin surfaces as previously described [10]. Collagenase-treated laminin surfaces supported equivalent levels of platelet- and fibrin-rich thrombus formation (data not shown). In contrast, fibrin formation was abrogated in the presence of the direct thrombin inhibitor, hirudin (20 μg mL^{-1}). The treatment of blood with the anti-FXI antibody 1A6, which blocks activation of FIX by FXIa, or 14E11, which interferes with FXI activation by FXIIa, or the FXIIa inhibitor, CTI, prior to perfusion over laminin surfaces drastically reduced both visible fibrin formation and D-dimer detection compared with vehicle (Fig. 3A).

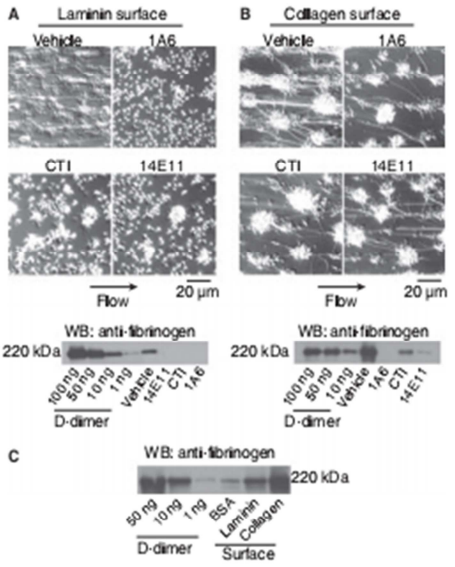

Fig. 3. Laminin supports thrombus formation under shear. Sodium citrate-anticoagulated whole human blood was co-perfused with a solution of Ca^{2+} / Mg^{2+} (7.5 mmol L^{-1}/3.75mmol L^{-1} final, respectively) over (A) laminin, (B) collagen or (C) BSA surfaces for 12 min at 250 s^{-1}, followed by perfusion of modified Tyrode buffer to remove non-adherent cells. In separate experiments, blood was pretreated with 20 μg mL^{-1} 1A6, 40 μg mL^{-1} CTI or 20 μg mL^{-1} 14E11 for 10 min at 37 °C prior to perfusion. Images were recorded using DIC microscopy prior to lysis and immunoblotting for the fibrin degradation product, D-dimer. Images and blots are representative of at least three experiments.

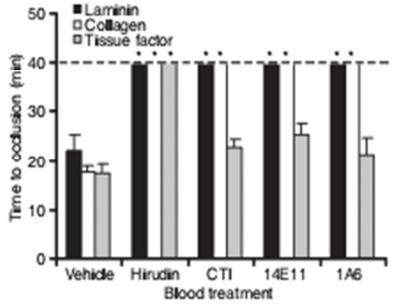

Fig. 4. Laminin supports occlusive thrombus formation under a constant pressure gradient. Whole human blood in 0.38% sodium citrate was recalcified and perfused through a laminin-, collagen- or tissue factor-coated glass capillary until occlusion. Blood flow was driven by a constant pressure difference. In selected experiments, blood was pretreated with either 40 μg mL^{-1} CTI, 20 μg mL^{-1} 14E11 or 20 μg mL^{-1} 1A6. Data are reported as mean ± SEM of at least three experiments. *P < 0.05 compared with occlusion time in the presence of vehicle on each respective surface.

We have previously demonstrated that inhibition of FXI with the anti-FXI mAb, 1A6, reduces fibrin deposition on collagen in both *in vitro* and *in vivo* models [14]. Our current study extends these findings to characterize the role of FXII in fibrin formation on collagen. Our results demonstrate that platelet aggregates and fibrin strands form on collagen following the perfusion of recalcified blood. The presence of 1A6 abrogated both visible fibrin formation and D-dimer detection, while the presence of either 14E11 or CTI reduced, but did not eliminate, fibrin formation and D-dimer detection (Fig. 3B).

Laminin supports occlusive thrombus formation under a constant pressure gradient

We next investigated whether laminin was capable of supporting occlusive thrombus formation. We have recently developed an *ex vivo* model of occlusive thrombosis formation that is driven by a constant pressure rather than constant volumetric flow rate [19]. In this *ex vivo* model, recalcified blood is driven by a constant pressure gradient at an initial shear rate through capillaries coated with laminin, collagen or tissue factor. The initial shear rate was adjusted to 300 s^{-1}, and flow through the capillary was monitored until occlusion. Our data demonstrate that an occlusive thrombus developed on laminin after 22.0 ± 3.2 min. Similar occlusion times were observed in collagen- or tissue factor-coated capillaries (Fig. 4), while occlusion in BSA-coated control capillaries occurred after 30.6 ± 1.7 min. Occlusive thrombus formation on laminin, collagen or tissue factor was dependent upon the activity of thrombin, as recalcified blood pretreated with the direct thrombin inhibitor, hirudin (20 μg mL^{-1}), failed to occlude in the capillary over the 40 min observation time.

FXI has been shown to play a critical role in thrombus growth and stability *in vivo*, and its inhibition prolongs occlusion times in animal models of thrombosis [14,22–25]. Our data demonstrate that pretreatment of blood with the anti-FXI mAb 1A6 or 14E11 or the FXIIa inhibitor CTI prolonged the time to occlusion on laminin past the 40 min observation period (Fig. 4). Similar results were observed in collagen-coated capillaries. In contrast, occlusion times in tissue factor-coated capillaries were equivalent in the presence of vehicle, CTI, 14E11 or 1A6 (Fig. 4).

Discussion

Despite the fact that laminin is a predominant member of the basement membrane, the contribution of laminin to hemostasis and thrombosis remains unclear. While expression of ECM proteins such as collagen types I, III, V and VI, as well as the adhesive proteins fibronectin and vitronectin, is enhanced in atherosclerotic plaques, laminin expression levels remain surprisingly constant [26–28]. Perhaps this is indicative of a physiological role for laminin in hemostasis. The current study

confirms and extends the recent studies by Inoue and colleagues demonstrating that laminin supports platelet recruitment, adhesion and activation under static and flow conditions in a manner analogous to collagen [10,12]. As collagen surfaces have been shown to support the initiation of coagulation in the presence of flow [9,14], our aim was to characterize (i) the ability of laminin to initiate coagulation by augmenting FXII activation and (ii) the ability of laminin surfaces to support platelet procoagulant activity in the presence of shear. Data presented here demonstrate that the presence of laminin accelerated clotting in recalcified plasma in a FXII-dependent manner. In a purified system, laminin accelerated the activation of FXII in the presence of HK and PK. Furthermore, immobilized laminin was able to stimulate platelet procoagulant activity and support thrombus formation and occlusion in the presence of flow.

The contribution of FXII to hemostasis is unclear as FXII deficiency in humans is not associated with a bleeding diathesis. Conversely, recent thrombosis studies in FXII-deficient mice have demonstrated an important role for FXII in thrombus formation and stability [24,29]. FXII has been shown to be activated by native materials that are not normally exposed to circulating blood, or that are exposed in the event of a vascular injury, such as collagen [9,29], RNA [30] and polyphosphates that are contained in platelet-dense granules [31]. The current study builds on previous work demonstrating that FXII binds to laminin surfaces [32], and adds laminin to the ever-increasing list of physiological surfaces that accelerate FXII activation. As FXIIa is a potent activator of FXI during contact activation, exposure of blood to one or more of these substances would result in the activation of FXI.

Epidemiological data indicate that men in the upper quintile of the normal distribution for plasma FXI levels have a ~2-fold increased risk of myocardial infarction compared with those in the lowest quintile, while severe deficiency of FXI is protective against ischemic stroke [33–36]. The mild bleeding risk in the absence of FXI, along with evidence that FXI and FXII each support thrombus formation, makes the intrinsic pathway of coagulation an attractive target for antithrombotic therapy. We have recently demonstrated that the administration of the FXI-blocking antibody, 1A6, which blocks FIX binding to FXIa, to non-human primates dramatically reduced both platelet and fibrin deposition on collagen [18]. Moreover, pharmacological inhibition of FXI with 1A6 prevented the occlusion of 2-mm diameter collagen-coated grafts without affecting template bleeding times [18]. The current study provides evidence that the blockade of FXI inhibits thrombus formation and occlusion on laminin-coated surfaces, identifying FXI as a common regulator of thrombus formation downstream of the basement membrane proteins collagen and laminin. Future studies using the anti-FXI mAb 14E11, which interferes with the ability of FXIIa to activate FXI, in these non-human primate models of thrombus formation and template bleeding should provide essential insights into the physiological role of FXII and FXI in hemostasis and thrombosis.

In conclusion, this study provides the first evidence that the ECM protein, laminin, is able to contribute to the initiation of coagulation and support thrombus formation in the presence of shear. Laminin joins fellow extracellular matrix protein, collagen, and a growing list of physiological materials that are exposed to circulating blood in the event of an injury, and have been shown to contribute to FXII activation [9,30,31]. Further studies are warranted to more fully describe the role that extracellular matrix proteins may play in other coagulation reactions.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Biochemical characterization of laminin.
Fig. S2. Collagen supports procoagulant processes.
Fig. S3. Effects of decreasing doses of collagen on PRP clotting times.
Fig. S4. Laminin does not induce platelet shape change or aggregation.

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APPENDIX D

PROMOTION OF EXPERIMENTAL THROMBUS FORMATION BY THE PROCOAGULANT ACTIVITY OF BREAST CANCER CELLS MANUSCRIPT

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

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

Abbreviations

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Reagents

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

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

2.2. Collection of human blood and preparation of plasma

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

2.3. Cell preparation for experiments

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

2.4. Clotting times and OG-488 thrombin binding

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

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

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

2.5. Flow cytometry

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

2.6. Capillary occlusion assay

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

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

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

2.7. Statistical analysis

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

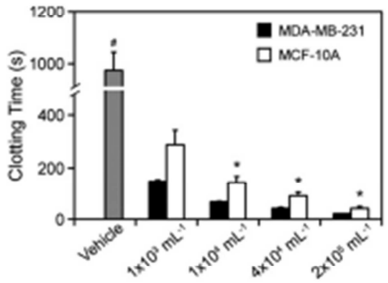

Figure 1. Breast epithelial cells promote coagulation. Human sodium citrate-anticoagulated plasma was incubated with vehicle or suspensions of cultured MDA-MB-231 or MCF-10A cells (1×10^3–2×10^5 mL^{-1}) for 3 min at 37 °C. Coagulation of plasma was initiated by recalcification using 16.7 mM $CaCl_2$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

3. Results

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

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

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

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

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

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

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

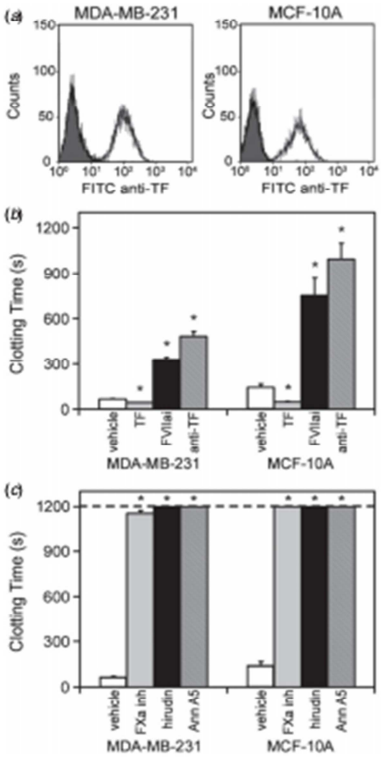

Figure 2. Characterization of the procoagulant activity of breast epithelial cells. (*a*) Cultured MDA-MB-231 or MCF-10A cells (1×10^6 mL^{-1}) were labeled with a FITC-conjugated anti-TF antibody (1 μg mL^{-1}) and analyzed by flow cytometry. Shaded curves represent background fluorescence of unlabeled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

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

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

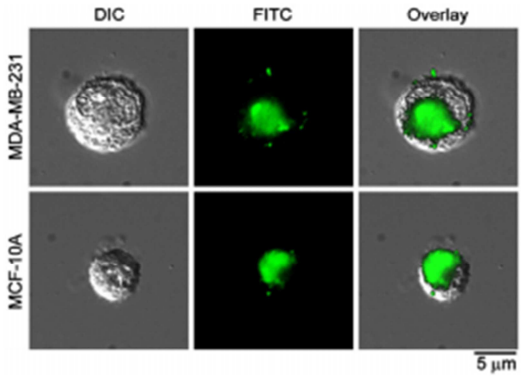

Figure 3. Cultured breast epithelial cells bind thrombin under procoagulant conditions. Human sodium citrate-anticoagulated plasma was incubated with suspended MDA-MB-231 or MCF-10A cells (2×10^5 mL^{-1}) for 3 min at 37 °C in the presence of OG-488 active-site-labeled thrombin (1 μM). Plasma was pretreated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

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

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

4. Discussion

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

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

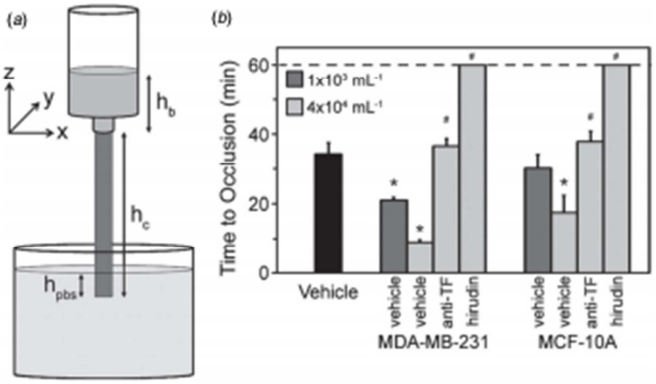

Figure 4. Cultured breast epithelial cells promote TF-dependent occlusive thrombus formation in flowing blood, *ex vivo*. Human sodium citrate-anticoagulated whole blood was mixed with vehicle, MDA-MB-231 or MCF-10A cells (4×10^4 or 1×10^3 mL^{-1}) for 5 min at room temperature. In selected experiments,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

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

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

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

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