Novel Mechanisms Mediating Platelet, Coagulation and Extracellular Matrix Interactions in the Presence of Shear

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To my grandma Jackie, my inspiration, for always being strong, independent and resolute. I would not be the person I am today without you.

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List of Abbreviations

A1	apple 1 domain
A2	apple 2 domain
A3	apple 3 domain
A4	apple 4 domain
ACD	acid / citrate / dextrose
ADP	adenosine diphosphate
ANOVA	analysis of variance
APC	activated protein C
ApoER2	apolipoprotein E receptor 2
APS	antiphospholipid antibody syndrome
β_2GPI	beta-2 glycoprotein I
BD1	low density liproprotein binding domain 1
BD2	low density liproprotein binding domain 2
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CTI	corn trypsin inhibitor
Dab-1	disabled-1
DIC	differential interference contrast
ECM	extracellular matrix
EGF	epidermal growth factor
EPCR	endothelial cell protein C receptor
FG	fibrinogen
FIIa	activated factor II
FITC	fluorescein isothiocyanate
FIX	factor IX
FIXa	activated factor IX
FV	factor V
FVa	activated factor V
FVIIa	activated factor VII

FVIIai	inactivated form of 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
GD	lacking the γ-carboxyglutamic acid domain
Gla	γ-carboxyglutamic acid domain
GP	glycoprotein
GPIb/IX/V	glycoprotein Ibα, glycoprotein Ibβ, glycoprotein IX, glycoprotein V receptor complex
HK	high molecular weight kininogen
HRP	horseradish peroxidase
LC	light chain
LDL	low-density lipoprotein
LDLR	low-density lipoprotein
LRP	low-density lipoprotein receptor-related protein
LRP8	low-density lipoprotein receptor-related protein 8
LRR	leucine-rich repeats
MAP	mitogen activated protein
NO	nitric oxide
PAR	protease-activated receptor
PBS	phosphate buffered saline
PGI_2	prostaglandin I_2
PI	phosphatidylinositol
PK	plasma prekallikrein
PKA2	plasma prekallikrein apple 2 domain

PL	phospholipase
PPACK	D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone
PS	phosphatidylserine
RAP	receptor-associated protein
RBC	red blood cell
RNA	ribonucleic acid
RT	room temperature
TF	tissue factor
tPA	tissue-type plasminogen activator
Tx	thromboxane
vWF	von Willebrand factor
WT	wild-type

Abstract

Novel Mechanisms Mediating Platelet, Coagulation and Extracellular Matrix Interactions in the Presence of Shear

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The prevention and treatment of cardiovascular disease complications, including occlusive blood clots responsible for heart attacks and strokes, are projected to cost Americans more than $500 billion in 2010. While therapeutic strategies have been employed to prevent occlusive events, the most effective antithrombotic agents are accompanied by unintended bleeding side effects. In order to develop new approaches that distinguish pathological events (thrombosis) from physiological clotting (hemostasis), there exists a need for a deeper understanding of the interactions between the major players involved in hemostasis and thrombosis: platelets, coagulation factors and extracellular matrix (ECM) components. In this thesis, we employed flow chamber studies, clotting assays and biochemical and microscopy techniques in order to identify the mechanisms governing the molecular interactions between platelets, coagulation factors and the ECM.

The ECM participates in the crucial event of platelet recruitment to the vascular wall after injury. Recent studies have described the ability of the ECM component collagen to accelerate coagulation factor XII (FXII) activation, thus contributing directly to blood coagulation. Due to the parallel mechanisms employed by collagen and fellow ECM protein, laminin, in platelet interactions, we hypothesized that laminin contributes to coagulation in an analogous manner. Results showed that laminin shortened plasma clotting times in a FXII-dependent manner and accelerated FXII activation in the presence of prekallikrein and high molecular weight kininogen.

Factor XI (FXI) has been shown to play a critical role in clot growth and stability *in vivo*. While FXI binding to platelet glycoprotein (GP)Ib/IX/V has been demonstrated, its ability to localize FXI to the growing clot or promote its activity has yet to be demonstrated. Results here identify a second platelet receptor for FXI: apolipoprotein E receptor 2 (ApoER2), which exists in a complex with GPIb/IX/V.

Protein C is the zymogen precursor to activated protein C (APC), which plays a regulatory role in blood coagulation by inactivating cofactors essential for thrombin production. These pro-coagulant cofactors assemble onto the platelet surface, and although APC has been shown to bind to the platelet surface, results described here extend these findings by identifying two receptors, GPIb/IX/V and ApoER2, that mediate platelet-protein C/APC interactions.

Chapter I: Background

1.1 Rationale

Cardiovascular disease (CVD) kills an American every 38 seconds.(Lloyd-Jones et al., 2009) The prevention and treatment of CVD complications, which include the occlusive blood clots responsible for heart attacks and strokes, are projected to cost Americans more than $500 billion in the year 2010 (Lloyd-Jones et al., 2009). While many pharmaceutical strategies have been employed to prevent occlusive events, the most effective agents are accompanied by bleeding side effects. There exists a need for deeper understanding of the interactions that occur during blood clotting in order to develop new approaches for targeting pathological events, while leaving physiological clotting intact. This chapter discusses the cellular and biochemical processes known to be involved in hemostasis and thrombosis and introduces the molecules involved in the newly identified interactions presented in the remainder of this thesis.

1.2 Platelets

Platelets are 2-3 μm in diameter, discoid, anucleate blood cells that are released into circulation from mature megakaryocytes in the bone marrow. Platelet count in healthy adults ranges between 150,000 and 400,000 per μL of blood. In intact blood vessels, platelets circulate in a quiescent state. Platelets respond rapidly to injuries at the vessel wall by adhering to the site of injury via surface receptors and subsequently undergoing activation (Fig. 1.1). Importantly, platelets contain granules which are secreted upon platelet activation, the

contents of which contribute to sustained platelet activation and cohesion. Dense granule contents include adenosine diphosphate (ADP), thromboxane (Tx)A_2, Zn^{2+} and Ca^{2+} that potentiate platelet activation and support blood coagulation. α-granules contain adhesive proteins, chemokines and coagulation components, as well as negative regulators of blood coagulation. The essential role of platelets and platelet receptors in injury response is illustrated by the profound bleeding exhibited by patients whose major glycoproteins contain defects or are insufficiently expressed, including the glycoprotein (GP)Ib/IX/V complex (Bernard-Soulier syndrome) and integrin $\alpha_{IIb}\beta_3$ (Glanzmann thrombasthenia), respectively (Nurden, 2005; Rao et al., 2004).

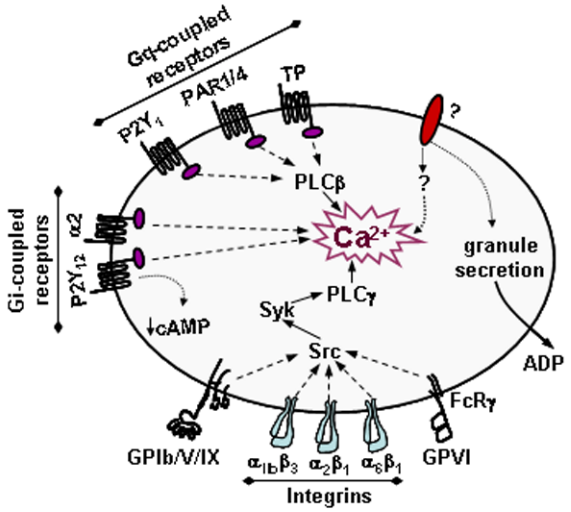

Figure 1.1 Platelet Receptors and Signaling Molecules. Platelets interact with ligands through surface receptors. Ligand binding initiates intracellular signal transduction via various pathways, resulting in platelet activation and granule secretion.

GPIIb/IIIa

Platelets express several integrins, which are formed by the noncovalent association of two integral membrane subunits (Fig. 1.1). Integrins are comprised of one α and one β subunit, which in quiescent cells assume a low binding affinity conformation. “Inside-out” signaling in response to a platelet-activating stimulus can promote a conformational change into an open, or high binding affinity conformation. The platelet-specific integrin, GPIIb/IIIa ($\alpha_{IIb}\beta_3$), can bind plasma

proteins such as fibrinogen and von Willebrand Factor and is important for platelet-platelet interactions. The cytosolic tail of the β_3 subunit contains one NPxY and one NxxY motif, which become phosphorylated upon activation, presumably by the Src family kinase, Fyn (Law et al., 1999; Phillips et al., 2001). These phosphotyrosine motifs support the docking of proteins with phosphotyrosine binding domains (Calderwood et al., 2003; Cowan et al., 2000; Garcia et al., 2004), which, upon ligand binding or receptor clustering, play an important role in propagating "outside-in" signaling, which proceeds through the tyrosine kinases, Src and Syk, as well as phosphatidylinositol (PI)3-kinase and phospholipase (PL) Cγ2, resulting in lamellipodia formation (Watson et al., 2005).

Protease-Activated Receptors

Protease-activated receptors (PARs), which belong to the G-protein coupled receptor superfamily, are also present on the platelet surface. Human platelets express two variants, PAR-1 and PAR-4. Although PARs can be activated by more than one protease, thrombin appears to be the most potent physiological activator of these receptors. PAR-1 is responsive to much lower levels of thrombin than PAR-4, while PAR-4 is thought to play a critical role in stabilizing platelet aggregates through sustained platelet activation (Covic et al., 2002; Hirano, 2007). Activation of PARs on murine platelets, which express PAR-3 and PAR-4, is thought to proceed through the cleavage of PAR-3, which does not readily transmit intracellular signals, but is presumed to maintain thrombin at the platelet surface and act as a cofactor for the thrombin-activation of PAR-4 (Coughlin, 2005). The GPIbα subunit of the GPIb/IX/V complex interacts with exosite II of thrombin via its sulfated tyrosine region (De Cristofaro et al., 2000; Li et al., 2001), and is presumed to perform a similar cofactor function for PAR-1 cleavage on human platelets (De Candia et al., 2001). In response to cleavage, the newly-formed N-terminus of PAR-1 acts as the agonist that tethers to the receptor and leads to outside-in signaling through members of the G12/13, Gi and Gi/z families (Coughlin, 2005). Signaling via this wide range of molecules proceeds through Rho kinases, PLCβ, protein kinase C and mitogen-activated

protein (MAP) kinase, resulting in a reduction in cAMP, facilitation of Ca^{2+} mobilization, shape change, granule secretion and platelet aggregation (Coughlin, 2005; Martorell et al., 2008).

Glycoprotein Ib/IX/V

The glycoprotein (GP)Ib/IX/V receptor complex is present at 25,000 copies per platelet (Coller et al., 1983) and is comprised of four separate gene products: GPIbα, GPIbβ, GPIX and GPV, in a 2:2:2:1 stoichiometry, respectively. GPIbα and β are covalently linked by a disulfide bond in their extracellular domains. GPIX is tightly, but not covalently associated with the GPIb complex, while GPV coprecipitates with GPIbα and may contribute to the efficient expression of the complex on platelets (Li et al., 1995; Meyer and Fox, 1995). While the bulk of ligand binding potential resides near the N-terminal of GPIbα, the absence of or a mutation in just one of the GPIX, GPIbα or GPIbβ subunits has been shown to disrupt proper complex formation and function, leading to abnormally large platelets, thrombocytopenia and moderately-to-severely prolonged bleeding times known as Bernard-Soulier Syndrome (Bernard and Soulier, 1948; Lanza, 2006).

GPIbα is the largest subunit of the GPIb/IX/V complex and contains binding sites for several proteins involved in response to injury such as von Willebrand Factor (vWF) (Michelson et al., 1986), α-thrombin (Takamatsu et al., 1986), high molecular weight kininogen (Joseph et al., 1999), and the coagulation factors VII (Weeterings et al., 2008), XI (Baglia et al., 2002; Baglia et al., 2004a; Baglia et al., 2004b), and XII (Bradford et al., 2000). GPIb/IX/V may transmit intracellular signals in response to ligand binding through its intracellular interactions with 14-3-3ζ and PI3-kinase (Mu et al., 2008).

Apolipoprotein E Receptor 2

GPIbα has been shown to form a complex with apolipoprotein E receptor 2 (ApoER2, LRP8) on the platelet surface (Pennings et al., 2007b). ApoER2 is a member of the low density lipoprotein (LDL) receptor superfamily. The ligand-binding region of ApoER2 has been described to be structurally similar to the very low density lipoprotein receptor (VLDLR). The N-terminal region contains seven 40-residue repeats, dubbed the LDL binding domains, followed by 3 epidermal growth factor (EGF) homology precursor repeats and an O-linked sugar domain (Kim et al., 1996). The transmembrane region is followed by a cytoplasmic tail containing 115 residues (Kim et al., 1996). Within this tail is the Tyr phosphorylation motif, ΨxNPxY (Ψ is hydrophobic), which is important for intracellular signal transmission (Kim et al., 1996; Riddell et al., 1999). ApoER2 is known to be highly expressed in the brain, and is essential for neuronal migration, brain development and synaptic transmission (May et al., 2005).

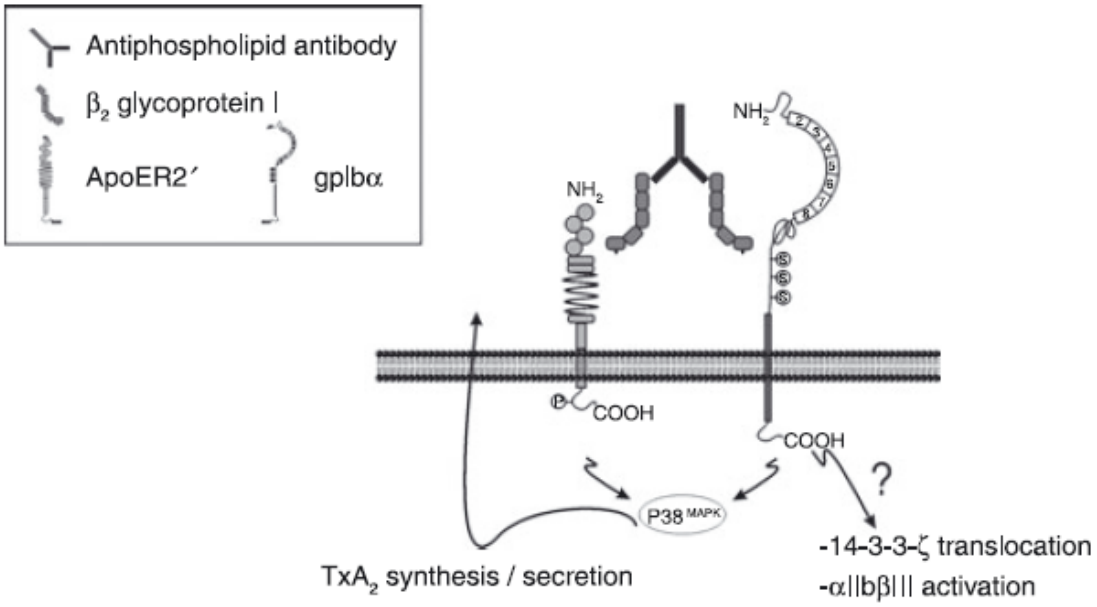

Figure 1.2 GPIbα and ApoER2 are present in a complex on the platelet surface. The GPIbα-ApoER2 axis binds antiphospholipid syndrome antibody-dimerized β_2GPI, leading to signal transduction via 14-3-3ζ and Disabled (Dab)-1. (Pennings, MT. *J Thromb Haemost*. 2007 Feb;5(2):369-77.)

The expression of ApoER2 on platelets was discovered in 1999 (Riddell et al., 1999). Platelets express three alternatively spliced forms of ApoER2, each containing the LDL binding domain 1 (Pennings et al., 2007a). ApoER2' (shApoER2Δ5) results from the omission of exon 5 during mRNA splicing and lacks the LDL binding domains 4, 5 and 6 (Pennings et al., 2007a). The ApoER2-GPIbα axis has been shown to bind antibody-associated, as well as chimeric β_2 glycoprotein (GP)I, which has been dimerized by fusing the apple 4 (dimerization) domain of factor XI to the N-terminal region of β_2GPI (Fig. 1.2). This interaction is particularly interesting because the presence of β_2GPI auto-antibodies is highly correlated with complications associated with antiphospholipid antibody syndrome (APS). Patients with APS suffer from recurrent arterial and venous thrombosis, as well as early and late pregnancy loss. Intriguingly, anti-β_2GPI antibodies can impair the anticoagulant activity of the protein C system (Galli et al., 1998). Furthermore, recent studies describe a role for ApoER2 in mediating pro-thrombotic effects in response to administration of APS antibodies or dimeric β_2GPI *in vivo* (Romay-Penabad et al., 2009).

1.3 The Vessel Wall

1.3.1 Endothelial Cells

The lumen of all blood vessels is lined with a monolayer of endothelial cells, known collectively as the endothelium. Importantly, the endothelium forms a selective barrier between flowing blood and other components of the vessel wall. The endothelium plays a significant role in the maintenance of vascular structure and tone, vascular homeostasis and permeability, resistance of leukocyte adhesion and invasion, and oxidative stress. Healthy, intact endothelial cells will release substances such as nitric oxide (NO), prostacyclin (PGI_2) and tissue type plasminogen activator (tPA) into the bloodstream, which downregulate both platelet activation and blood coagulation (Ruggeri, 2002).

1.3.2 Extracellular Matrix

Beneath the endothelium lies the extracellular matrix (ECM), a meshwork that behaves as both a scaffold and a filter (Fig. 1.3). ECM composition influences many cellular processes, including survival, proliferation and permeability. This is particularly important in the development of atherosclerotic plaques, which can cause vessel stenosis, and upon rupture can lead to occlusive clotting events. Two of the most abundant ECM proteins, collagen IV and laminin, are known to bind platelet surface receptors, leading to intracellular signaling events, and thus platelet activation (Inoue et al., 2006; Jarvis et al., 2002).

Laminins

Laminins are a major component of the ECM. They are present as heterotrimers, each subtype comprised of one each of the known α, β and γ chains (Miner et al., 1997). The characteristic structure of a laminin molecule is cross-shaped, in which the N-terminus of each subunit comprises one of the short arms. The subunits merge in a coiled-coil conformation that extends down the long arm, which contains a globular domain at the C-terminus (Engel, 1992). The short arms are critical for reversible, divalent cation-dependent laminin polymerization, while the long arm can interact with the α_3 or β_1 integrin subunit on the cellular surface (Fig. 1.3) (Colognato et al., 1999). Laminin networks have been shown to associate with collagen IV in the ECM both directly and via linkage through fellow ECM protein, nidogen (Fig. 1.3) (McKee et al., 2007).

Collagen

Another ECM protein known to self-polymerize is collagen IV. Heterotrimetic collagen molecules can assemble into dimers or tetramers via their noncollagenous domain 1 or 7S domains, respectively. These multimers are interwoven into an elaborate 3-dimensional fibrous network (Fig. 1.3) (Khoshnoodi et al., 2008). A defining feature of the chains comprising the heterotrimetic collagen IV molecule is a ~1400 residue domain containing Gly-

Xaa-Yaa triple repeats (Khoshnoodi et al., 2008). Collagen interacts with the A3 domain of plasma von Willebrand Factor, which comprises a critical step in vascular injury response in the presence of shear.

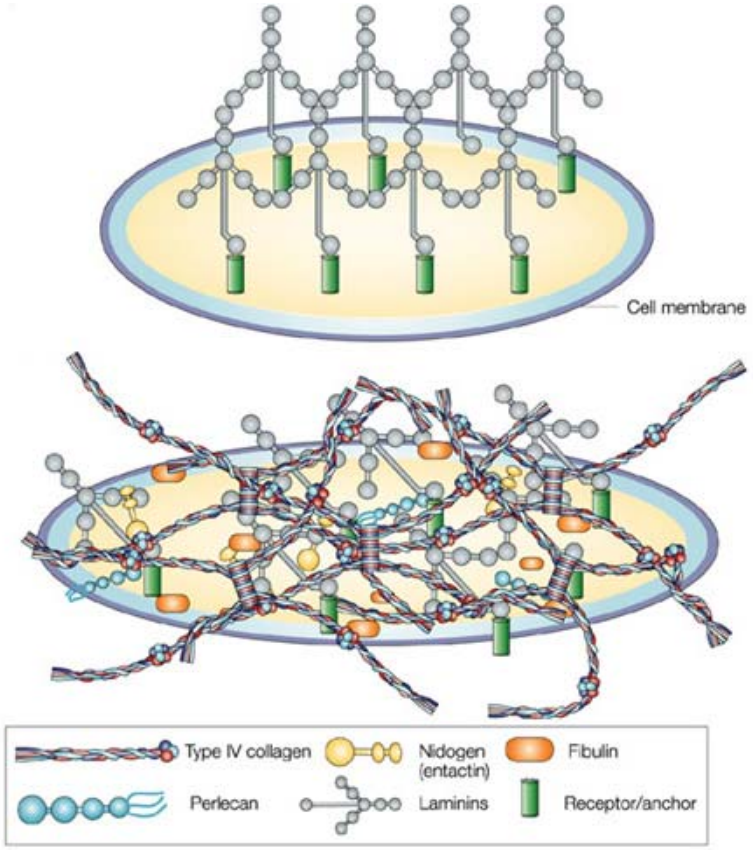

Figure 1.3 Extracellular Matrix Components and Structure. Beneath the endothelial cell layer is a supportive meshwork comprised of polymerized laminin **(top)**, which is bound to the cell membrane by integrins, and a network of collagen fibers **(bottom)**. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Kalluri, R. *Nat Rev Cancer*. 2003 Jun;3(6):422-33.), copyright 2003.

1.4 Hemostasis

1.4.1 Platelet Response

Damage to the vessel wall leads to the exposure of flowing blood to the subendothelial ECM, which contains platelet agonists such as collagen, laminin and fibronectin. In response to such an injury, plasma von Willebrand Factor (vWF) rapidly binds to exposed collagen and laminin (Cruz et al., 1995a; Cruz et

al., 1995b; Inoue et al., 2008; Ruggeri, 2002). The GPIbα subunit of the platelet receptor complex, GPIb/IX/V, interacts with matrix-bound vWF, and as a result of the rapid on-rate of this interaction, platelets tether to the injury surface (Fig. 1.4) (Miura et al., 2000). The rapid off-rate of GPIbα-vWF interactions results in platelet rolling at the site of injury, allowing adhesive interactions of receptors with slower binding kinetics to mediate firm platelet adhesion (Fig. 1.4) (Kumar et al., 2003).

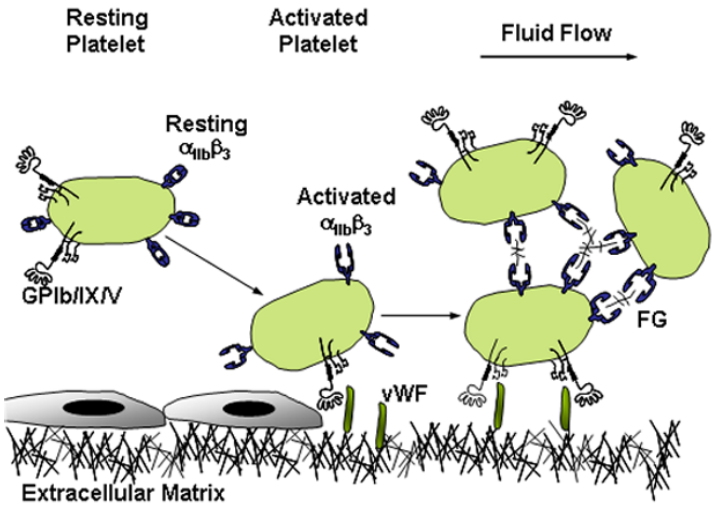

Figure 1.4 Platelet Response to Injury. Denudation of the endothelium can expose extracellular matrix components to the flowing blood. Platelet GPIbα interacts with matrix-bound vWF, leading to platelet rolling and eventual arrest by activated integrins. The resulting platelet activation leads to further recruitment of platelets, which become crosslinked by fibrinogen (FG) and vWF to platelets within the hemostatic plug.

Further interactions between platelet receptors and the subendothelial matrix [i.e. GPVI- and integrin $\alpha_2\beta_1$-collagen (Kunicki et al., 1988; Moroi et al., 1989; Santoro, 1986), integrin $\alpha_5\beta_1$-fibronectin (Wayner et al., 1988), integrin $\alpha_6\beta_1$-laminin (Hindriks et al., 1992; Sonnenberg et al., 1988), integrin $\alpha_{IIb}\beta_3$-fibronectin/vWF (Gardner and Hynes, 1985; Ruggeri et al., 1983)] lead to outside-in signaling. This signaling results in an increase in intracellular Ca^{2+} concentration (Fig. 1.1) (Heemskerk et al., 2002). Sustained, elevated cytosolic Ca^{2+} potentiates platelet activation, subsequent release of α- and dense

granules, the contents of which recruit nearby platelets into the growing hemostatic plug (Figs. 1.1 and 1.4), and exposure of negatively-charged phosphatidylserine (PS) on the platelet surface (Bevers et al., 1985; Heemskerk et al., 2002; Packham, 1994; Varga-Szabo et al., 2008). PS on the platelet surface supports the assembly of procoagulant complexes (Fig. 1.5B), thus serving as a platform for the promotion of coagulation (Zwaal et al., 1998).

1.4.2 Coagulation Response

Vitamin K-Dependent Coagulation Factors

Coagulation is propagated by the sequential activation of zymogens to their serine protease forms by limited proteolysis. Several coagulation factors contain structural features that allow for more efficient progression of this activation cascade. Factors II (prothrombin), VII, IX, X, as well as the anticoagulant precursor, protein C, are vitamin K-dependent proteins. Vitamin K-dependent post-translational modification of these coagulation factors results in the carboxylation of 9-12 glutamic acid (Gla) residues within the first 40 residues of the N-terminal region, known as the Gla domain (Vermeer, 1990). Importantly, Ca^{2+}-dependent docking via the Gla domain to negatively-charged phospholipid surfaces (i.e. the activated platelet surface) leads to the assembly of coagulation factor-activating complexes (Fig. 1.5B) (Zwaal et al., 1998). Importantly, this characteristic promotes the rapid progression of coagulation at the site of vascular injury.

Extrinsic Coagulation

Concomitant with platelet adhesion, damage to the endothelium exposes tissue factor (TF) to the flowing blood and initiates blood coagulation through the injury or extrinsic pathway (Fig. 1.5A). TF acts as a cofactor to the serine protease, activated factor VII (FVIIa), and as a complex, TF:FVIIa activates, by limited proteolysis, zymogens factor IX (FIX) and factor X (FX) to their serine protease forms, activated FIX and FX (FIXa and FXa, respectively).

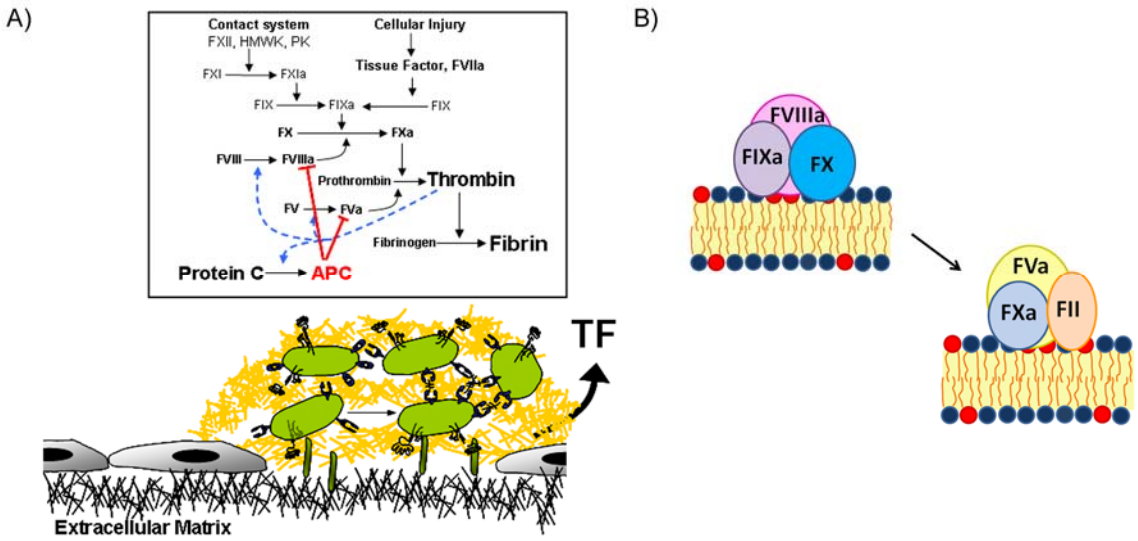

Figure 1.5 Platelet and Coagulation Interplay. (A) In response to a vascular injury, tissue factor (TF) is exposed to blood and initiates the injury/extrinsic arm of the coagulation cascade, while the exposed ECM protein, collagen, supports contact activation, culminating in thrombin production and fibrin formation (yellow mesh). **(B)** Procoagulant complexes form on the phosphatidylserine (red) expressing activated platelet surface, which catalyzes the activation of factor X (FX; top left) and prothrombin (factor II/FII; bottom right).

As illustrated in Fig. 1.5B, FIXa assembles with its non-proteolytically active cofactor, activated factor VIII (FVIIIa), to activate FX in the tenase complex. Subsequently, FXa joins its cofactor, activated factor V (FVa), in the prothrombinase complex (Fig. 1.5B) to convert the zymogen prothrombin to its active serine protease form, thrombin (FIIa). Not only does thrombin cleave fibrinogen to fibrin, but it also activates factor XIII (FXIII), which crosslinks fibrin into an insoluble mesh that stabilizes the growing platelet plug at the site of injury. Thrombin also activates upstream components of coagulation, such as factor XI, which resides in the intrinsic arm of coagulation, and cofactors V and VIII (Fig. 1.5A) (Esmon, 1979; Kravtsov et al., 2009). This feedback results in an explosion of thrombin generation at the site of injury.

Intrinsic Coagulation and FXI Contributions to Hemostasis

A second mechanism for the initiation of coagulation is activation of the intrinsic pathway. Traditionally, intrinsic, or contact activation is triggered by the presence of a foreign substance exposed to flowing blood, such as RNA or polyphosphates released from dense granules following platelet activation (Kannemeier et al., 2007; Smith et al., 2006). This leads to the cleavage of factor XII (FXII) into activated FXII (FXIIa). When bound to a surface, FXIIa is a potent activator of factor XI (FXI). While FXII appears to be dispensable in physiological hemostasis, about a third of patients with severe FXI deficiency experience a mild to moderate bleeding diathesis, usually in response to a hemostatic challenge such as surgery. Thus, the feedback activation of FXI by thrombin may be crucial for sustained thrombin generation in response to a vascular injury (Gailani and Broze, 1991; Kravtsov et al., 2009).

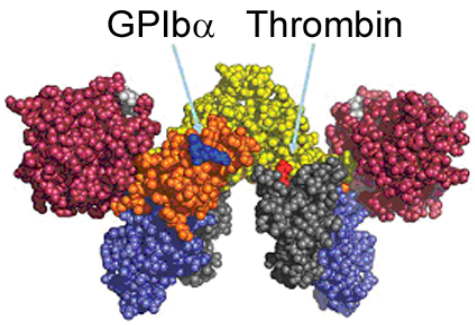

Figure 1.6 Space-filling representation of the FXI dimer. White side chains: Arg^{369} and Ile^{370} in the activation loop; blue side chain: $Lys^{252-253}$ – GPIbα binding site; red side chain: Glu^{66} – thrombin binding site. Adapted by permission from Macmillan Publishers Ltd: Nature Structural & Molecular Biology (*Nat Struct Mol Biol*. 2006 Jun;13(6):557-8.), copyright 2006.

FXI is a 160 kDa homodimer that circulates in a complex with high molecular weight kininogen. The heavy chain of each monomer consists of four tandem apple domains (A1-A4), a trait shared with plasma prekallikrein (PK), and dimerization occurs as a result of a disulfide bond between Cys^{321} in adjacent A4 domains (Fig. 1.6) (Papagrigoriou et al., 2006). Activated FXI (FXIa) is generated

by cleavage of FXI in the light chain between Arg^{369}-Ile^{370} by FXIIa during contact activation, by autoactivation, or by thrombin (Gailani and Broze, 1991). Thrombin binding to FXI has been mapped to the A1 domain, while the A3 domain contains binding sites for GPIbα and FIX (Baglia et al., 2004a; Baglia and Walsh, 1996; Sun and Gailani, 1996). FXIa activation of FIX leads to the convergence of the two arms of coagulation at FX activation (Fig. 1.5A). The essential role for intrinsic coagulation in hemostasis is evidenced by the severe bleeding associated with hemophilic patients, who are deficient in factor VIII (hemophilia A) or factor IX (hemophilia B) (Moll and White, 1995; Valentino and Scheiflinger, 2006).

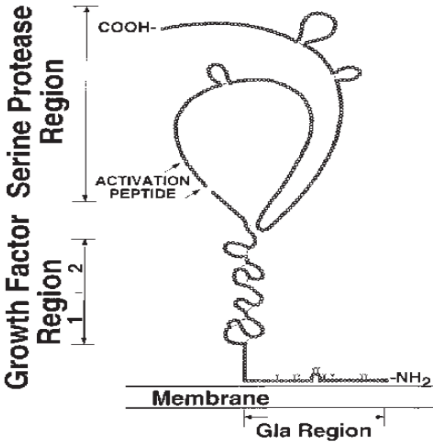

Figure 1.7 Diagram of Protein C. Major structural components of protein C include the Gla region, two epidermal growh factor repeats and the serine protease light chain. Obtained from *Haematologica*. (Esmon, C.T. (1999). Inflammation, Sepsis and Coagulation. *Haematologica*, 84:254-259)

Activated Protein C Regulation of Coagulation

Thrombin also plays a role in the downregulation of coagulation by activating protein C. Protein C is a vitamin K-dependent protein, containing a Gla domain, two epidermal growth factor (EGF) domains, and a serine protease domain with an activation peptide (Fig. 1.7). Protein C is the zymogen precursor to the trypsin-like serine protease, activated protein C (APC). APC is responsible for inactivating cofactors VIIIa and Va. While its substrates assemble into

complexes on the platelet surface, the mechanisms for APC recruitment to the platelet surface are unknown. Protein C contains the Gla domain that allows Ca^{2+}-dependent interaction of several coagulation factors with phospholipid surfaces (Fig. 1.7) (Zwaal et al., 1998), however the saturable binding sites for APC on the platelet surface described in the literature suggest that APC binding to platelets is receptor-mediated (Harris and Esmon, 1985). Vascular cells, such as endothelial cells (Fukudome and Esmon, 1994) and monocytes (Galligan et al., 2001), express the endothelial cell protein C receptor (EPCR), which binds APC and protein C with similar affinity. However, EPCR has not been shown to be expressed on platelets.

1.5 Thrombosis

While the activation of platelets and coagulation are essential for normal hemostasis, these events can be triggered in disease states such as atherosclerotic plaque rupture or the presence of APS antibodies. The presence of these pathologic stimuli, or an imbalance in pro- and anti-coagulant processes in response to a more typical injury, can lead to thrombus formation. Thrombus formation is a pathologic event that may lead to diseases, including myocardial infarction, pulmonary embolism, or ischemic stroke, which are the leading causes of death and disability in industrialized nations (Gawaz et al., 2005; Ruggeri, 2002).

To combat the formation of pathologic thrombi, an anti-platelet or anticoagulation agent, such as aspirin, warfarin or heparin, is administered to individuals who are considered to be at high, long-term risk of thrombotic disease, while $\alpha_{IIb}\beta_3$-blockers are used in acute situations in the clinic (Mousa, 2003; Phillips et al., 2005). Further, anticoagulants and thrombolytic agents (such as heparins, tissue-type plasminogen activator) are used to prevent or to break up thrombi in various diseases, including ischemic stroke (Albers et al., 2004; Hacke et al., 1995).

Although current antithrombotic agents are effective in preventing thrombus formation by reducing platelet aggregation and clot formation, or removing thrombi from the circulation, they can have severe hemorrhagic side effects. Plasminogen activator treatment, currently the only approved therapy for ischemic stroke in the USA, has been shown to increase the risk of brain hemorrhage, has only a 3-hour time window of efficacy, and is capable of directly damaging neurons (Benchenane et al., 2004); the same properties of aspirin that inhibit the activation and aggregation of platelets also predispose patients to bleed (Gorelick and Weisman, 2005; Kimmey, 2004); and oral $\alpha_{IIb}\beta_3$-blockers cause a paradoxical increase in cardiovascular disease mortality (Chew et al., 2001; Cox et al., 2000; Holmes et al., 2000; Quinn et al., 2003; Quinn et al., 2002; Topol et al., 2003), presumably by activating the FcγRIIa receptor on platelets (Gao et al., 2009). Therefore, there exists a need for a safer therapeutic approach for targeting thrombus formation without affecting hemostasis.

The role for the initiation of the intrinsic pathway in hemostasis appears to be minimal: FXII-deficiency is not associated with a bleeding diathesis, while approximately a third of FXI-deficient patients exhibit moderate bleeding in response to hemostatic challenge. Recent *in vivo* injury models implicate a role for the intrinsic pathway of coagulation in stable thrombus growth (Furie and Furie, 2005; Gruber and Hanson, 2003; Kleinschnitz et al., 2006; Renne et al., 2005; Tucker et al., 2009). In humans, a high plasma FXI level appears to be a risk factor for venous thromboembolism and myocardial infarction (Doggen et al., 2006; Meijers et al., 2000), while severe FXI deficiency is protective against ischemic stroke (Salomon et al., 2008; Yang et al., 2006). The emerging role for intrinsic coagulation in thrombosis, paired with its apparently minimal role in hemostasis, make FXI and FXII attractive antithrombotic targets.

1.6 Summary

Formation of a hemostatic plug requires an orchestrated series of receptor-mediated and biochemical events, leading to the rapid cessation of bleeding. A disruption or an imbalance in pro- and anti-coagulant events can lead to severe bleeding or thrombosis. Current antithrombotic treatments typically target key hemostatic mechanisms and can cause severe bleeding or, paradoxically, thrombosis in some patients. It is critical to gain further understanding of the interactions that occur after the presentation of injury, and to distinguish between the mechanisms that contribute to hemostasis and those that promote thrombosis.

Members of the intrinsic pathway of coagulation have recently emerged as antithrombotic targets based on the contributions of FXI and FXII to the growth and stabilization of thrombi. The ECM protein, collagen, has been shown to contribute to the initiation of intrinsic coagulation through FXII activation (van der Meijden et al., 2009; Wilner et al., 1968). Chapter III addresses the potential of another ECM protein, laminin, to support coagulation in a FXI- and FXII-dependent manner.

The modest role for FXI in hemostasis is evidenced by the challenge-induced bleeding tendencies of some FXI-deficient patients. FXIa may be important for promoting sustained thrombin generation, as thrombin can provide positive feedback by activating FXI in plasma (Kravtsov et al., 2009). FXI does not contain a Gla domain that can anchor the protein to the activated platelet membrane, but it has been shown to interact with the GPIbα subunit on the platelet surface. Chapter IV discusses a series of studies identifying a second platelet receptor with potential for maintaining FXI on the platelet surface, which may provide a mechanism for sustained thrombin generation at the site of injury.

Regulatory mechanisms of coagulation are integral in the prevention of thrombosis. APC plays a critical role in limiting thrombin formation at the site of

injury. While the coagulation components that APC targets assemble on the activated platelet surface, the mechanism for APC recruitment to the platelet surface is unknown. Protein C does contain a Gla domain, however, whether this is sufficient and/or necessary for recruitment to the site of injury is unknown. Chapter V describes the identification of two platelet surface receptors that support protein C / APC interactions in the presence of shear. Determining whether these receptors promote APC activity on the platelet surface or simply provide a mechanism for APC recruitment to the PS-exposing platelet membrane will require further studies.

Concluding remarks in Chapter VI describe the future directions for each of the studies described in Chapters III, IV and V.

Chapter II: Common Materials and Methods

2.1 Blood Collection and Purification Processes

2.1.1 Blood Collection

Blood was collected from healthy adult volunteers according to OHSU eIRB protocol IRB00001673. Blood was taken from donors by venipuncture into syringes containing anticoagulants that were appropriate for the desired preparation, as described below.

2.1.2 Platelet Rich Plasma Preparation

Blood was collected into a ratio of 1:10 vol/vol 3.8% (wt/vol) sodium citrate. The first two mL of blood were drawn into a separate syringe and discarded in order to minimalize tissue factor contamination. The anticoagulated blood was then added to 5 mL polypropylene tubes (VWR 60818-576) and centrifuged at 200 × *g* for 20 min, and the plasma fraction was removed from the tubes and kept at room temperature prior to use.

2.1.3 Platelet Poor Plasma Preparation

Blood was collected from at least three donors into a ratio of 1:10 vol/vol 3.2% (wt/vol) sodium citrate. The first two mL of blood were drawn into a separate syringe and discarded in order to minmalize tissue factor contamination. The anticoagulated blood was then added to 5 mL polypropylene tubes (VWR

60818-576) and centrifuged at 2150 × *g* for 10 min. The plasma fraction from each donor was then placed into a separate 50 mL tube and centrifuged for 10 min at 2150 × *g*. The supernatant from all donors was collected and combined prior to aliquotting and storing at -80˚C until use. Aliquots were thawed in a 37˚C water bath just prior to use and plasma was kept at room temperature throughout each experiment.

2.1.4 Blood Cell Purification

Blood was drawn into a ratio of 1:10 vol/vol 3.8% (wt/vol) sodium citrate. Acid/citrate/dextrose (ACD; 71 mM citric acid, 85 mM sodium citrate, 111 mM glucose) was then added to the blood at 1:10 vol/vol prior to centrifugation in 5 mL polypropylene tubes (VWR 60818-576) at 200 × *g* for 20 min.

Washed Platelets

Plasma fractions were then transferred to a 50 mL tube, followed by the addition of 1 μg/mL PGI_2 (Cayman Chemical Co., Ann Arbor, MI, USA) and centrifugation at 1000 × *g* for 10 min. The supernatant was discarded and the platelet pellet was resuspended in a solution of 1 mL modified Tyrode buffer (in mM: 129 NaCl, 0.34 Na_2HPO_4, 2.9 KCl, 12 $NaHCO_3$, 20 HEPES, 5 glucose, 1 $MgCl_2$) and 150 μL ACD. Following resuspension, 25 mL of modified Tyrode buffer, 3 mL ACD and 1 μg/mL PGI_2 were added to the tube and platelets were pelleted once more by centrifugation at 1000 × *g* for 10 min. After discarding the supernatant, the pellet was resuspended in 1 mL modified Tyrode buffer and washed platelets were allowed to rest for 30 min prior to appropriate dilution and use in experiments.

Washed Red Blood Cells

Following the removal of plasma, the red blood cell (RBC) fractions were combined in a 50 mL tube and centrifuged at 2000 × *g* for 10 min. The supernatant and buffy coat were aspirated and discarded, and RBCs were

washed 3 times by the addition of 15 mL RBC buffer (10 mM HEPES, 140 mM NaCl, 5 mM glucose, pH 7.4) followed by centrifugation at 2000 × *g* for 10 min and supernatant aspiration. The resulting packed RBCs were stored at room temperature prior to use in experiments.

2.2 Flow Experiments

2.2.1 Design and Theory

The interactions leading to hemostasis and thrombosis occur in the dynamic environment of the vasculature. While valuable mechanistic information can be acquired by performing experiments under static conditions, it is essential to analyze these interactions in the presence of physiological shear. We utilized a parallel plate flow model to mimic the *in vivo* environment of the vasculature (Fig. 2.1) (McCarty et al., 2004a). Shear rates vary widely throughout the circulation, and with the parallel plate flow system, initial wall shear rates can be manipulated to mimic those observed in both venous and arterial environments.

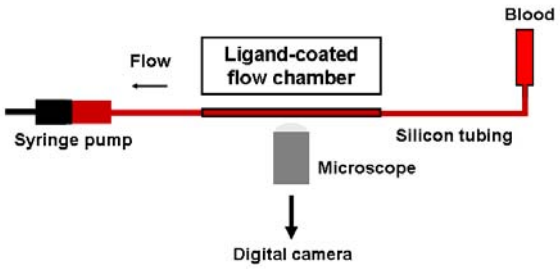

Figure 2.1 Flow chamber schematic. A syringe pump was utilized to maintain blood perfusion through a protein-coated flow chamber at a constant volumetric flow rate. After washing with modified Tyrode buffer, differential interference contrast (DIC) images were acquired using a Zeiss AxioCam connected to Slidebook imaging software (Intelligent Imaging Innovations, Inc., Santa Monica, CA, USA).

Initial shear rates were calculated using several approximations. Due to the height of the chamber being much smaller than the width, one can assume that the flow chamber is composed of two infinite, parallel plates (Lawrence et al.,

1987). Furthermore, initial laminar flow conditions ensure a parabolic velocity profile, and shear calculations are made based on the assumption that the fluid perfused through the system is Newtonian and incompressible (Lawrence et al., 1987). The resulting shear rate is calculated as:

$$\gamma_{wall} = \frac{6Q}{h^2 w} \qquad \text{(Eq. 2.1)}$$

where γ_{wall} is wall shear rate, Q is the volumetric flow rate, h and w are the height and width of the chamber, respectively. Note that the shear rate of a Newtonian fluid is a measure of the difference in velocity between two subsequent layers of flow, and is independent of viscosity.

2.2.2 Experimental Procedure

Flow experiments were performed on the stage of a Zeiss Axiovert 200M microscope (Carl Zeiss, Thornwood, NY). As shown in Fig. 2.1, a ligand-coated glass capillary tube (0.2 × 2 mm, VitroCom Inc., Mountain Lakes, NJ) or 32 mm diameter coverslip (No. 1.5 thickness, Fisher Scientific, Pittsburgh, PA) attached to a parallel plate flow chamber (Cat.#31-001: gap width .25 cm, gasket height .254 mm, Glycotech Corp., Gaithersburg, MD), was inserted into a silicone line (VWR Cat. #60985-716 and #60985-708, Bridgeport, NJ) connecting a sample reservoir to a programmable syringe pump (Harvard Apparatus, Holliston, MA). Flow through the ligand-coated chamber was initiated with modified Tyrode buffer to eliminate air bubbles between the reservoir and pump. Subsequently, perfusion samples, including anticoagulated whole blood or reconstituted blood (washed red blood cells recombined with washed platelets, 50% vol/vol, final platelet count 3×10^8 /mL), were pipetted into the reservoir and perfused across the ligand-coated surface for a designated time at a constant volumetric flow rate corresponding to the selected initial shear rate. Blood samples were chased with a solution of modified Tyrode buffer for 10 minutes prior to imaging slides.

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

3.1 Abstract

Laminin is the most abundant noncollagenous 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. The objective here was to investigate the ability of laminin to initiate the coagulation cascade and support thrombus formation under shear. Data presented here show that soluble laminin accelerated factor XII activation in a purified system, and shortened the clotting time of recalcified plasma in a factor XI (FXI) and factor XII (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 factor XII (FXIIa), or an antibody that blocks activated factor XI (FXIa) activation of factor IX (FIX). 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.

This work has been accepted for publication in the *Journal of Thrombosis and Haemostasis*.

3.2 Introduction

The extracellular matrix (ECM) plays a critical role in primary hemostasis: the recruitment of platelets to the vascular wall after injury. A study published in 2009 implicated the ECM component, collagen, in the direct activation of the intrinsic pathway of coagulation (van der Meijden et al., 2009). Laminin, which is abundantly expressed in the ECM, has been shown to interact with platelets in an analogous manner to collagen (Inoue et al., 2006; Inoue et al., 2008), but the ability of laminin to directly contribute to coagulation has not been investigated. Here, we provide evidence that laminin is able to contribute to contact activation in two ways: by stimulating phophatidylserine exposure on the outer leaflet of laminin-bound platelets and by accelerating contact activation.

3.3 Background

When vessel walls are damaged, exposed ECM proteins trigger a series of events that lead to the formation of a hemostatic plug (Jackson, 2007; Jackson et al., 2003). 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 (Watson et al., 2005). 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 (McCarty et al., 2006; Ozaki et al., 2005; Ruggeri, 2002). 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 (Jurk and Kehrel, 2005).

Concomitant with platelet recruitment and activation are the first steps of blood coagulation, triggered by the exposure of blood to tissue factor (Mackman et al., 2007). 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 XIII and XI (FXI) and cofactors V and VIII, which in concert contribute to the rapid and efficient arrest of bleeding from sites of vascular injury.

Recent work has highlighted a dual role of exposed ECM proteins in contributing to thrombus formation. van der Meijden and colleagues demonstrated that the ECM protein collagen triggers the activation of coagulation via factor XII (FXII), and thus subsequent FXI activation, in addition to the well-defined role for collagen in mediating platelet recruitment and activation (van der Meijden et al., 2009). Until now, the ability of other ECM proteins, such as laminin, to contribute to the initiation of coagulation and thrombus formation in a similar fashion has not been investigated.

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 $\alpha_6\beta_1$ (Hindriks et al., 1992; Inoue et al., 2006). Further studies by Inoue and colleagues revealed that immobilized laminin supports platelet recruitment under shear flow in a GPIbα-vWF dependent manner (Inoue et al., 2008). 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.

3.4 Materials and Methods

3.4.1 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). Murine monoclonal anti-factor XI antibodies 1A6 and 14E11 were cloned, expressed and purified as described (Kravtsov et al., 2009; Tucker et al., 2009). Oregon Green488 labeled annexin V was purchased from Invitrogen Corp. (Carlsbad, CA, USA). Laminin (L6274) from human placenta was purchased from Sigma-Aldrich (St. Louis, MO, USA). The predominant laminin isoform present in this preparation has been shown to be $\alpha 5\beta 1\gamma 1$, as characterized elsewhere (Wondimu et al., 2006). All other reagents were from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

3.4.2 Clotting times

Clotting times of human PRP or PPP were measured with a KC4 Coagulation Analyzer (Trinity Biotech, Bray, Co Wicklow, Ireland). Samples were pre-treated 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 mM final), and clotting time was recorded.

3.4.3 Activation of FXII

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 nM), laminin (50 μg/mL), CTI (4 μM), prekallikrein (30 nM), and high-molecular-weight kininogen (30 nM) in HEPES buffer (136 mM NaCl, 5 mM HEPES, 2.7 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, pH 7.45). Solutions were incubated for 120 min at 37°C. After addition of 0.8 mM Pefachrome FXIIa substrate, the rate of increase in absorption at 405 nm was determined for a period of 10 min.

3.4.4 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 in a binding buffer (in mM: 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.

3.4.5 Recalcified blood flow

Glass capillary tubes (0.2 × 2 mm, VitroCom, Mountain Lakes, NJ, USA) were incubated for 1 hr at RT with a solution of laminin (50 μg/mL), collagen (100 μg/mL). In selected experiments, immobilized laminin or collagen was treated with 0.1 mg/mL collagenase for 120 min, followed by blocking with 5 mg/mL denatured BSA for 1 hr and assembly into a flow system on the stage of a Zeiss Axiovert 200M microscope (Carl Zeiss, Thornwood, NY). Sodium citrate (0.38% w/v) anticoagulated whole blood was perfused through the capillary for 12 min at an initial wall shear rate of 250 s^{-1}. A solution of 37.5 mM Ca^{2+} and 18.75 mM 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 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% NP-40 and 5 mM PMSF), followed by treatment with 1 μM 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) to detect the 220 kDa fibrin degradation product, D-dimer. Sample D-dimer levels were compared to known amounts of purified D-dimer on the same blot (1 ng – 100 ng).

3.4.6 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 mM Ca^{2+} and 3.75 mM 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 (Berny et al., 2010):

$$\gamma_{wall} = -a\left[\frac{\rho_b g(h_c + h_b) - \rho_{pbs} g h_{pbs}}{h_c \mu}\right] \qquad \text{(Eq. 3.1)}$$

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, 2a is the width of the capillary. The time to capillary occlusion was recorded, within a maximum observation period of 40 min.

3.4.7 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.

3.5 Results

3.5.1 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 (Inoue et al., 2006; Inoue et al., 2008). Our study was designed to determine the role of laminin in the initiation of coagulation. The presence of 40 μg/mL laminin shortened the clotting time of recalcified platelet-rich plasma (PRP) compared to vehicle (Fig. 3.2A). Pre-treating PRP with the FXIIa inhibitor, CTI (4 μM) ; 14E11 (20 μg/mL), which binds to the second apple (A2) domain of FXI and inhibits FXI activation by FXIIa (Fig. 3.1) (Kravtsov et al., 2009); or the anti-FXI mAb 1A6 (20 μg/mL), which binds to the third apple (A3) domain of FXI and blocks FIX binding to FXIa (Fig. 3.1) (Kravtsov et al., 2009), prior to incubation with laminin prolonged clotting times compared to the presence of laminin alone (Fig. 3.2A). The presence of CTI, 14E11 or 1A6 did not affect clotting times in the presence of laminin plus 1 pM tissue factor (Fig. 3.2A). Similar trends were observed in platelet-poor plasma (PPP, Fig. 3.2C), suggesting that the presence of platelets plays a minimal role in the mechanism by which laminin shortens clotting times. Furthermore, the addition of 40 μg/mL laminin to PRP failed to initiate either shape change or aggregation (Fig. 3.2D), providing evidence that laminin is capable of affecting coagulation in a manner not dependent upon platelet activation.

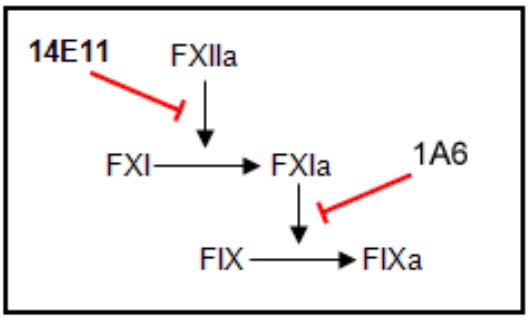

Figure 3.1 Anti-FXI monoclonal antibodies. The anti-human FXI mAb, 1A6, blocks the ability of FXIa to activate factor IX (FIX), while the anti-murine FXI mAb, 14E11, blocks the ability of FXII to activate FXI.

Collagen has been shown to activate FXII and promote coagulation in recalcified PRP (van der Meijden et al., 2009). In experiments performed here, PRP was incubated with either collagen (40 μg/mL) or vehicle, followed by the initiation of coagulation by adding $CaCl_2$. In accord with van der Meijden, et al (van der Meijden et al., 2009), the presence of collagen significantly shortened clotting time compared to vehicle (Fig. 3.2B). Clotting times in the presence of collagen, but not collagen plus 1 pM tissue factor, were prolonged by the addition of 1A6, 14E11 or CTI (Fig. 3.2B).

3.5.2 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 to the presence of HK and PK alone (Fig. 3.2E). Chromogenic activity was negligible in the presence of CTI (Fig. 3.2E).

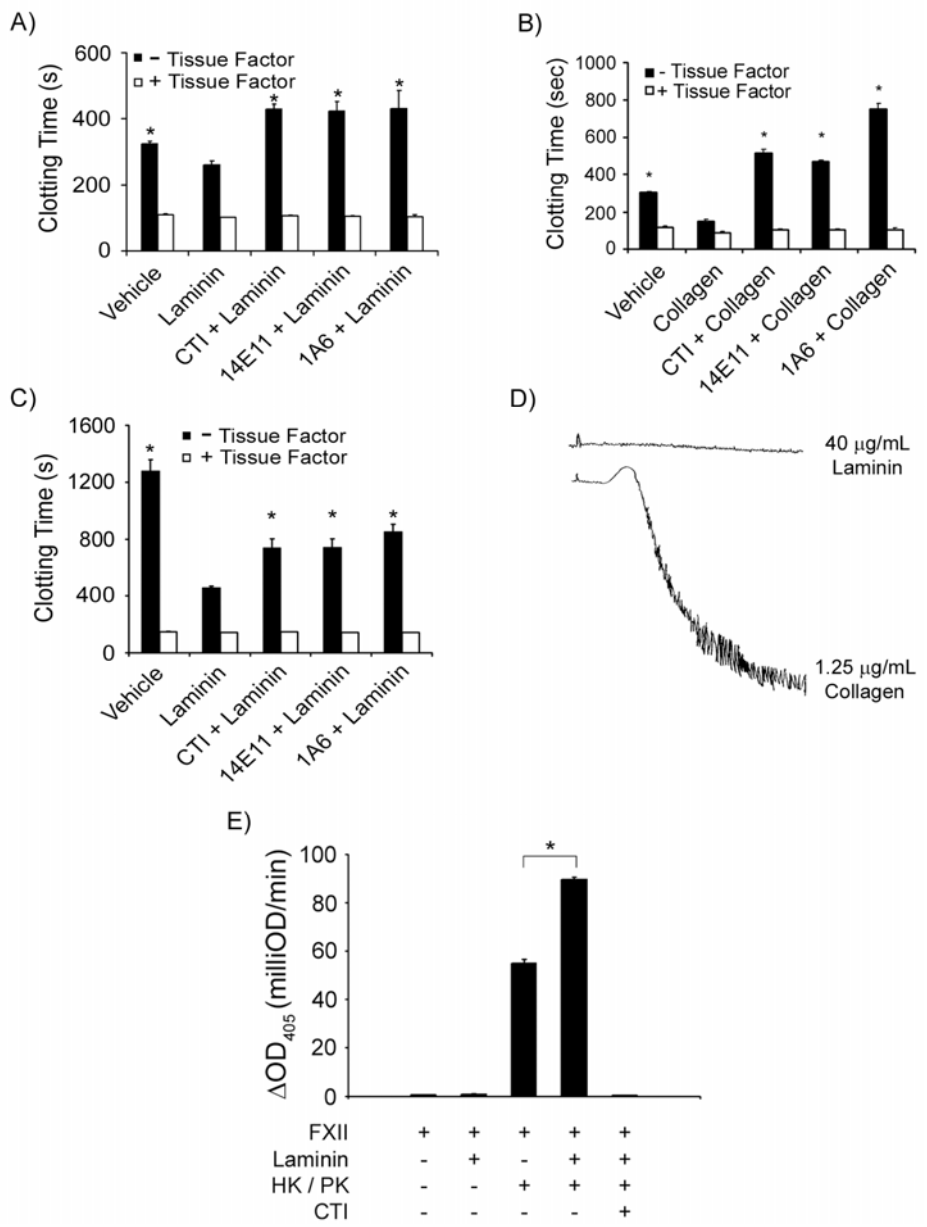

Figure 3.2 Laminin enhances coagulation. Human sodium citrate-anticoagulated **(A-B)** platelet-rich plasma (PRP) or **(C)** platelet-poor plasma (PPP) was pre-treated with 4 μM CTI, 20 μg/mL 14E11 or 20 μg/mL 1A6. Laminin or collagen was then added to the plasma (40 μg/mL in PRP, 10 μg/mL in PPP) in the absence (black bars) or presence (white bars) of 1 pM tissue factor. Following a 3 min incubation, coagulation was initiated by the addition of 16.6 mM $CaCl_2$, and clotting times were recorded. Data reported are mean ± SEM of at least 3 experiments; * $P < 0.05$ compared to clotting times in the presence of laminin or collagen alone. **(D)** Aggregation in human PRP was initiated with either 40 μg/mL laminin or 1.25 μg/mL collagen. **(E)** FXII activation was monitored in the presence of 50 μg/mL laminin. Experiments were performed in the presence of high molecular weight kininogen/prekallikrein (HK/PK, 30 nM each) or CTI (4 μM) as indicated. FXIIa was detected by measuring cleavage of its chromogenic substrate, Pefachrome FXIIa. Data are mean ± SEM of 3 experiments. (* $P < 0.05$)

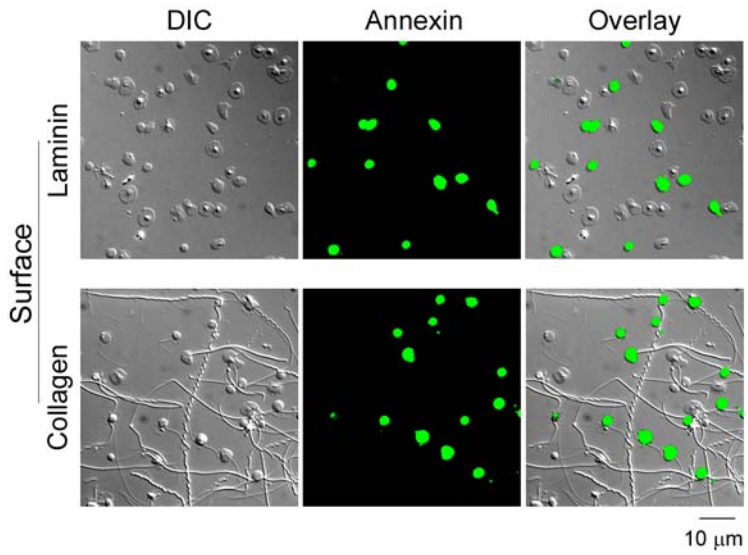

Figure 3.3 Laminin- and Collagen-bound platelets expose phosphatidylserine (PS). Washed human platelets were exposed to a surface of immobilized laminin or collagen. Bound platelets were incubated with a solution of fluorescently-labeled annexin V to visualize PS exposure. Images were recorded using DIC and fluorescence microscopy. Corresponding brightfield and fluorescent images are shown alone and in the overlay. *These experiments were performed by Michelle Berny in the McCarty laboratory.

3.5.3 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 (Heemskerk et al., 2002; Heemskerk et al., 2000). 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. 3.3). The portion of collagen-bound platelets that supported annexin V binding was 49% (Fig. 3.3). Conversely, BSA surfaces supported less than 2% of the platelet adhesion observed on laminin or collagen, and only 8.6% of the platelets adherent on BSA bound annexin V.

3.5.4 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. 3.4A). Fibrin strands were visible throughout the laminin-coated capillary (Fig. 3.4A), and D-dimer was readily detected in clot lysates (Fig. 3.4A). The degree of fibrin deposition, platelet aggregation and D-dimer detection was visibly higher on laminin as compared to BSA coated surfaces (Fig. 3.4C). 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 (Inoue et al., 2006). Collagenase-treated laminin surfaces supported equivalent levels of platelet- and fibrin-rich thrombus formation (Fig. 3.5). In contrast, fibrin formation was abrogated in the presence of the direct thrombin inhibitor, hirudin (20 μg/mL). 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 to vehicle (Fig. 3.4A).

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 (Tucker et al., 2009). 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. 3.4B).

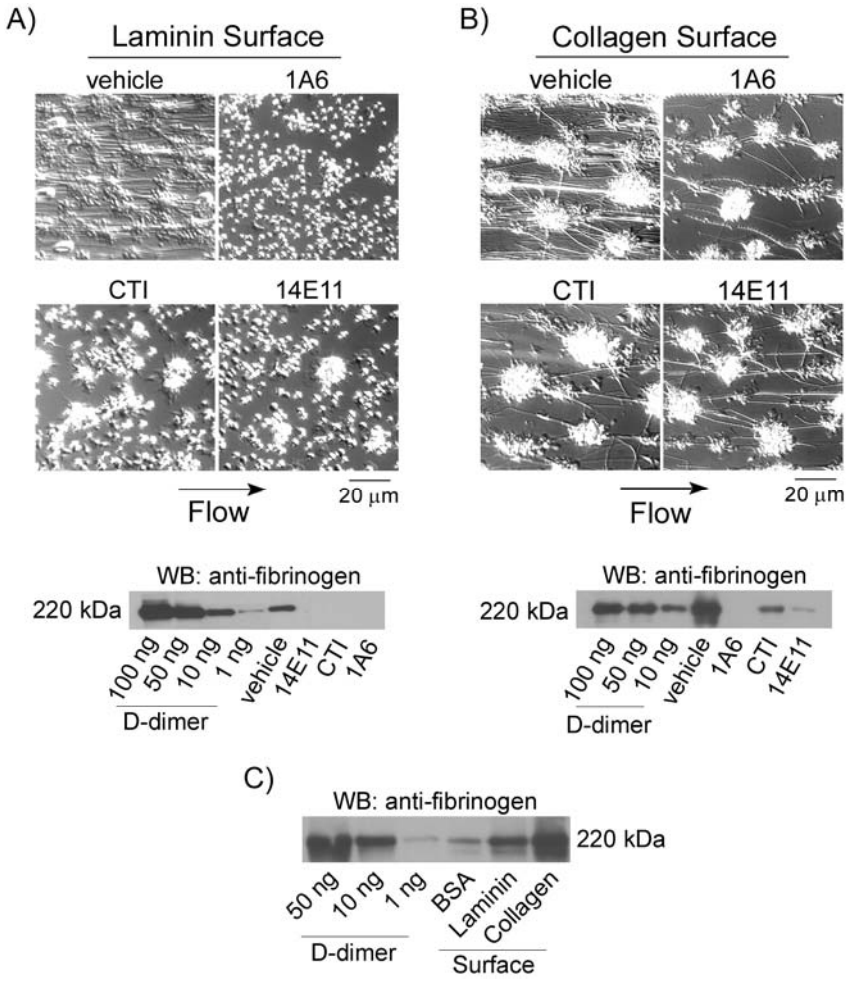

Figure 3.4 Laminin supports thrombus formation under shear. Sodium citrate-anticoagulated whole human blood was co-perfused with a solution of Ca^{2+}/Mg^{2+} (7.5 mM/3.75mM final, respectively) over **(A)** laminin, **(B)** collagen or **(C)** BSA surfaces for 12 min at 250 s^{-1}, followed by perfusion of modified Tyrode buffer to remove non-adherent cells. In separate experiments, blood was pre-treated with 20 μg/mL 1A6, 40 μg/mL CTI or 20 μg/mL 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 3 experiments. *Western blots were performed by Jiaqing Pang in the McCarty laboratory.

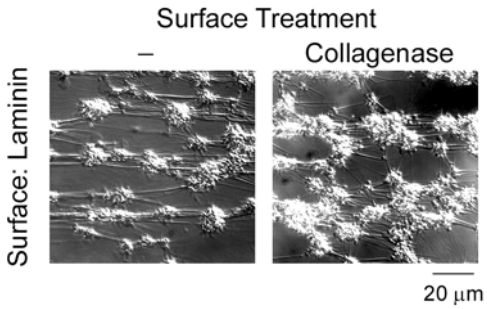

Figure 3.5 Laminin supports fibrin deposition following collagenase proteolysis. Laminin surfaces were treated with either vehicle (–) or collagenase (0.1 mg/mL) for 120 min at 37°C prior to perfusion studies. Recalcified blood was perfused over laminin surfaces for 12 min. at 37°C, followed by washing with modified Tyrode buffer prior to imaging using DIC microscopy. *These experiments were performed by Brian Fuchs in the McCarty laboratory.

3.5.5 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 (Berny et al., 2010). 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. 3.6), 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), 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

(Gruber and Hanson, 2003; Rosen et al., 2002; Tucker et al., 2009; Wang et al., 2005; Yamashita et al., 2006). Our data demonstrate that pretreatment of blood with 20 μg/mL of the anti-FXI mAbs 1A6 or 14E11 or the FXIIa inhibitor CTI prolonged the time to occlusion on laminin past the 40 min observation period (Fig. 3.6). 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. 3.6).

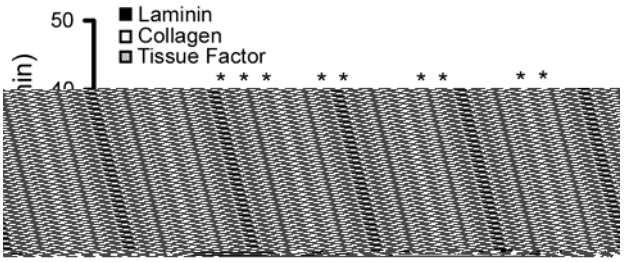

Figure 3.6 Laminin supports occlusive thrombus formation under a constant pressure gradient. Whole human blood in 0.38% sodium citrate was recalcified and perfused through a laminin-, collagen-, or tissue factor-coated glass capillary until occlusion. Blood flow was driven by a constant pressure difference. In selected experiments, blood was pre-treated with either 40 μg/mL CTI, 20 μg/mL 14E11 or 20 μg/mL 1A6. Data are reported as mean ± SEM of at least 3 experiments. * $P < 0.05$ compared to occlusion time in the presence of vehicle on each respective surface. *These experiments were performed by Ishan Patel in the McCarty laboratory.

3.6 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 are enhanced in atherosclerotic plaques, laminin expression levels remain surprisingly constant (Stenman et al., 1980; Stoop et al., 2000; van Zanten et al., 1994). Perhaps this

kinases (PP2, 20 μM), PI3-kinases (wortmannin, 100 nM) or an intracellular Ca^{2+} chelator (BAPTA-AM, 10 μM) inhibited platelet spreading on immobilized FXI (Fig. 4.2). Similar results were seen on FXIa surfaces (Fig. 4.2).

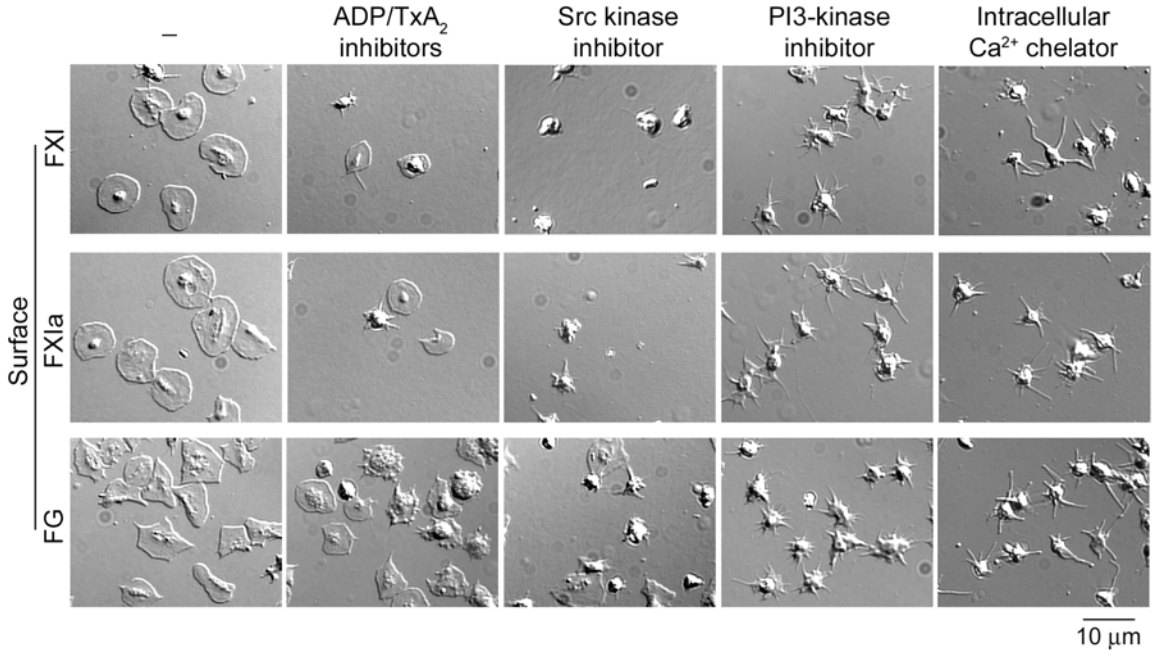

Figure 4.2 The effect of inhibitors on platelet spreading on immobilized FXI and FXIa. Purified human platelets (2×10^7/mL) were gently pipetted onto surfaces of immobilized FXI, FXIa or fibrinogen (FG), incubated for 45 min at 37°C and imaged using DIC microscopy. In selected experiments, platelets were pretreated with the following: vehicle (–), 2 U/mL of the ADP scavenger apyrase and 10 μM of the cyclo-oxygenase inhibitor indomethacin (ADP/TxA_2 inhibitors), the Src-kinase inhibitor PP2 (20 μM), the PI3-kinase inhibitor wortmannin (100 nM), or the intracellular calcium chelator BAPTA-AM (10 μM). Images are representative of at least three experiments. *These data were produced by Ion Melinte and Michelle Berny in the McCarty laboratory.

4.5.2 ApoER2 mediates platelet adhesion to FXI

FXI has been shown to bind specifically and reversibly to high affinity sites on the platelet surface. To characterize the molecular mechanisms of platelet-FXI binding, adhesion assays were performed in the presence of receptor-associated protein (RAP), which universally inhibits ligand binding to the LDL receptor family. Our data show that RAP eliminated platelet binding to immobilized FXI, but not to fibrinogen (Fig 4.3). Furthermore, the presence of soluble recombinant

ApoER2' (sApoER2') abrogated platelet binding to FXI (Fig 4.3). Moreover, the presence of the LDL-binding domain 1 or 2 of ApoER2 reduced platelet adhesion to FXI by 78% and 55%, respectively (Fig. 4.3B).

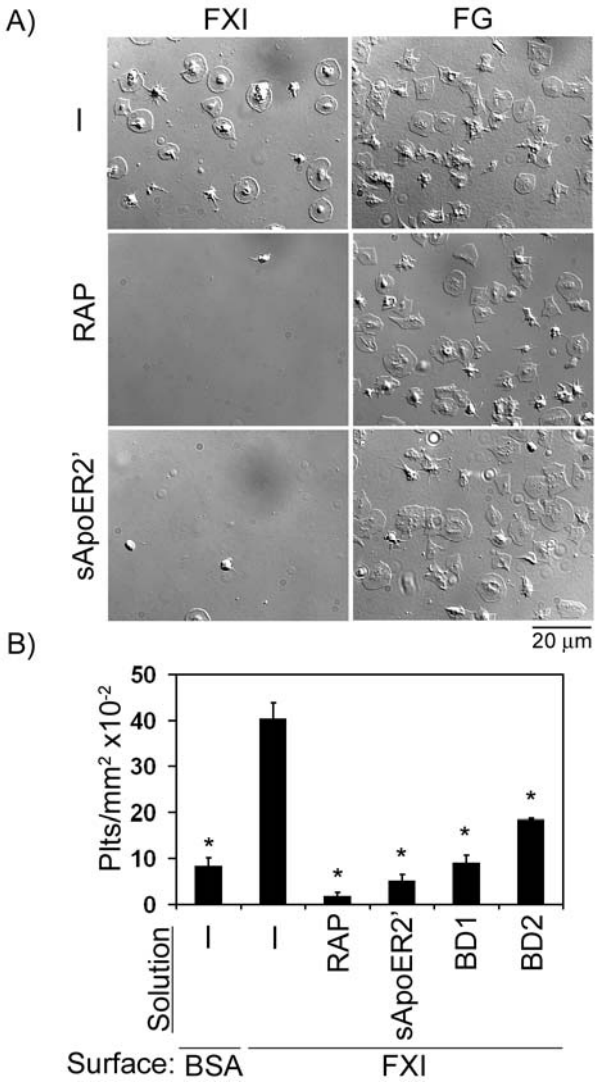

Figure 4.3 The role of ApoER2 in platelet adhesion to FXI. (A) Purified human platelets (2×10^7/mL) were pipetted onto surfaces coated with FXI or fibrinogen (FG) in the absence or presence of receptor-associated protein (RAP, 40 μg/mL) or soluble ApoER2' (sApoER2', 40 μg/mL), followed by incubation at 37°C for 45 min. **(B)** Additional adhesion experiments were performed on immobilized FXI in the presence of the LDL binding domains 1 or 2 of ApoER2 (BD1 or BD2, respectively, 40 μg/mL). Adherent platelets were analyzed for each suspension treatment and reported as adherent cells/mm^2 $\times$ 10^{-2}. Values are reported as mean $\pm$ SEM of three experiments. *P < 0.05 compared to adhesion on FXI in the presence of vehicle (–, lane 2).

In order to verify the ability of ApoER2 to mediate platelet binding to FXI, platelets from wild-type C57Bl/6 or ApoER2-deficient mice were incubated over surfaces coated with FXI or fibrinogen. As shown in Fig. 4.4, a greater than 90% reduction in platelet adhesion on FXI was observed for ApoER2-deficient platelets compared to wild-type (13.6 ± 1.4 vs. 1.2 ± 0.5 × 10^2 platelets/mm^2 on FXI for wild-type vs. ApoER2$^{-/-}$, respectively). A similar level of reduction in binding was observed for wild-type platelets in the presence of binding domain 1 of ApoER2 (Fig. 4.4). Equivalent levels of adhesion were observed on fibrinogen for wild-type and ApoER2$^{-/-}$ platelets (155 ± 15 vs. 150 ± 12 × 10^2 platelets/mm^2 on fibrinogen for wild-type vs. ApoER2$^{-/-}$, respectively).

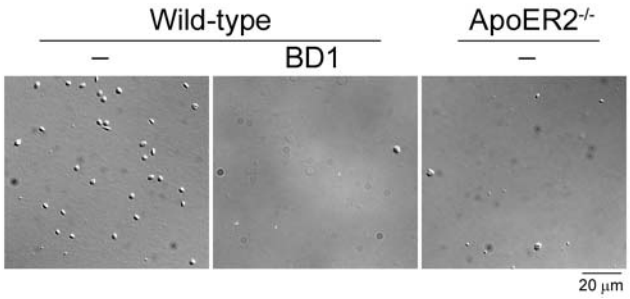

Figure 4.4 ApoER2-deficient platelets do not bind to FXI. Purified platelets (2 × 10^7/mL) from wild-type or ApoER2-deficient mice were incubated over immobilized FXI at 37°C for 45 min in the presence of vehicle (–) or the LDL binding domain 1 of ApoER2 (BD1, 40 μg/mL). Representative images from 3-4 experiments are shown. *Experiments with ApoER2$^{-/-}$ mice were performed by Owen McCarty in the laboratory of Philip G de Groot at Utrecht University.

4.5.3 Mechanisms of FXI-platelet interactions under flow.

In order to evaluate the interaction between platelets and FXI in the presence of shear, washed human platelets were reconstituted with purified red blood cells (50% vol/vol, final platelet count 3 × 10^8/mL) and perfused over immobilized FXI for 3 min at 600 s^{-1}. In the presence of shear, platelets in reconstituted blood formed aggregates on immobilized FXI (Fig. 4.5). Addition of the anti-GPIbα mAb, AK2, or LDL-binding domain 1 of ApoER2 blocked platelet

adhesion to FXI under shear conditions (Fig. 4.5). Similar results were observed on FXIa surfaces in the presence of shear (Fig. 4.5).

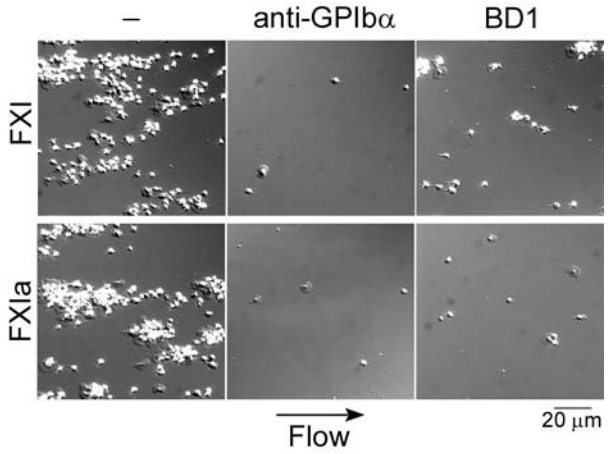

Figure 4.5 Platelet binding to FXI under physiological shear. Human reconstituted blood (autologous packed red blood cells and washed human platelets combined to 50% vol/vol, yielding a final platelet concentration of 3×10^8/mL) was perfused over a surface of FXI or FXIa at a shear rate of 600 s^{-1} for 3 min in the presence of 25 μM Zn^{2+}. Separate experiments were performed in the presence of either an anti-GPIbα antibody (AK2, 20 μg/mL) or LDL-binding domain 1 of ApoER2 (BD1, 50 μg/mL). Images are representative of at least 3 separate experiments.

4.5.4 Effects of Zn^{2+} and HK on platelet adhesion to FXI surfaces.

FXI circulates in the plasma in complex with HK, and earlier studies have shown that FXI-platelet interactions in solution require both HK and the divalent cation, Zn^{2+} (Baird and Walsh, 2002a; Greengard et al., 1986). As shown in Fig. 4.6A, FXI surfaces supported platelet adhesion in the presence of either vehicle or the combination of Zn^{2+} and HK. The presence of RAP abrogated platelet adhesion to FXI in the absence or presence of Zn^{2+} and HK (Fig. 4.6A). The degree of platelet adhesion to surfaces of the active site mutant FXI-Ala^{557}, which lacks proteolytic activity, in the presence of vehicle was similar to adhesion to surfaces of native FXI (31.4 ± 4.2 vs. 33.9 ± 3.6 × 10^2 platelets/mm^2 on FXI-Ala^{557} vs. native FXI, respectively).

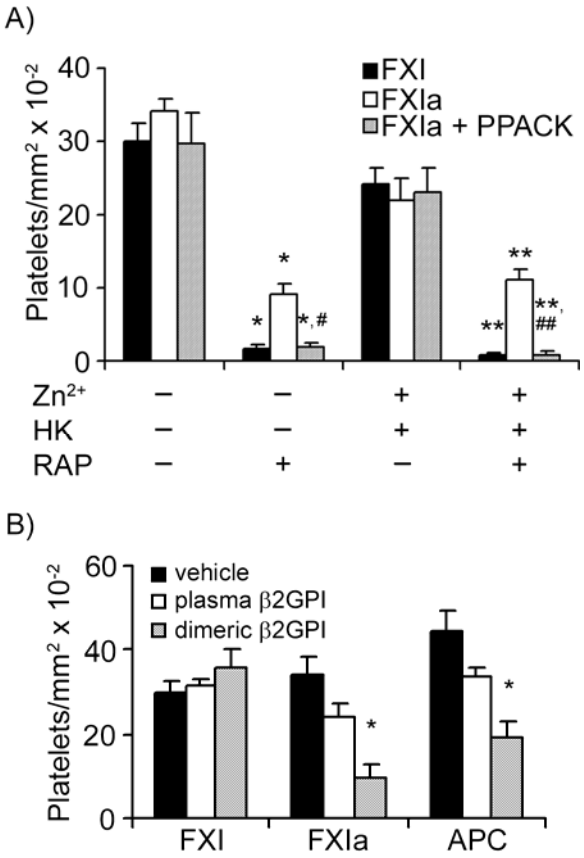

Figure 4.6 Platelet-FXI interactions in the presence of Zn^{2+}, high molecular weight kininogen or β_2 glycoprotein I. Washed human platelets (2×10^7/mL) were incubated over surfaces of FXI or FXIa in the absence or presence of **(A)** Zn^{2+} (25 μM) and high molecular weight kininogen (HK, 42 nM). Selected experiments were performed in the presence of PPACK (40 μM) or RAP (40 μg/mL). $P < 0.05$ compared to adhesion on each respective surface in the presence of vehicle (*) or Zn^{2+} and HK (**). $P < 0.05$ compared to adhesion on FXIa with RAP in the presence of vehicle (#) or Zn^{2+} and HK (##). **(B)** Additional adhesion experiments were performed over FXI, FXIa or activated protein C (APC) in the presence or absence of plasma-derived beta2-glycoprotein I (β_2GPI, 350 nM) or dimeric β_2GPI (350 nM). Adherent platelets were calculated for each treatment and are reported as adherent cells/$mm^2 \times 10^{-2}$. Adhesion numbers are reported as mean ± SEM of at least three experiments. *P < 0.05 compared to adhesion on each respective surface in the presence of vehicle.

FXIa surfaces supported platelet adhesion either in the presence of vehicle or the combination of Zn^{2+} and HK. Addition of RAP significantly, but incompletely, inhibited platelet adhesion to FXIa in the absence or presence of Zn^{2+} and HK (Fig. 4.6A). The remaining platelet adhesion on FXIa in each case was eliminated in the presence of RAP in combination with the serine protease

inhibitor, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK, 40 μM).

4.5.5 Effects of known ApoER2 ligands on platelet adhesion to FXI.

ApoER2 has been shown to bind to plasma components such as antiphospholipid antibody-dimerized beta 2 glycoprotein I (β_2GPI) and activated protein C (APC, described in Chapter V) (Pennings et al., 2007b). As shown in Fig. 4.6B, platelet adhesion to FXI was unaffected by the presence of either plasma-derived or dimerized β_2GPI. Conversely, platelet adhesion to immobilized FXIa or APC was significantly reduced in the presence of dimeric β_2GPI (Fig. 4.6B).

4.5.6 Evaluation of soluble FXI binding to purified sApoER2'.

To quantify direct binding of FXI to ApoER2, prescribed dilutions of FXI were incubated with immobilized sApoER2', and the level of bound FXI was determined using anti-FXI antibodies as described in Section 4.4.4. As shown in Fig. 4.7, soluble FXI bound to immobilized sApoER2' with an apparent affinity of 61 nM. The presence of RAP significantly reduced FXI binding to sApoER2' (Fig. 4.7). Taken together, our data identify ApoER2 as a novel receptor for FXI.

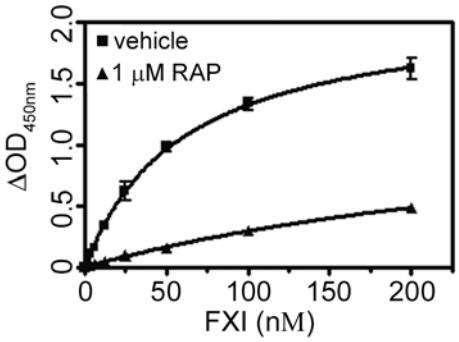

Figure 4.7 Soluble FXI binding to purified ApoER2'. FXI at the indicated concentrations was incubated over a surface of recombinant ApoER2'. Designated experiments were performed in the presence of either vehicle (squares) or 1 μM RAP (triangles). Bound FXI was detected by anti-FXI antibodies as described in section 4.4.4. Values are reported as mean ± SEM of 3 experiments. *These results were provided by Rolf T. Urbanus in the laboratory of Philip G de Groot at Utrecht University.

4.6 Discussion

The aim of this study was to investigate the molecular mechanisms of FXI-platelet interactions. We identified FXI as a novel ligand for the receptor ApoER2. Our initial data demonstrated that purified, recombinant forms of ApoER2 abrogated platelet binding to FXI, and that platelets from ApoER2-deficient mice did not adhere to immobilized FXI. Immobilized ApoER2' specifically bound FXI in a cell-free assay with an affinity of 61 nM. Our data identify FXI as a ligand for the platelet receptor ApoER2.

FXI binding to platelets was first described over 30 years ago (Walsh et al., 1975). GPIbα was identified as the major receptor for FXI on activated platelets (Baglia et al., 2004a; Baglia et al., 2004b; Ho et al., 2000), but studies have failed to show that GPIbα mediates FXIa binding to activated platelets under static conditions (Baird and Walsh, 2002b; Sinha et al., 1984). The data reported here support a role for GPIbα in mediating shear-dependent interactions between quiescent platelets and both FXI and FXIa. The reason for the discrepancy between our results and earlier studies is not clear, but it is noteworthy that ApoER2' has been shown to co-localize with the GPIb/IX/V complex on the platelet surface (Pennings et al., 2007b). As FXI and FXIa exist as homodimers, perhaps one FXI/FXIa molecule can simultaneously bind both GPIbα and ApoER2' on the platelet surface. Since ApoER2 is expressed on the platelet surface at far lower levels than the GPIb/IX/V complex (Pennings et al., 2007b), this may explain why a total of only 1500 FXI binding sites per platelet have been reported (Greengard et al., 1986), even though GPIb is present at 25,000 copies per platelet (Bergmeier et al., 2008).

Since the discovery of ApoER2 expression on platelets in 1999, platelet ApoER2 has been shown to bind a handful of plasma components (Berny et al., 2008; Korporaal et al., 2009; Korporaal et al., 2004; Pennings et al., 2007b), one of which is identified in Chapter V. Data reported here suggest that FXI has a similar affinity for ApoER2 as recently-described ligands such as dimeric β_2GPI

and activated protein C (APC, Chapter V) (Berny et al., 2008; van Lummel et al., 2005). Autoantibodies to β_2GPI correlate highly with recurrent arterial thrombosis and fetal loss associated with antiphospholipid antibody syndrome (APS) (Urbanus et al., 2008). The *in vitro* properties of APS patient β_2GPI-anti-β_2GPI antibody complexes are mimicked by dimeric β_2GPI (Lutters et al., 2001). While neither dimeric β_2GPI nor APC in our experiments inhibited platelet adhesion to FXI, the presence of dimeric β_2GPI significantly reduced platelet adhesion to immobilized FXIa. β_2GPI has been shown to inhibit the activation of FXI by FXIIa and thrombin, whereas cleavage of β_2GPI at Lys^{317}-Lys^{318} by FXIa eliminates this inhibitory effect (Shi et al., 2005). Because β_2GPI is able to interact directly with FXI and FXIa, and FXIa is able to cleave β_2GPI, further studies are required to determine the exact mechanisms leading to the differential effects of dimeric β_2GPI on platelet binding to FXI and FXIa (Shi et al., 2005; Shi et al., 2004). Perhaps the platelet receptor ApoER2 plays a role in mediating the interaction of β_2GPI with FXI on the surface of activated platelets and subsequent FXIa-mediated proteolytic cleavage of β_2GPI. It remains to be determined whether deregulation of this process by autoantibodies to β_2GPI may be an important mechanism for thrombosis in patients with APS.

While the universal LDL-receptor ligand-binding inhibitor RAP abolished platelet adhesion to FXI, its presence significantly, but incompletely inhibited platelet adhesion to FXIa. Previous studies have shown that the catalytic domain of FXIa is involved in platelet interactions (Miller et al., 2007). In this study, the remaining platelet adhesion to FXIa was eliminated with the addition of the serine protease inhibitor, PPACK, in combination with RAP. A parallel set of observations was made in the presence of HK/Zn^{2+}. Taken together, these data point to a possible role for the catalytic domain in platelet-FXIa interactions, suggesting that a distinct, RAP-insensitive, site exists on the platelet surface that is capable of interacting with FXIa. One such possibility is that FXIa is capable of cleaving PAR receptors. Perhaps FXIa, once bound to the platelet surface via

ApoER2, contributes to platelet activation via proteolytic cleavage of platelet PAR receptors. It remains to be determined if FXI binding to monocytes and endothelial cells, which express ApoER2 (Berny et al., 2008; Yang et al., 2009), is mediated by ApoER2, and whether FXIa ligation of ApoER2 promotes a Reelin-like signaling pathway in these cells.

Recent findings by Tucker, et al. support the hypothesis that FXI activity is necessary for thrombus stability, as inhibition of FXI led to instability and dissolution of platelet-rich thrombi in a nonhuman primate thrombosis model (Tucker et al., 2009). FXIa bound to activated platelets has been shown to be resistant to inhibition by exogenous protease nexin 2 (Baird and Walsh, 2002b). It could be that ApoER2', in concert with GPIbα, plays a critical role in localizing FXI/FXIa to the growing platelet plug, subsequently facilitating FXIa activation of FIX, accelerating thrombin formation and thus promoting thrombus stability. Future work will be focused on identifying the significance of ApoER2 in FXI-dependent thrombus formation.

4.7 Conclusion

This study is the first to describe that FXI binding to platelets is mediated by the GPIbα-ApoER2 axis. FXI binding to the GPIbα subunit of GPIb/IX/V has been established (Baglia et al., 2004a; Baglia et al., 2004b; Ho et al., 2000); however the number of GPIbα copies on the platelet surface far outnumbers the number of measured FXI binding sites (Bergmeier et al., 2008; Greengard et al., 1986). ApoER2 exists in a complex with GPIbα on the platelet surface (Pennings et al., 2007b) and is expressed at far lower levels than the GPIb/IX/V complex. Our results may reconcile the discrepancy between the number of FXI binding sites on the platelet surface with the expression levels of the previously-identified FXI receptor, GPIbα.

Chapter V: Protein C supports platelet binding and activation under flow: role of glycoprotein Ib and apolipoprotein E receptor 2

5.1 Abstract

Activated protein C (APC) regulates thrombin generation and inhibits apoptosis. Endothelial protein C receptor (EPCR)-bound protein C is activated by thrombomodulin-bound thrombin. APC inactivates activated coagulation factors V and VIII (FVa and FVIIIa, respectively) and generates cytoprotective signaling downstream of protease activated receptor-1 (PAR-1). Binding of APC to EPCR both modifies and induces PAR-1 signaling, but it is unknown if protein C interacts with cells in an alternative manner. The aim of this study was to determine whether platelets possess receptors for protein C that can generate intracellular signals. Immobilized protein C or APC supported platelet adhesion, lamellipodia formation and elevation of intracellular Ca^{2+}. Adhesion of platelets to protein C or APC was inhibited by soluble recombinant apolipoprotein E receptor 2' (ApoER2') and by receptor-associated protein (RAP), an inhibitor of the low-density lipoprotein receptor family. Under shear, surface-bound protein C supported platelet adhesion and aggregation in a glycoprotein (GP)Ibα-dependent manner, and adhesion of platelets to immobilized protein C was abrogated by the addition of soluble forms of ApoER2' or RAP. APC bound to purified recombinant ApoER2' or GPIbα. Data presented here demonstrate that activation of platelets with rapid intracellular signaling caused by binding to immobilized protein C or APC occurs via mechanisms that require ApoER2 and

GPIbα and that APC directly binds to purified ectodomains of the receptors ApoER2 and GPIbα. These findings imply that protein C and APC may directly promote cell signaling in other cells by binding to ApoER2 and/or GPIbα.

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5.2 Introduction

When bound to the site of vascular injury, activated platelets secrete molecules that accelerate coagulation and promote further platelet activation and cohesion. Additionally, Chapter IV identified a platelet receptor capable of mediating factor XI (FXI) interactions in the absence and presence of shear. This receptor-ligand interaction could conceivably play a role in the activation of or activity of FXI, which is thought to promote sustained thrombin generation at the site of injury. The studies presented in Chapter V discuss another possible function of platelets as regulators of blood coagulation. The anti-coagulant serine protease, activated protein C (APC), was shown to bind to the platelet surface more than 20 years ago (Harris and Esmon, 1985). Furthermore, platelet-bound APC was nearly 8000 times more efficient at inactivating activated factor V (FVa), and thus pro-coagulant prothrombinase complex (Harris and Esmon, 1985). Data presented here identify two platelet receptors that interact with both APC and its zymogen, protein C.

5.3 Background

Hemostasis requires platelet adhesion, rapid cellular activation, and the subsequent accumulation of fibrin and additional platelets into a growing hemostatic plug (Watson et al., 2005). Concomitant with platelet recruitment and activation, coagulation is initiated by exposure of blood to tissue factor (Mackman

et al., 2007; Renne et al., 2006). This process leads to the sequential conversion of other coagulation factors into their corresponding active serine proteases, culminating with the local generation of thrombin. Thrombin mediates the feedback activation of the cofactors, factor V (FV) and VIII (FVIII), which results in additional thrombin formation, leading to fibrin production and clot formation (Adams and Huntington, 2006). Excessive thrombin generation and thrombosis are regulated by negative feedback in the coagulation cascade, in which thrombin proteolytically activates protein C (Esmon and Owen, 2004). Activated protein C (APC), together with its anticoagulant cofactor, protein S, degrades FVa and FVIIIa, which are required to sustain thrombin formation via the coagulation cascade. Protein C activation is slowly catalyzed by thrombin in solution, but the binding of thrombin to its cofactor, thrombomodulin, increases more than a thousand-fold the rate of this reaction (Esmon and Owen, 2004). Activation of protein C is further enhanced (~5-fold) by its binding to the endothelial cell protein C receptor (EPCR) (Esmon, 2004). Importantly, in addition to its anticoagulant function, APC can activate endothelial and other cells via outside-in signaling, leading to the activation of intracellular G-proteins and the generation of multiple protective cellular responses (anti-apoptotic, anti-inflammatory, etc.) (Mosnier et al., 2007). Bovine platelets exhibit protein S-dependent binding of bovine APC (Harris and Esmon, 1985). However, it is currently unknown if human platelets possess receptors for protein C or APC or whether protein C or APC can activate platelets, so we undertook the studies reported here of interactions between human platelets and ligands, protein C and APC.

Genome-wide linkage scans of 428 nuclear families have identified a significant genetic susceptibility locus for premature myocardial infarction and artery disease involving a nonconservative substitution, R952Q, in the LRP8 gene encoding for the LRP8 protein, also known as apolipoprotein E receptor 2 (ApoER2) (Shen et al., 2007). As a member of the low-density lipoprotein receptor family (LDLR), ApoER2 can regulate intracellular signaling cascades in

brain and endothelial cells, and three alternatively spliced forms of ApoER2 are the only members of the LDLR family known to be expressed on platelets (Riddell et al., 1999; Stolt and Bock, 2006). ApoER2 and GPIbα mediate platelet binding to a dimeric form of beta2-gylcoprotein I (β_2GPI), a major pathological antigen implicated in antiphospholipid syndrome that is characterized by the presence of arterial and/or venous thrombotic complications (Arnout and Vermylen, 2003; Pennings et al., 2007b). It has been proposed that, analogous to the activation of platelets by thrombin, GPIbα serves as a docking site for dimeric β_2GPI and that binding of the pathological ligand β_2GPI to ApoER2 leads to subsequent signaling. These findings and the general nature of LRP family members to bind multiple different ligands suggest that there may be multiple endogenous ligands for platelet ApoER2 and for GPIbα, and data reported here indicate that protein C and APC are two such ligands.

5.4 Materials and Methods

5.4.1 Reagents

The anti-GPIbα monoclonal antibody (mAb), SZ2, was purchased from Beckman Coulter (Fullerton, CA). The anti-GPIbα mAb, 6D1, was a generous gift from Barry Coller (Rockefeller University, NY). Prostaglandin I_2 was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA), and PP2 and PPACK were from Calbiochem, Inc. (La Jolla, CA, USA). Recombinant S360A-protein C was expressed, purified, and characterized as described (Gale et al., 1997). Recombinant human soluble GPIbα was expressed and purified as described (Huizinga et al., 2002). Plasma-derived wild-type and γ-carboxyglutamic acid-domainless human protein C and APC, as well as fibrinogen were purchased from Enzyme Research Laboratories (South Bend, IN). ApoER2' and LDL-binding domains 1 and 2 of ApoER2' and RAP were expressed and purified as described (Pennings et al., 2007a). All other reagents were purchased from Sigma Aldrich.

5.4.2 Static adhesion assays

Coverslips were incubated with a suspension of 50 μg/mL protein C, APC, thrombin, or fibrinogen for 1 h at RT. Surfaces were then blocked with denatured BSA (5 mg/mL) for 1 hr at RT followed by washing with PBS before use in spreading assays. Quiescent platelets failed to bind to surfaces coated with denatured BSA (Table 5.1).

For spreading experiments, washed platelets (2 x 10^7/mL) were incubated on protein-coated coverslips at 37°C for 45 min. Subsequently, coverslips were gently washed with modified Tyrode buffer to remove unbound cells. Platelet adhesion and spreading were imaged using Köhler illuminated Nomarski differential interference (DIC) contrast optics with a Zeiss 63× oil immersion 1.40 NA plan-apochromat lens on a Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) (McCarty et al., 2006). Time-lapse events were captured by a Zeiss AxioCam using Slidebook 4.0 (Intelligent Imaging Innovations, Inc., Denver, CO). For immunofluorescence microscopy, adherent platelets were fixed for 10 min with 4% paraformaldehyde, permeabilized using 0.1% Triton-X 100, incubated overnight with FITC-conjugated phalloidin, and imaged with a Zeiss Axiovert inverted fluorescent microscope.

5.4.3 Single platelet Ca^{2+} measurement

Washed human platelets were loaded with the Ca^{2+} sensitive dye Oregon Green BAPTA 1-AM (15 μM) for 30 min at 30°C. Loaded platelets were washed and allowed to sediment for a period of 30 min at 37°C in the presence or absence of calcium chelator, BAPTA-AM (40 μM). Fluorescence changes in single platelets were measured as described (McCarty et al., 2004b).

5.4.4 Flow adhesion studies

Human blood was drawn into PPACK (40 μM) or sodium citrate (0.38% wt/vol final). 32 mm glass coverslips were coated as described above before being mounted onto a parallel plate flow chamber (Glycotech, Gaithersburg, MD) on the stage of a Zeiss Axiovert microscope (McCarty et al., 2004a). Blood samples were then perfused through the chamber for 3 min at a wall shear rate of 300 s^{-1}. Platelet interactions were imaged in real-time with a Zeiss AxioCam.

5.4.5 Solid phase binding assay

96 well Maxisorp plates (Nunc, Rochester, NY) were coated with soluble ApoER2' or GpIbα overnight at 10°C. Wells were washed and blocked with Seablock blocking reagent (Pierce, Rockford, IL). Dilutions of recombinant APC were added to the wells and incubated for 2 hr at RT. Following washing, HRP-labeled-anti-protein C antibody was added to the plates and incubated for 1 hr at RT. After washing, the assay was developed by addition of OPD substrate (Sigma) and changes in absorbance at 450 nm were measured for 10 min using an Optimax reader (Molecular Devices, Sunnyvale, CA). The apparent Kd value was obtained from fitting the binding data using Prism 4 software (GraphPad Software, Inc., San Diego, CA).

5.4.6 Data analysis

Images were quantitated by determining the number of pixels within each outline using a Java plug-in for ImageJ software as previously described (McCarty et al., 2004b). Statistical significance of differences between the means was determined by analysis of variance, while multiple comparisons were performed by the Tukey test.

5.5 Results

5.5.1 Evaluation of platelet binding to protein C

To determine the ability of platelets to bind protein C and APC and the functional consequences of such binding, washed human platelets were incubated on surfaces of immobilized protein C and APC, and platelet responses were monitored in real-time with Normarski differential interference contrast (DIC) microscopy. Platelets adhered to and formed lamellipodia on both protein C and APC in less than 10 min (Fig. 5.1A). Fluorescent labeling of the actin cytoskeleton revealed that stress fibers were formed in fully spread platelets on all surfaces (Fig. 5.1B). Minimal platelet adhesion was observed on BSA surfaces (Table 5.1).

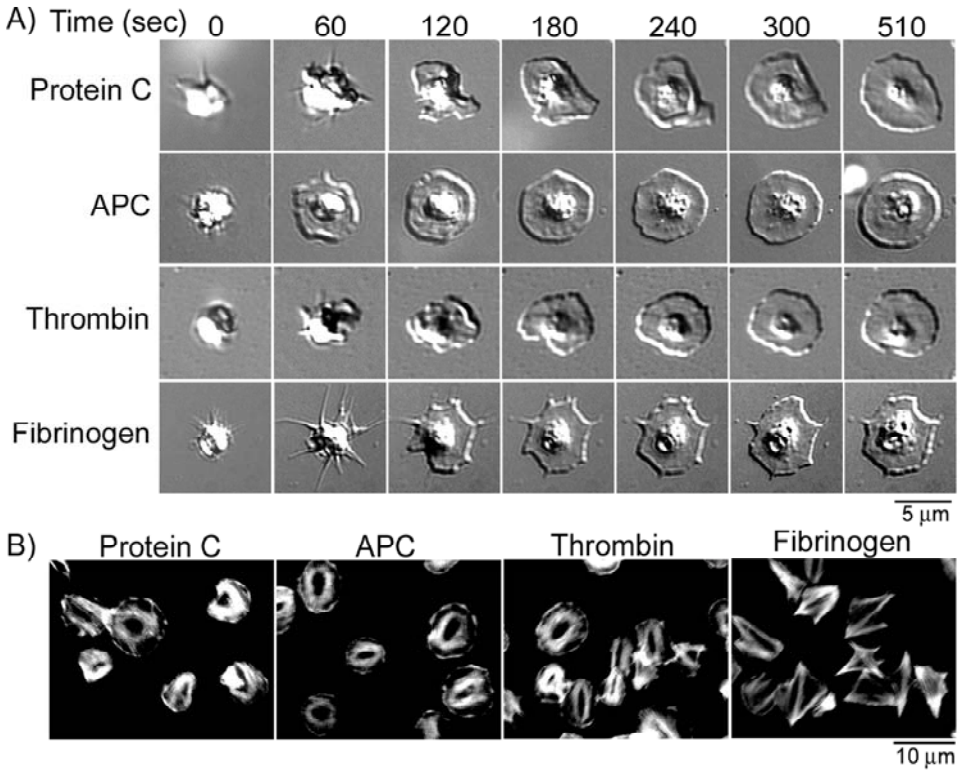

Figure 5.1 Real time imaging of platelet spreading and activation. Purified human platelets (2×10^7/mL) were exposed to surfaces coated with protein C, APC, thrombin or fibrinogen at 37°C and observed in real time using DIC microscopy. **(A)** A representative time course of a single platelet spreading on each surface is shown. **(B)** Adherent platelets were fixed, permeabilized and stained for F-actin using FITC-conjugated phalloidin. Results representative of 3-5 experiments.

Table 5.1 Platelet binding to immobilized protein C and APC.

Surface	Treatment	Platelet adhesion (cells $mm^{-2} \times 10^{-2}$)
BSA	–	4.5 ± 0.3
Protein C	–	50.1 ± 4.6
Protein C	Protein C	3.8 ± 1.8 *
Protein C	apy/indo	11.2 ± 2.0 *
Protein C	RAP	5.7 ± 4.0 *
Protein C	sApoER2'	9.1 ± 1.9 *
Protein C	sApoER2'-BD1	12.4 ± 2.3 *
Protein C	sApoER2'-BD2	22.1 ± 1.0 *
Protein C	$\alpha_{IIb}\beta_3$ antagonist	32.8 ± 5.2
Protein C	sGPIb	46.9 ± 3.5
Protein C	anti-GPIbα mAb SZ2	45.1 ± 3.8
Protein C	anti-GPIbα mAb 6D1	47.9 ± 4.2
APC	–	53.4 ± 5.0
APC	RAP	2.9 ± 0.9 *
APC	sApoER2'	8.2 ± 2.2 *
APC	sApoER2'-BD1	10.3 ± 2.5 *
APC	sApoER2'-BD2	23.9 ± 5.7 *

Purified platelets were pipetted onto BSA, protein C or APC-coated coverslips for 45 min at 37°C. In designated experiments, washed platelets were resuspended in buffer containing exogenously added 20 μg/mL protein C, apyrase and indomethacin (2 U/mL apy; 10 μM indo), RAP (40 μg/mL), 50 μg/mL sApoER2', sApoER2'-BD1 or sApoER2'-BD2, the $\alpha_{IIb}\beta_3$ antagonist epitifibatide (20 μg/mL), sGPIb (50 μg/mL), or the anti-GPIbα mAb SZ2 or 6D1 (20 μg/mL). Adherent platelets are reported as mean ± SEM of 3-10 experiments. * $P < 0.05$ with respect to untreated samples on each respective surface.

5.5.2 Role of Ca^{2+} mobilization on platelet spreading on protein C and APC

To evaluate the ability of surface-associated protein C to induce platelet activation, we examined intracellular Ca^{2+} flux of adherent platelets. Platelets were loaded with the Ca^{2+}-sensitive dye, Oregon Green BAPTA 1-AM, before being pipetted onto protein C or APC and imaged in real-time with fluorescence microscopy. Following a delay of up to 120 s, platelet adhesion to protein C or APC generated a rapid and sustained Ca^{2+} spike, which subsequently declined over a period of 3-10 min (Fig. 5.2). Minor oscillations were observed during the sustained elevation of Ca^{2+}. A distinct pattern of intracellular Ca^{2+} was observed in platelets on thrombin or fibrinogen. An initial, rapid elevation in intracellular Ca^{2+}, followed by a declining phase of Ca^{2+} levels was observed in platelets on thrombin, while a series of rhythmic spikes of Ca^{2+} was observed in platelets on

fibrinogen (Fig. 5.2). The presence of the intracellular Ca^{2+} chelator, BAPTA-AM, abrogated intracellular Ca^{2+} elevations in platelets on all surfaces (Fig. 5.2).

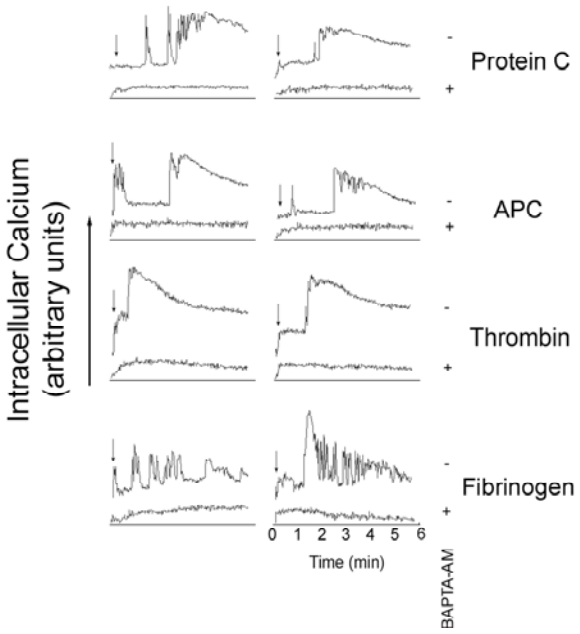

Figure 5.2 Intracellular Ca^{2+} mobilization in platelets adherent on protein C and APC surfaces. Washed human platelets loaded with the Ca^{2+}-sensitive dye Oregon Green BAPTA 1-AM were imaged as they made contact with each surface. In select experiments, platelets were incubated with the intracellular Ca^{2+} chelator, BAPTA-AM. The Ca^{2+} traces were analyzed using Matlab software. The graph shows the platelets arriving at the region of interest as denoted by an arrowhead, followed by subsequent Ca^{2+} fluctuations that are shown. The scale is in arbitrary units of fluorescence emission. Traces representative of 3-6 experiments.

5.5.3 ApoER2 regulates platelet spreading on protein C and APC

Inhibitors and blocking antibodies were utilized to investigate the molecular mechanisms governing platelet binding to protein C. Addition of the ADP-scavenger apyrase and cyclooxygenase inhibitor indomethacin resulted in a >60% reduction in platelet adhesion on immobilized protein C and APC (Fig. 5.3 and Table 5.1), as well as a substantial reduction in the degree of platelet spreading (platelet surface area = 30.2 ± 1.1 μm^2 vs. 16.2 ± 0.9 μm^2 in the absence and presence of apyrase/indomethacin; mean±SEM of 300 cells, n=6).

In contrast, apyrase and indomethacin had no effect on platelet adhesion or spreading on thrombin or fibrinogen. The addition of protein C in solution abrogated the ability of platelets to bind to immobilized protein C surfaces (Table 5.1). These competitive inhibition results suggest that a saturable protein C receptor(s) exists on the platelet surface.

To test whether ApoER2 was involved in platelet adhesion to protein C, adhesion assays were repeated in the presence of receptor-associated protein (RAP), which is an inhibitor of ligand binding to members of the LDLR family, or soluble (s) ApoER2'. Addition of either RAP or sApoER2' eliminated platelet adhesion to protein C and APC (Fig. 5.3 and Table 5.1). Moreover, the soluble form of ApoER2 containing only the LDL-binding domain 1 (sApoER2'-BD1) significantly reduced platelet binding to protein C, while the soluble form of ApoER2 containing only the LDL-binding domain 2 (sApoER2'-BD2) reduced platelet binding to protein C by over 50% (Table 5.1). Equivalent results were observed on immobilized APC (Table 5.1). Platelet lamellipodia formation, but not adhesion, on immobilized protein C, APC or thrombin was abrogated in the presence of the αIIbβ3 receptor inhibitor peptide, eptifibatide (Fig. 5.3 and Table 5.1). The presence of either soluble GPIbα or a blocking mAb against GPIb did not affect platelet adhesion to immobilized protein C under static conditions (Table 5.1). Platelets treated with the Src kinase inhibitor, PP2, failed to form full lamellipodia on immobilized protein C, APC and fibrinogen, while platelets retained full lamellipodia formation on thrombin (Fig. 5.3). Platelets treated with the intracellular Ca^{2+} chelator, BAPTA-AM (Fig. 5.3), were able to form filopodia but lacked the ability to form lamellipodia on all surfaces.

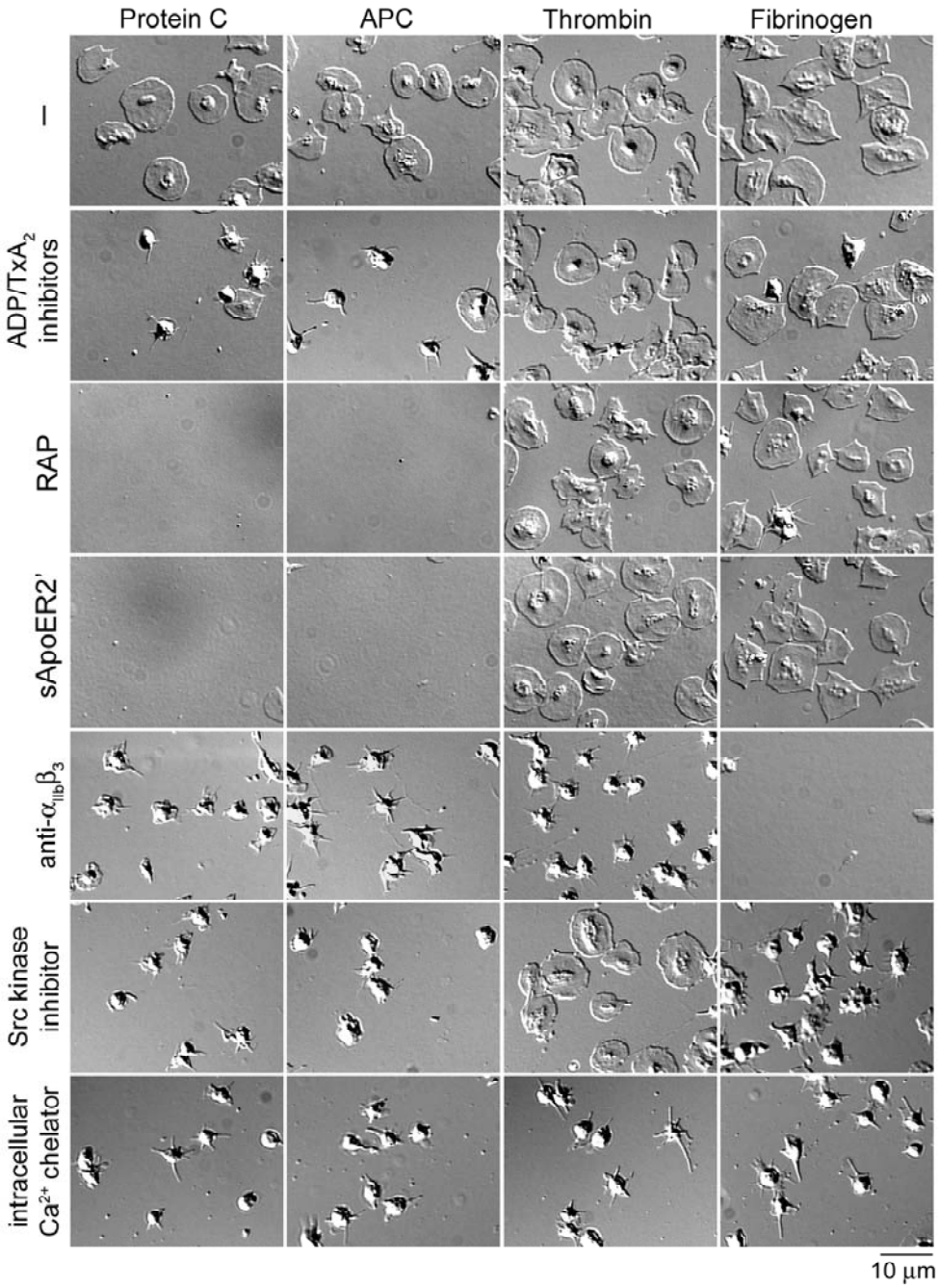

Figure 5.3 Effects of inhibitors, receptor blockade and competition on platelet adhesion and spreading in response to surface-immobilized protein C. Purified human platelets (2×10^7/mL) were gently pipetted onto surfaces of immobilized protein C, APC, thrombin or fibrinogen, incubated for 45 min at 37°C and imaged using DIC microscopy. In selected experiments, platelets were pretreated with the following: 2 U/mL apyrase and 10 μM indomethacin (ADP/TxA_2 inhibitors); RAP (40 μg/mL); soluble ApoER2' (50 μg/mL); integrin $\alpha_{IIb}\beta_3$ antagonist eptifibatide (anti $\alpha_{IIb}\beta_3$,20 μg/mL); the Src-kinase inhibitor PP2 (20 μM); the intracellular calcium chelator BAPTA-AM (10 μM). Images representative of 3-10 experiments.

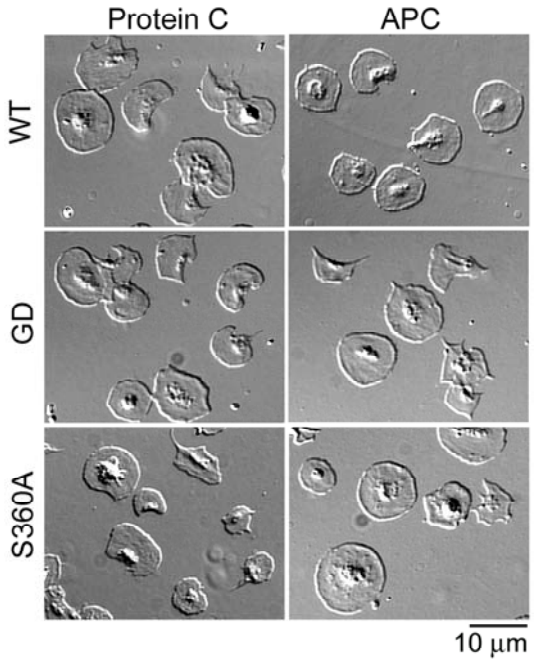

Figure 5.4 Role of key regions of protein C and APC on platelet adhesion and spreading. Purified human platelets (2×10^7 /mL) were incubated for 45 min at 37°C on surfaces of immobilized protein C or APC, each with the following mutations: native (WT), absence of the γ-carboxylated glutamic acid region (GD) or active site mutation serine to alanine (S360A).

5.5.4 Activation of platelets on immobilized protein C or APC variants

To investigate the role that the various regions of protein C or APC play in supporting platelet interactions, purified human platelets were exposed to surfaces containing wild-type (WT) or variant forms of protein C or APC. Platelets were able to adhere to and form lamellipodia on surfaces containing S360A-protein C or S360A-APC that have an active site serine to alanine mutation (Fig. 5.4). Moreover, platelets both adhered to and exhibited full lamellipodia formation on γ-carboxyglutamic acid-domainless protein C or APC, which lacks the N-terminal γ-carboxyglutamic acid domain which is required for binding to negatively-charged phospholipid surfaces.

5.5.5 Evaluation of protein C-platelet interactions under flow

In an attempt to identify the molecular mechanisms of platelet-protein C binding under flow, human platelets were reconstituted with purified red blood cells and perfused over immobilized surfaces of protein C, APC, thrombin, or fibrinogen at a shear rate of 300 s^{-1}. Platelets were recruited to and formed aggregates on protein C, APC, thrombin and fibrinogen (Fig. 5.5). Platelet aggregates on immobilized protein C and on APC consisted of 3-15 platelets that were loosely clustered and could be resolved as individual cells. In contrast, platelet aggregates on thrombin appeared as tightly clumped conglomerates, while a confluent layer of spread platelets formed on fibrinogen, which supported binding of a single layer of individual, non-spread platelets (Fig. 5.5).

Addition of an anti-GPIbα mAb eliminated platelet adhesion on protein C and APC under flow (Fig. 5.5). Presence of the anti-GPIbα mAb, SZ2, reduced platelet adhesion to immobilized thrombin under flow, whereas in controls, SZ2 did not block the ability of platelets to form a confluent layer on fibrinogen (Fig. 5.5). The presence of the $\alpha_{IIb}\beta_3$ antagonist, eptifibatide, eliminated platelet aggregate formation, but not primary single-platelet adhesion, on protein C, APC, or thrombin. In contrast, the presence of eptifibatide abrogated platelet adhesion to fibrinogen (Fig. 5.5). Platelet adhesion and aggregation were altogether eliminated on immobilized protein C or APC in the presence of either RAP or the soluble form of ApoER2 containing only the LDL-binding domain 1 (sApoER2'-BD1), or the ADP-scavenger apyrase and cyclooxygenase inhibitor indomethacin. In contrast, addition of apyrase and indomethacin did not affect the degree of platelet aggregation on thrombin or the formation of a confluent platelet layer on fibrinogen; however, the number of secondary layer adherent platelets on fibrinogen was reduced (Fig. 5.5).

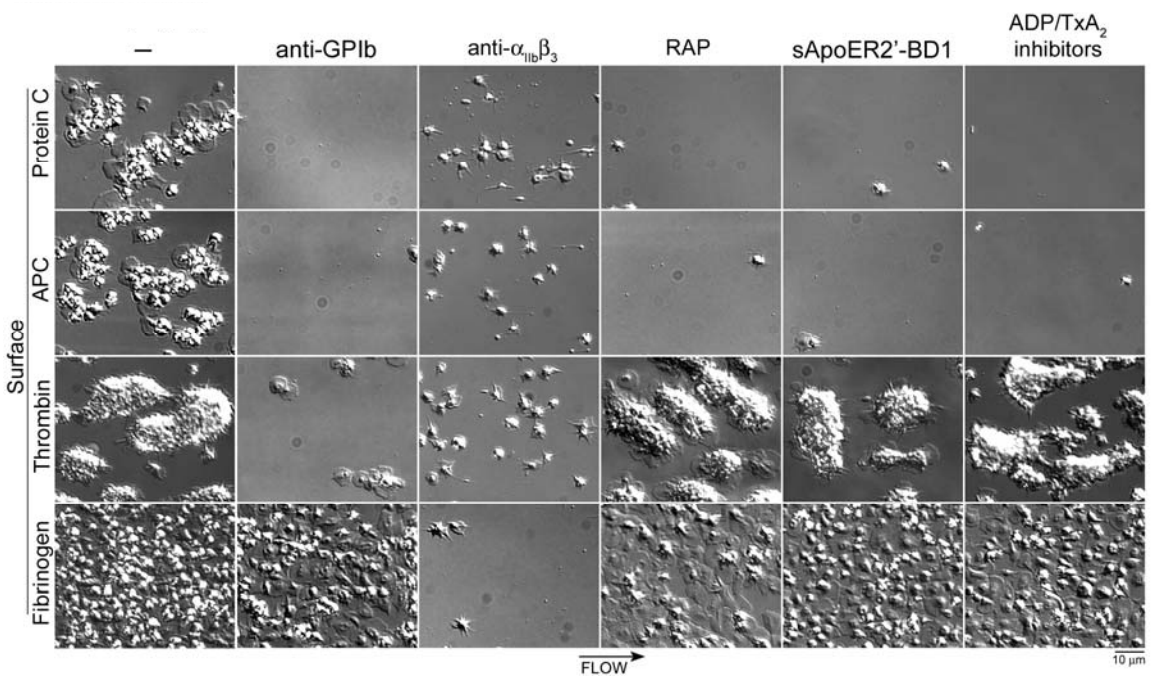

Figure 5.5 Platelet adhesion to protein C and APC under flow. Reconstituted blood (3×10^8 platelets/mL) was perfused at 300 s^{-1} over surfaces of immobilized protein C, APC, thrombin, or fibrinogen for 3 min followed by modified Tyrode buffer for 3 min to remove nonadherent cells. In separate experiments, the reconstituted blood was pre-treated with either the anti-GPIbα mAb, SZ2 (20 μg/mL), $\alpha_{IIb}\beta_3$ antagonist eptifibatide (20 μg/mL), RAP (40 μg/mL), sApoER2'-BD1 (50 μg/mL), or 2 U/mL apyrase and 10 μM indomethacin (ADP/TxA_2 inhibitors). Images representative of 3-8 experiments.

To evaluate the ability of protein C and APC to support platelet interactions in whole blood, we perfused PPACK-anticoagulated whole blood over immobilized protein C and APC and monitored platelet interactions under flow conditions using real-time fluorescence microscopy. Immobilized protein C and APC supported platelet tethering and rolling at a shear rate of 300 s^{-1} (Table 5.2). Presence of the anti-GPIbα mAb, SZ2, reduced platelet tethering and rolling on protein C and APC to below background levels. A decreasing trend in the number of tethering platelets was observed in the presence of the anti-GPIbα mAb, 6D1, however this trend was not statistically significant (Table 5.2).

Table 5.2 Interactions between protein C and platelets under flow.

Surface	Treatment	Platelet adhesion (cells mm^{-2} min^{-1} × 10^{-2})
BSA	–	20.5 ± 6.4
Protein C	–	56.5 ± 10.9
Protein C	SZ2	19.3 ± 3.7*
Protein C	6D1	39.3 ± 4.9
APC	–	72.4 ± 12.1
APC	SZ2	18.2 ± 3.0*
APC	6D1	43.8 ± 5.4

PPACK-anticoagulated whole blood was perfused over surfaces containing immobilized protein C or APC. In designated experiments, platelets in whole blood were pre-treated with either the anti-GPIbα mAb SZ2 or 6D1 (20 μg/mL). Interacting platelets (those that either tether to or roll along the surface) are reported as mean ± SEM of 3-6 experiments. * $P < 0.05$ with respect to untreated platelets on the corresponding surface.

5.5.6 Evaluation of activated protein C binding to ApoER2 and GPIbα

To demonstrate direct binding of APC to GPIbα and ApoER2, APC was incubated in a microtiter plate over immobilized purified recombinant sApoER2' or sGPIbα ectodomains and the level of bound APC was determined using anti-protein C antibodies. Data in Fig. 5.6 demonstrate that APC specifically bound to immobilized sApoER2' and sGPIbα with an affinity of 221 nM and 668 nM, respectively. The presence of RAP or the anti-GPIbα mAb, SZ2, significantly reduced APC binding to sApoER2' and sGPIbα, respectively, demonstrating specificity for ApoER2 and GPIbα. Thus, our data suggest that ApoER2 and GPIbα on the platelet surface are receptors for APC and that binding to these receptors mediates cell signaling.

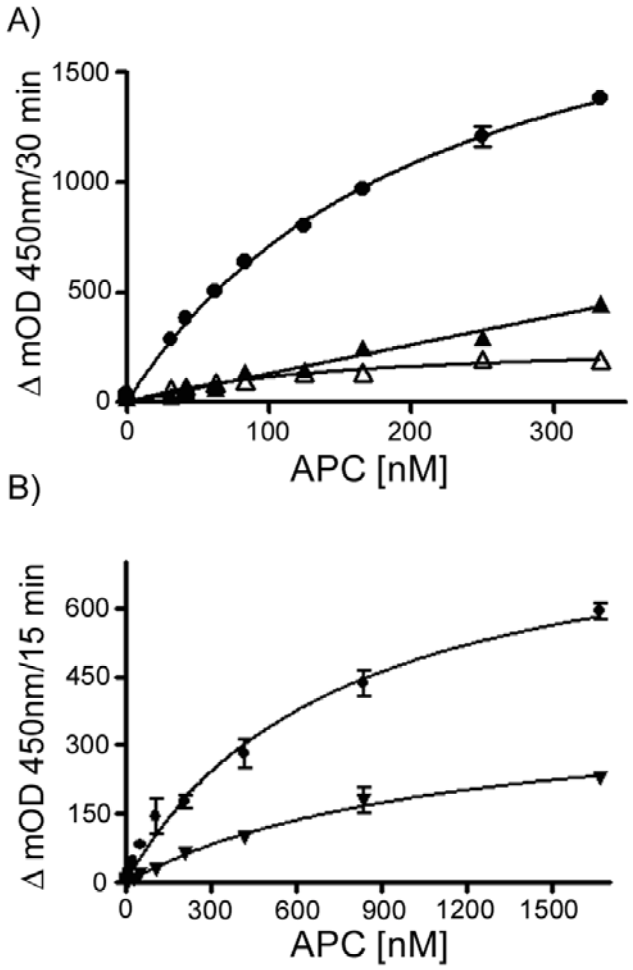

Figure 5.6 APC binding to purified ApoER2 and GPIbα. Recombinant APC at indicated concentrations was incubated over **(A)** surface-bound sApoER2 in the presence vehicle (closed circles), 1 μM RAP (solid triangles) or 2 μM RAP (open triangles) or over **(B)** surface-bound sGPIbα. Bound APC was quantified by anti-protein C antibodies as described in Section 5.3.5. *These results were generated by José A. Fernández in the laboratory of John Griffin at The Scripps Research Institute.

5.6 Discussion

Recent studies have highlighted the pleiotropic direct effects of protein C on various cells, including changes in gene expression profiles, and anti-inflammatory, cytoprotective, and anti-apoptotic activities (Mosnier et al., 2007). The current study characterizes the ability of immobilized protein C and APC to interact directly with platelets, facilitating platelet activation under shear

conditions, and points toward specific roles for two receptors, apoER2 and GPIbα, in these phenomena.

Platelet ApoER2 is identified as a receptor for protein C/APC, and the LDL-binding domain 1 of ApoER2 appears to play a role in protein C binding to platelets, as evidenced by the ability of the soluble form of ApoER2'-BD1 to block platelet binding to immobilized protein C and APC under both static and shear flow conditions. The putative interactions of protein C with ApoER2 resulted not only in the adhesion of platelets but also in the activation of platelets as platelet spreading was observed on immobilized protein C. Platelet spreading on protein C was abrogated in the presence of a Src kinase inhibitor, congruent with the fact that Src family kinases play a critical role in regulating Reelin-dependent neuron activation downstream of the integrin-like intracellular NPxY motif of ApoER2 (May et al., 2005).

The multipotent platelet receptor subunit GPIbα plays a key role in orchestrating platelet interactions with thrombin, highlighting a major role in hemostasis and arterial thrombosis for GPIbα beyond serving as a VWF-receptor (Bergmeier et al., 2006). The present study would seem to extend the ligands for GPIbα to include protein C and APC. We show that platelet binding to immobilized protein C/APC may require the anionic sulfated tyrosine region of GPIbα, as the monoclonal antibody, SZ2, which binds to this region (Shen et al., 2000) and inhibits thrombin binding to GPIbα (Berny et al., 2008), eliminated platelet rolling on and adhesion to immobilized protein C and APC under shear flow conditions. Additionally, SZ2 significantly reduced APC binding to immobilized-sGPIbα in a solid-phase binding assay. The anti-GPIbα mAb, 6D1, which recognizes the fourth leucine-rich repeat (Leu^{105}-Glu^{128}) of GPIbα which is essential for VWF binding (Shen et al., 2000), did not significantly inhibit platelet rolling on protein C or APC under flow conditions. However, as a trend of inhibition was observed in the presence of 6D1, further investigation is warranted to map the binding of protein C to GPIbα.

As formation of stable hemostatic plugs requires coordinated and sustained platelet activation (Cosemans et al., 2006), perhaps protein C plays a role in hemostasis by contributing to platelet activation. This would be analogous to the role that thrombin plays following assembly of the prothrombinase complex on the platelet surface. Independent of thrombin's procoagulant role of fibrin generation and anticoagulant role in the generation of APC (Heemskerk et al., 2005), thrombin contributes to hemostasis by activating platelets via outside-in signaling. The ability of platelets to bind protein C leads us to hypothesize that a protein C or APC complex with ApoER2 and GPIbα forms on the platelet surface during hemostasis and that this complex may play a dual role by initially stimulating platelet activation via ApoER2 to help achieve hemostasis and subsequently limiting thrombus growth by localizing the anticoagulant actions of the protein C system to the platelet aggregate surface.

ApoER2 can assemble into a complex with GPIbα on the platelet membrane (Pennings et al., 2007b), which may play a critical role in mediating platelet activation following binding of β_2GPI, a highly relevant antigen in antiphospholipid antibody syndrome (Pennings et al., 2007a; Pennings et al., 2007b). Antibody-induced dimerization of β_2GPI results in the activation of platelets, as well as several other cell types, such as monocytes (Bohgaki et al., 2004) and endothelial cells (Raschi et al., 2003). Together, these three cell types participate in the thromboembolic events and obstetric complications associated with the antiphospholipid antibody syndrome (Arnout and Vermylen, 2003; Urbanus et al., 2008). Intriguingly, anti-β_2GPI antibodies can impair the anticoagulant activity of the protein C system (Galli et al., 1998), and antibodies directed toward several domains of β_2GPI share the ability to block specifically the phospholipid-dependent enhancement of APC activity (Safa et al., 2005), A history of thrombotic events in patients with antiphospholipid antibody syndrome is associated with a stronger resistance to the anticoagulant effect of APC, independent of factor V Leiden mutation (Liestol et al., 2007), and anti-β_2GPI

antibodies that seem to impair APC action on factor V were linked to recurrent miscarriages in women (Gris et al., 2000). At this time, the molecular mechanism by which anti-β_2GPI antibodies might contribute to impair APC anticoagulant actions, i.e., to cause APC resistance, is ill-defined, but this deserves attention. It is not known whether the β_2GPI-anti-β_2GPI complex could compete with APC for a membrane receptor. Dimeric β_2GPI binds in close proximity of the sulfated thrombin binding site present on GPIbα with a K_d of 180 nM and to the LDL-binding domain 1 of ApoER2 with a K_d of 22 nM (Pennings et al., 2007b). Interestingly, the affinity of dimeric β_2GPI for ApoER2 and GPIb is 4-10 fold higher than for the binding of APC to these receptors. It is unclear whether or not the receptors' binding sites for the β_2GPI-anti-β_2GPI antibody complex are the same or similar to the putative binding sites for APC; however, if this were true, we would speculate that competitive inhibition between the protein C/APC and the β_2GPI-anti-β_2GPI antibody ligands could play some role in the pathogenesis of anti-β_2GPI antibodies in antiphospholipid antibody syndrome patients.

5.7 Conclusion

Our work is the first to identify two platelet receptors, namely ApoER2 and GPIbα, for the ability of protein C/APC to bind and stimulate platelets. Further studies are needed to determine either the physiologic or pharmacologic relevance of these receptors for multiple potential activities of protein C or APC acting on platelets. Moreover, our data showing that APC directly binds to purified ectodomains of the receptors ApoER2 and GPIbα imply that protein C and APC may promote signaling in other cells (eg., endothelial cells, monocytes, immune systems cells, neurons, smooth muscle cells, etc.) that involves binding to ApoER2 and/or GPIbα.

Chapter VI: Future Work

6.1 Describe laminin contributions to coagulation independent of the intrinsic pathway

Studies described in Chapter III were designed to determine whether laminin plays a role in initiating or accelerating contact activation. While laminin was shown to accelerate clotting in a FXII- and FXI-dependent manner, these results may not fully describe the role of laminin in coagulation. Additional studies have indicated that laminin may be contributing to the activation of coagulation by an additional mechanism. The addition of an inactivated form of FVIIa (FVIIai), a competitive inhibitor to FVIIa, prolonged PPP clotting times in the presence of laminin (Fig. 6.1). Conversely, FVIIai did not affect clotting times when coagulation was triggered by the addition of FXIa (Fig. 6.1).

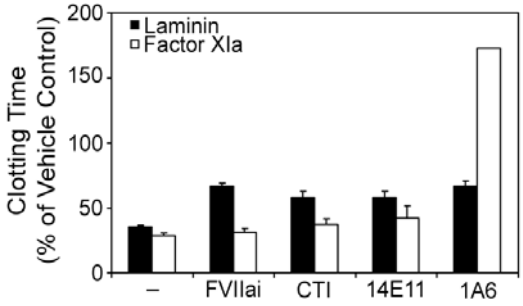

Figure 6.1 FVIIai prolongs clotting times in the presence of laminin. Human sodium citrate-anticoagulated platelet-poor plasma (PPP) was pre-treated with 20 μg/mL FVIIai, 4 μM CTI, 20 μg/mL 14E11 or 20 μg/mL 1A6. 10 μg/mL laminin (white bars) or 25 ng/mL factor XIa (black bars) was then added to the plasma. Following a 3 min incubation, coagulation was initiated by the addition of 16.6 mM $CaCl_2$, and clotting times were recorded.

Reports in the literature have shown that specific fragments of FXII are capable of activating FVII (Radcliffe et al., 1977). Thus further studies are required to determine whether the prolonged clotting times observed in the presence of FVIIai are 1) related to direct laminin interactions with members of the extrinsic pathway, or 2) a consequence of crosstalk between the intrinsic and extrinsic pathways.

6.2 Describe the molecular mechanisms mediating FXI interactions with ApoER2

We have begun work in collaboration with David Gailani, MD, at Vanderbilt University, to elucidate the binding site for ApoER2 on FXI. Due to the structural homology shared by FXI and plasma prekallikrein (PK), initial experiments utilized FXI / PK chimeras. Chimeras consisted of either 1) FXI with PK heavy chain (HC – tandem apple domains) or light chain (LC – serine protease domain) substitutions or 2) FXI or PK with a substitution for one of the four apple (A) domains from the other molecule. For instance, FXI-PKA2 is the FXI molecule whose A2 domain has been replaced with the PK A2 domain. Preliminary studies revealed that the binding capacity for resting platelets is contained within A2 of the FXI heavy chain (Fig. 6.2). Importantly, PK-FXIA2 supported similar levels of platelet adhesion as seen on native FXI, thus FXI A2 appears to be both necessary and sufficient for supporting platelet interactions in this system. These initial studies provide evidence that the binding site for platelet ApoER2 resides within the A2 domain of FXI.

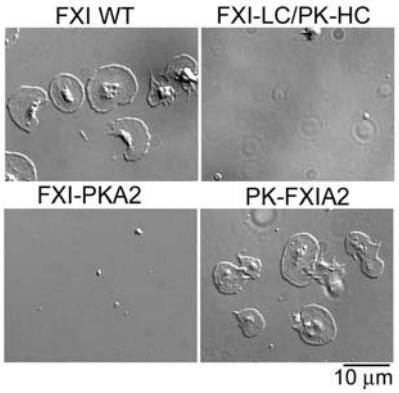

Figure 6.2 The platelet binding capacity of FXI resides in A2. Washed human platelets (2×10^7 /mL) were incubated for 45 min at 37˚C over surfaces of immobilized native FXI (WT) or FXI/PK chimeras containing domain substitutions.

In collaboration with Drs. Gailani and Jonas Emsley (University of Nottingham), further comparison of the exposed residues on the surface of the FXI A2 domain with the PK A2 domain has led to the identification of five residues in FXI that are not conserved in PK, and thus represent candidates for the ApoER2 binding site on FXI (Fig. 6.3). Site-directed mutagenesis of these residues was performed in which the residues Arg^{136}, Gln^{137}, Glu^{120}, Gln^{116}, or Lys^{165} were replaced with Ala or oppositely charged residues (Glu for Lys; Gln for Glu). As shown in Fig. 6.3, human platelets bound to recombinant (native) FXI, while the degree of platelet adhesion was reduced on each of the FXI A2 mutants, with the greatest degree of inhibition observed with mutations in FXI at residues Gln^{137} and Glu^{120}. Control experiments showed that, indeed, similar levels of each FXI mutant become immobilized onto the glass slides in these studies. It is important to note the proximity of the potential ApoER2 binding residues we identified in the FXI A2 domain to Ser^{248}, Arg^{250}, and Lys^{255}, which are components of the purported GPIbα binding site on the A3 domain of FXI (Fig. 6.3) (Baglia et al., 2004a).

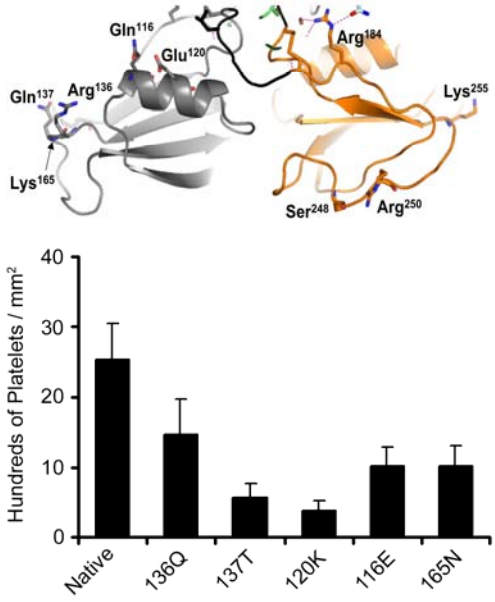

Figure 6.3 Potential binding sites for platelets within FXI A2. (Top) Shown are the α-helices and portions of the β-sheets of the A2 domain (gray) and A3 domain (orange). Positions of surface residues on FXI A2 that are not conserved in PK A2 are shown. Ser^{248}, Arg^{250}, and Lys^{255} are components of the GPIbα binding site on the A3 domain. **(Bottom)** Purified platelet binding to recombinant FXI mutants with residues Arg^{136}, Gln^{137}, Glu^{120}, Gln^{116}, or Lys^{165} that have been replaced with Ala or oppositely charged residues (Glu for Lys; Gln for Glu). Mean $\pm$ SEM; n=3-4.

Additional experiments are required to map the FXI binding region on ApoER2. Data presented in Chapter IV (Fig. 4.3) showed that LDL-binding domain 1 (BD1) inhibited platelet adhesion to FXI to a similar degree as the entire extracellular domain of ApoER2', while BD2 interfered with platelet adhesion to a lesser extent. Direct interactions between ApoER2' and FXI were initially detected in a solid phase binding assay (Fig. 4.7). In order to directly determine the domain within ApoER2' that contains the highest affinity for FXI, parallel experiments should be performed in which the different binding domains of ApoER2 (Fig. 6.4) are immobilized into the wells of a 96-well plate, and binding of soluble FXI to immobilized ApoER2 fragments is detected by a HRP-conjugated anti-FXI antibody. Once the FXI binding site within ApoER2 is determined, this purified system should be used to verify the ApoER2 binding site within FXI using the FXI/PK chimeras and FXI A2 mutants described above.

Figure 6.4 ApoER2 Variants. Schematic of the three isolated splice variants and two constructed domain deletion mutants of ApoER2. (Pennings, MT. *J Thromb Haemost*. 2007 Feb;5(2):369-77.)

6.3 Determine the role of platelet-FXI interactions in coagulation

Activated platelets express ~1500 binding sites for FXI (Baglia et al., 2004a). In contrast, activated platelets only present ~200 FXIa binding sites, which are distinct from FXI binding sites (Miller et al., 2007). While previous studies suggest that FXI binds to a subpopulation of the 25,000 copies of GPIb/IX/V on the activated platelet, the discovery of ApoER2 as a receptor for FXI on quiescent platelets described in Chapter IV could alter this view. Studies have shown that the existence of FXIa in a dimer is required for FXIa activation of FIX in the presence of platelets (Gailani et al., 2001). Furthermore, experimental evidence suggests that an intermediate of FXIa in which one monomer remains inactive while the other subunit is proteolytically active (1/2-FXIa) can exist in plasma, and could possibly be generated *in vivo* (Smith et al., 2008). During hemostasis, the 1/2-FXIa intermediate could conceivably bind the GPIbα-ApoER2 axis via its inactive monomer, while presenting the FIX binding and activation sites on its proteolytically active subunit (Smith and Gailani, 2008).

In order to determine whether the interaction of FXI with the GPIbα-ApoER2 axis contributes to FIX activation and thus sustained thrombin

generation, the recalcified-reconstituted blood flow experiments we presented in *Blood* (Tucker et al., 2009) should be repeated in which $FXI^{-/-}$ plasma is supplemented with either recombinant native FXI or FXI-PKA2. Preliminary experiments (see 6.2) suggest that the ApoER2 binding site resides in the A2 domain of FXI, and should the FXI-ApoER2 interaction contribute to FIX activation on the platelet surface, experiments performed with FXI-PKA2 would hypothetically yield less fibrin deposition than those with supplemented native FXI.

6.4 Identify the molecular mechanisms governing platelet interactions with APC

In an attempt to map the binding of APC to ApoER2, John Griffin, Ph.D., our collaborator at The Scripps Research Institute, has begun production of recombinant mutants of ApoER2. Once these mutants have been purified and characterized, solid phase assays (like those described in Fig. 4.7) should be performed to determine the binding capacity of each mutant for APC. Furthermore, the plethora of APC mutants that have been generated by the Griffin lab will be assessed for their affinity toward ApoER2.

6.5 Determine the contribution of ApoER2 and GPIbα to APC anticoagulant activity

In collaboration with Dr. Griffin, we were able to show that the binding of APC to U937 monocytic cells was mediated by ApoER2, which initiated Dab-1 phosphorylation and downstream signaling events reminiscent of those elucidated for ApoER2 signal transduction in neurons (Yang et al., 2009). Furthermore, the binding of APC to endothelial protein C receptor and ApoER2 is required for APC to blunt endotoxin-induced upregulation of tissue factor expression on the surface of these cells (Yang et al., 2009). However, the functional consequence of platelet ApoER2/GPIbα-APC interactions is unknown.

Data presented in Chapters IV and V suggest that not only does the pro-thrombotic dimeric β_2GPI interact with the same two receptors as APC, but its presence is able to disrupt platelet-APC interactions (Fig. 4.6B). Dimeric β_2GPI has been shown to mimic the *in vitro* effects of plasma β_2GPI in the presence of antiphospholipid syndrome (APS) antibodies (Lutters et al., 2001). Interestingly, patients suffering from APS exhibit what is known as acquired APC resistance, in which activated factor V is resistant to inactivation by APC. Thus, determining the significance of APC interactions with the ApoER2-GPIbα axis will likely shed light on the pathologic mechanisms of β_2GPI in APS.

Initially, the ability of immobilized platelets to recruit protein C or APC in the presence of physiological shear must be assessed. This can be accomplished by forming platelet aggregates on a surface such as fibrinogen or collagen at venous ($300s^{-1}$) to stenosed arterial shear ($3000s^{-1}$), and chasing with a solution of labeled protein C or APC in the absence or presence of Ca^{2+}. These experiments should be repeated in the presence of RAP or anti-GPIbα antibodies to determine whether protein C / APC is interacting with these receptors in the presence of flow. Further experiments in the presence of dimeric β_2GPI would assess the ability of this pathologic molecule to disrupt APC-platelet interactions.

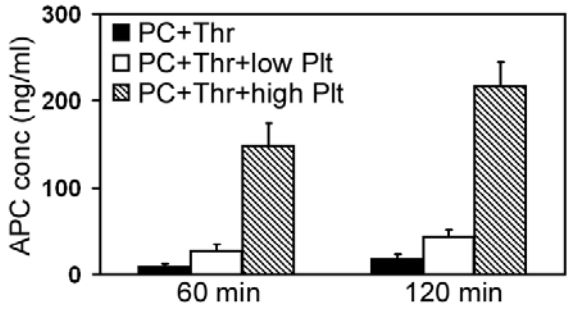

Figure 6.5 Activated Platelets Promote protein C activation. Protein C (PC, 100 nM) was incubated with α-thrombin in the presence or absence of thrombin-activated purified human platelets at 7×10^8 /mL (low plt) or 4.5×10^9 /mL (high plt), and APC was detected with a HAPC 1555 assay. APC production is reported at time points of 60 and 120 minutes. These data were produced by Erik Tucker Gruber laboratory.

In vivo, activated platelets secrete molecules that promote additional platelet recruitment and coagulation, and the activated platelet surface acts as a catalyst for thrombin generation. Conversely, preliminary studies showed that activated platelets supported the anticoagulant process of APC generation by α-thrombin (Fig. 6.5). These studies should be repeated in the presence of dimeric β_2GPI or inhibitors to ApoER2 or GPIbα in order to determine the relative roles of these two receptors in supporting APC generation. Elucidation of the ability of dimeric forms of β_2GPI to interfere with protein C activation could possibly explain the thrombotic phenotype of patients suffering from APS.

6.6 Identify the role for ApoER2 in hemostasis and thrombosis

The studies presented in this thesis have added protein C, APC, FXI and FXIa as ligands for ApoER2 (Fig. 6.6). Yet, the physiological significance of these interactions remains to be determined. The study of ApoER2 in the vasculature is a relatively young field, as platelet ApoER2 was only discovered in 1999 (Riddell et al., 1999).

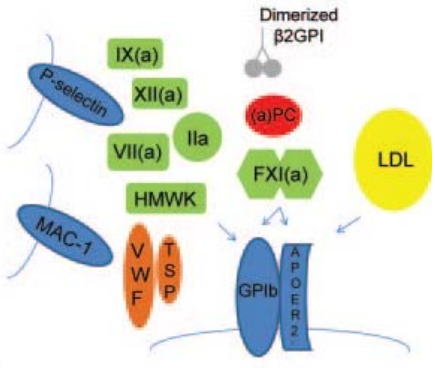

Figure 6.6 Identified ligands for platelet ApoER2 and GPIbα. The interactions of zymogen and serine protease forms of protein C and FXI with the platelet surface have been more fully described by the work presented in this thesis. (*Arterioscler Thromb Vasc Biol*. 2009;29:1409-10.) Copyright American Heart Association 2009.

Evidence suggesting that ApoER2 (LRP8) plays a role in thrombosis is demonstrated in a recent epidemiological study in which the nonconservative substitution, R952Q, in the *LRP8* gene encoding for ApoER2, presents an additive effect to the APOE ε2/ε3/ε4 genotype for risk of myocardial infarction (Martinelli et al., 2009). This polymorphism has been shown to be associated with familial and premature coronary artery disease and myocardial infarction and the potential for an increase in platelet aggregation activity, possibly through the sensitization of platelets (Shen et al., 2007). Sensitization of platelets for activation has been previously demonstrated downstream of LDL or dimeric β_2GPI binding to ApoER2 (Korporaal et al., 2004; Lutters et al., 2003).

Romay-Penabad and colleagues have demonstrated that ApoER2 mediates thrombosis and inflammation in the presence or dimeric β_2GPI or APS antibodies (Romay-Penabad et al., 2009). In an *in vivo* injury model, ApoER2 deficiency protected mice from thrombosis in the presence of APS patient antibodies (Romay-Penabad et al., 2009). Furthermore, wild-type, but not ApoER2-deficient, mice treated with the dimeric form of β_2GPI exhibited increased thrombotic and inflammatory markers such as VCAM-1 expression in the aorta and tissue factor activity in carotid artery homogenates and peritoneal macrophages (Romay-Penabad et al., 2009). While this study was based on the hypothesis that ApoER2 plays a critical prothrombotic role in the presence of antiphospholipid antibodies directed toward β_2GPI, future work should focus on determining the role of ApoER2 under native conditions.

ApoER2-deficiency does not appear to affect hemostasis in a mouse tail-bleeding model (Robertson et al., 2009). Conversely, a moderate benefit for ApoER2-deficient mice has been demonstrated in a ferric chloride injury model of thrombosis (Robertson et al., 2009). However, thrombosis in these studies was initiated with a relatively high (12.5%) concentration of $FeCl_3$, as other labs have shown that FIX- and FXI-deficiency in mice is protective in response to 5%, but not 10% $FeCl_3$ (Wang et al., 2005). A more robust role for ApoER2 in thrombosis

may be revealed by inducing vascular injury in wild-type and ApoER2$^{-/-}$ mice with $FeCl_3$ doses from 5% to 10%, or by initiating thrombus formation via other established *in vivo* injury models.

6.7 Conclusions

The work presented in this thesis has unearthed mechanisms further linking platelets and the ECM with blood coagulation. Additional characterization of these interactions will provide insight into their physiological significance in hemostatic or thrombotic processes. For example, the critical importance of platelet-ECM interactions is well-established. While laminin and collagen have been shown to contribute to the activation of the intrinsic pathway of coagulation, these mechanisms may play a supporting role in hemostasis or, due to the growing evidence that FXI and FXII, members of the intrinsic pathway, contribute to thrombus growth and stability, stimulate thrombus propagation *in vivo*.

Additionally, the mapping studies discussed in this chapter will determine whether ApoER2 is physically able to interact with more than one ligand simultaneously, or if there exists competition between APC, FXI and antibody-associated β_2GPI for ApoER2 binding under physiological or pathophysiological conditions. Our studies show that the pathologic dimeric β_2GPI was able to interfere with platelet-APC and platelet-FXIa interactions (Fig. 4.6B). Platelets, leukocytes and endothelial cells all express ApoER2. Whether the *in vivo* thrombotic effects observed in the presence of dimeric β_2GPI or APS antibodies are a result of interactions with one or all of these cell types remains to be determined. Additional studies will shed light on the source of the thrombotic complications induced by β_2GPI antibody complexes, which could be acting via ApoER2 signal transduction, interference with a regulatory process such as APC generation or activity, or some yet unidentified mechanism.

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Biographical Sketch

Tara Chantal White was born on 18 February 1983 and raised in Southwest Kansas by her parents, Greg and Denise White. She was joined by Riley Adams in marriage on 8 November 2008.

As a high school student, Tara earned All-League honors in volleyball, basketball and track, was a member of the National Honor Society and competed at the state and national levels in Future Business Leaders of America competitions. After graduating at the top of her class from Lewis High School in 2001, she attended Fort Hays State University to study physics and compete for the Tiger Track & Field team. She enjoyed the continuing challenge of balancing academics and athletics, even setting aside time weekly for tutoring and intramural sports. Tara won two Rocky Mountain Athletic Conference Championships in the javelin, was named to the All-Conference team her four years of eligibility, and garnered Academic All-American honors three times in the javelin and once in the discus. Sparked by the excitement of her research project with her undergraduate advisor, Dr. Kayvan Aflatooni, she applied to participate in two summer Research Experience for Undergraduates internships sponsored by the National Science Foundation. Through this program, Tara was exposed to exciting projects in theoretical solid state research at Auburn University and experimental optics at the Air Force Institute of Technology and presented her results in the 2004 National Conference for Undergraduate Research. Tara graduated Summa Cum Laude from the FHSU physics department in 2005 and was excited to continue her education while applying her skill set in a biological setting.

Tara joined the OHSU Biomedical Engineering department in September 2005, and during her second research rotation, found her home as the very first graduate student in the lab of Dr. Owen McCarty. She has since spent her time studying the interactions of blood platelets with plasma and extracellular matrix proteins. During her graduate tenure, Tara has been awarded the Achievement Rewards for College Scientists Scholarship, an NIH Predoctoral Fellowship in Molecular Hematology, an American Heart Association Predoctoral Fellowship, the International Society on Thrombosis and Haemostasis Young Investigator Award, the OHSU-OGI Award for Significant Achievement in Research, and was named a Vertex Scholar. She has presented her research at several national and international conferences and has summarized her work in a handful of peer-reviewed publications. A list of publications and abstracts to date follows:

Publications

1. **White TC**, Berny MA, Robinson DK, Yin H, DeGrado WF, Hanson SR, McCarty OJT, “The leech product saratin is a potent inhibitor of platelet integrin $\alpha_2\beta_1$ and von Willebrand factor binding to collagen” *FEBS Journal* 2007 Mar 15; 274(6): 1481-1491.

2. Berny MA, **White TC**, Tucker EI, Bush-Pelc LA, Di Cera E, Gruber A, McCarty OJ, "The thrombin mutant W215A/E217A acts as a platelet GPIb antagonist" *Arterioscler Thromb & Vasc Biol*. 2008 Feb; 28(2): 329-34. (with editorial; awarded 2009 Karl Link New Investigator Award in Thrombosis)

3. **White TC**, Berny MA, Tucker EI, Urbanus RT, de Groot PG, Fernandez JA, Griffin JH, Gruber A, McCarty OJT, "Protein C supports platelet binding and activation under flow: role of glycoprotein Ib and apolipoprotein receptor 2" *J Thromb Haemost* 2008 Jun;6(6):995-1002. (cover)

4. Lovely RS, Rein CM, **White TC**, Jouihan SA, Boshkov LK, Bakke AC, McCarty OJ, Farrell DH, "γA/γ' fibrinogen inhibits thrombin-induced platelet aggregation" *Thromb Haemost.* 2008 Nov;100(5):837-46.

5. Tucker EI, Marzec UM, **White TC**, Hurst S, Rugonyi S, McCarty OJT, Gailani D, Gruber A, Hanson SR, "Prevention of vascular graft occlusion and thrombus-associated thrombin generation by inhibition of factor XI" *Blood.* 2009 Jan 22;113(4):936-44.

6. Yang XV, Banerjee Y, Fernandez JA, Deguchi H, Xiao X, Mosnier L, Urbanus RT, de Groot PG, **White-Adams TC**, McCarty OJT, Griffin J, "Activated Protein C Ligation of ApoER2 (LRP8) Causes Dab1-dependent Signaling in U937 cells" *Proc Natl Acad Sci U S A.* 2009 Jan 6;106(1):274-9.

7. **White-Adams TC**, Berny MA, Tucker EI, Gertz JM, Gailani D, Urbanus RT, de Groot PG, Gruber A, McCarty OJ, "Identification of Coagulation Factor XI as a Ligand for Platelet Apolipoprotein E Receptor 2 (ApoER2)" *Arterioscler Thromb & Vasc Biol* 2009 Oct; 29:1602-1607. (with editorial)

8. Berny MA, Patel IA, Simonson P, **White-Adams TC**, Rugonyi S, McCarty OJT, "Rational design of an *ex vivo* model of thrombosis" (in press: *Cellular and Molecular Bioengineerin*g)

9. **White-Adams TC**, Tucker EI, Berny MA, Gailani D, Gruber A, McCarty OJT, "Laminin supports the initiation of coagulation and thrombus formation in the presence of flow" (in press: *J Thromb Haemost*)

Abstracts

1. **TC White**, DK Robinson, H Yin, WF DeGrado, SR Hanson, OJT McCarty, "The leech product saratin is a potent inhibitor of both VWF and integrin $\alpha_2\beta_1$ binding to collagen" *Annual Meeting of the American Society of Hematology*, Orlando, FL (Dec, 2006).

2. **TC White**, MA Berny, JA Fernandez, JH Griffin, A Gruber, OJT McCarty, "Glycoprotein Ib mediates platelet recruitment, activation and aggregation on immobilized protein C and activated protein C under shear" *XXIst Congress of the International Society on Thrombosis and Haemostasis*, Geneva, Switzerland (July, 2007).

3. **TC White**, MA Berny, DK Robinson, H Yin, WF DeGrado, SR Hanson, OJT McCarty, "The leech product saratin is a potent inhibitor of platelet integrin $\alpha_2\beta_1$ and VWF binding to collagen" *XXIst Congress of the International Society on Thrombosis and Haemostasis*, Geneva, Switzerland (July, 2007).

4. MA Berny, **TC White**, EI Tucker, E Di Cera, A Gruber, OJT McCarty, "The thrombin mutant W215A/E217A acts a platelet antagonist through inhibition of thrombin activation and GPIb binding to VWF" *XXIst Congress of the International Society on Thrombosis and Haemostasis*, Geneva, Switzerland (July, 2007).

5. OJT McCarty, **TC White**, EI Tucker, CE Swanson, DK Robinson, SA Mousa, A Gruber, "Factor XI mediates platelet lamellipodia formation and tethering and rolling under shear conditions" *XXIst Congress of the International Society on Thrombosis and Haemostasis*, Geneva, Switzerland (July, 2007).

6. **TC White**, MA Berny, DK Robinson, A Gruber, OJT McCarty, "GPIb mediates platelet recruitment leading to activation and aggregation on protein C under shear" *Annual Meeting of the Biomedical Engineering Society,* Los Angeles, CA (Sep, 2007).

7. MA Berny, **TC White**, EI Tucker, CE Swanson, DK Robinson, J Gertz, A Gruber, OJT McCarty, "Factor XI mediates platelet lamellipodia formation and tethering and rolling under shear conditions" *9th UK – 1st Netherlands Platelet Meeting*, London (Sept., 2007)

8. **TC White**, MA Berny, DK Robinson, A Gruber, OJT McCarty, "GPIb mediates platelet recruitment leading to activation and aggregation on protein C under shear" *Tenth Annual Heart Research Center Retreat*, Portland, OR (Jan, 2008).

9. **TC White**, T Eshel, A Gruber, OJT McCarty, "Identification of apolipoprotein E receptor 2 as a novel platelet receptor for protein C" *2008 Biomedical Engineering Society Annual Fall Meeting*, St. Louis, MO (Sept., 2008).

10. **TC White**, MA Berny, EI Tucker, J Pang, D Gailani, RT Urbanus, PG de Groot, A Gruber, OJT McCarty, "Identification of a role for apolipoprotein E receptor 2 as a platelet receptor for factor XI" *Platelets 2008 International Symposium*, Woods Hole, MA (Oct, 2008).

11. XV Yang, Y Banerjee, JA Fernandez, H Deguchi, X Xu, LO Mosnier, RT Urbanus, PG de Groot, **TC White**, OJT McCarty, JH Griffin, “Activated protein C ligation of ApoER2 (LRP8) causes Dab1-dependent signaling” *Annual Meeting of the American Society of Hematology*, San Francisco, CA (Dec, 2008).

12. **TC White**, MA Berny, EI Tucker, J Pang, D Gailani, RT Urbanus, PG de Groot, A Gruber, OJT McCarty, “Identification of a role for apolipoprotein E receptor 2 as a platelet receptor for factor XI” *Annual Meeting of the American Society of Hematology*, San Francisco, CA (Dec, 2008).

13. **TC White-Adams**, MA Berny, EI Tucker, JM Gertz, D Gailani, RT Urbanus, PG de Groot, A Gruber, OJT McCarty, “Apolipoprotein E receptor 2 is a platelet receptor for coagulation factor XI” *XXIInd Congress of the International Society on Thrombosis and Haemostasis*, Boston, MA (July, 2009).

14. **TC White-Adams** “Coagulation factor XI binds to the platelet receptors GPIbα and apolipoprotein E receptor 2 (ApoER2, LRP8)” *NIH National Graduate Student Research Forum*, Bethesda, MD (November, 2009).