Pathophysiology of Plasma Hypercoagulability in Thrombosis

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Abstract

Kellie Rae Machlus: Pathophysiology of Plasma Hypercoagulability in Thrombosis
(Under the direction of Dr. Alisa S. Wolberg)

Blood coagulation abnormalities are the leading cause of death world-wide. Elevated procoagulant factor levels (hypercoagulability) have been correlated with increased thrombin generation and increased risk of arterial and venous thrombosis. This dissertation explores the role of hypercoagulability on various aspects of coagulation and thrombosis *in vitro* and *in vivo*.

Thrombin generation tests are increasingly being employed as a high throughput, global measure of procoagulant activity. Thrombin generation was measured using calibrated automated thrombography (CAT) in platelet-free plasma (PFP) and platelet-rich plasma (PRP). The relative sensitivity of CAT parameters to elevated factors XI, IX, VIII, X, and prothrombin was: PFP initiated with 1 pM tissue factor (TF) > PFP initiated with 5 pM TF > PRP initiated with 1 pM TF. Monitoring the peak height following initiation with 1 pM TF in PFP was most likely to detect hypercoagulability (increased procoagulant factors).

Epidemiologic studies have correlated elevated plasma fibrinogen (hyperfibrinogenemia) with risk of arterial and venous thrombosis. However, it is unknown whether hyperfibrinogenemia is a biomarker of disease or causative in the etiology. In mice, hyperfibrinogenemia significantly shortened the time to occlusion (TTO) after $FeCl_3$ injury to the saphenous vein and carotid artery. Hyperfibrinogenemia increased thrombus fibrin

content, promoted faster fibrin formation, and increased fibrin network density, strength, and stability and increased thrombus thrombolysis resistance *in vivo*. These data indicate hyperfibrinogenemia directly promotes thrombosis and thrombolysis resistance via enhanced fibrin formation and stability.

Studies have correlated elevated plasma factor VIII (FVIII) with thrombosis. However, like hyperfibrinogenemia, it is unclear whether elevated FVIII is a biomarker or causative agent. In mice, elevated FVIII had no effect on 3-minute $FeCl_3$ carotid artery injury, but shortened the TTO after 2-minute injury. Additionally, elevated FVIII increased circulating thrombin-antithrombin complexes and stabilized clots after 2- but not 3-minute $FeCl_3$ injury. *In vitro*, elevated FVIII increased thrombin generation and accelerated platelet aggregation only when initiated by low TF. These results demonstrate dependence of FVIII thrombogenicity on extent of vascular injury.

These findings provide a better understanding of how plasma hypercoagulability impacts thrombogenesis. Specifically, these data suggest causative yet differential roles for hyperfibrinogenemia and elevated FVIII in thrombosis.

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List of Abbreviations and Symbols

α	Alpha
ADP	Adenosine Diphosphate
APC	Activated Protein C
β	Beta
BSA	Bovine Serum Albumin
°C	Degrees Celsius
$CaCl_2$	Calcium Chloride
CAT	Calibrated Automated Thrombography
CTI	Corn Trypsin Inhibitor
CV	Coefficient of Variation
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ETP	Endogenous Thrombin Potential
$FeCl_3$	Ferric Chloride
FV	Factor V
FVII	Factor VII
FVIII	Factor VIII
FIX	Factor IX
FX	Factor X
FXI	Factor XI
g	grams

HBS	HEPES Buffered Saline (20 mM Hepes, 150 mM NaCl, pH 7.4)
HEPES	4-(2-HydroxyEthyl)-1-PiperazineEthaneSulfonic acid
HSVEC	Human Saphenous Vein Endothelial Cells
HTF-1	Mouse anti-Human Tissue Factor Antibody
HUVEC	Human Umbilical Vein Endothelial Cells
KLF	Kruppel-Like transcription Factors
L	Liter
LSCM	Laser Scanning Confocal Microscopy
LT	Lag Time
μ	Micro
μL	Microliter
μM	Micromolar
M	Molar
min	Minute
mL	Milliliter
mM	Millimolar
NaCl	Sodium Chloride
nM	Nanomolar
NPP	Normal Pooled Plasma
PBS	Phosphate Buffered Saline (10 mM phosphate and 150 mM NaCl)
PCA	Procoagulant Activity
PFP	Platelet Free Plasma
pM	Picomolar

PPP	Platelet Rich Plasma
PRP	Platelet Rich Plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PTT	Partial Thromboplastin Time
SMC	Smooth Muscle Cells
TAFI	Thrombin Activatable Fibrinolysis Inhibitor
TNFα	Tumor Necrosis Factor α
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TM	Thrombomodulin
TNKase	Tenecteplase
tPA	tissue-type Plasminogen Activator
TRAP	Thrombin Receptor Activation Peptide (Serine-Phenylalanine-Leucine-Leucine-Arginine-Asparagine)
TTP	Time to Peak
U	Units
VTE	Venous Thromboembolism
vWF	von Willebrand Factor

Chapter 1

Introduction: Procoagulant Activity in Hemostasis and Thrombosis. Virchow's Triad Revisited

1.1 Abstract

Virchow's triad is traditionally invoked to explain pathophysiologic mechanisms leading to thrombosis, alleging concerted roles for abnormalities in blood composition, vessel wall components, and blood flow/shear in the development of arterial and venous thrombosis. Given the tissue-specific bleeding observed in hemophilia, it may be instructive to consider the principles of Virchow's Triad when interrogating mechanisms operant in hemostatic disorders, as well. Blood composition (procoagulant activity of circulating blood cells and plasma proteins) is the most well-studied component of the triad. For example, increased levels of plasma proteins such as prothrombin and fibrinogen are associated with thrombosis, and deficiencies in plasma factors VIII and IX result in bleeding (Hemophilia A and B, respectively). Second, vessel wall components contribute both cellular adhesion molecules that aid in recruiting circulating leukocytes and platelets to sites of vascular damage, and cellular tissue factor, which provides a procoagulant signal of vascular breach and a surface upon which to assemble procoagulant complexes. Third, although contributions of blood flow/shear to clotting are perhaps the least understood aspect of Virchow's Triad, it appears the shear rate affects several aspects of coagulation, including platelet activation/deposition and the kinetics of fibrin monomer formation and polymerization. Shear also affects vessel wall function by modulating endothelial cell procoagulant activity. That no one abnormality in any component of Virchow's Triad predicts coagulopathy *a priori* suggests coagulopathies are multifactorial in origin. This review focuses on the procoagulant contributions of blood, vasculature, and flow/shear to hemostasis and thrombosis and suggests interplay between the three components of Virchow's triad establishes hemostasis and determines propensity for thrombosis or bleeding. Understanding these relationships is essential for effectively

diagnosing and treating thrombotic and bleeding disorders.

1.2 Introduction

Abnormalities in blood coagulation are the leading cause of death world-wide, with treatment costs estimated at over $250 billion and projected to more than triple to $818.1 billion by 2030.[1] In addition to inherited bleeding and thrombotic disorders, therapeutic approaches to manage hemostatic and thrombotic episodes are expected to paradoxically increase the incidence of thrombotic and bleeding events, respectively. For example, while the hemostatic agent recombinant factor VIIa is highly effective at minimizing bleeding in hemophilic patients with inhibitors, its off-label use in non-hemophilic patients is associated with arterial thromboembolism.[2] Similarly, whereas the recently approved antithrombotic Dabigatran exhibits improved safety and efficacy compared to Coumadin, there is no known antidote or reversal agent, which may leave patients prone to bleeds.[3] Understanding mechanism(s) of blood coagulation and its associated disorders will allow us to design more targeted, effective and safe therapeutics to treat both bleeding and thrombosis.

1.3 Tissue factor (TF), thrombin, and fibrin(ogen) in clot formation and stability

Procoagulant activities have been traditionally separated into the extrinsic, intrinsic, and common pathways; however, the requirement to localize thrombin generation to a site of injury and complex presentation of certain factor deficiencies has led to the integration of these pathways.[4] Briefly, coagulation is initiated via extrinsic activity following exposure of cell-derived TF, formation of the factor VIIa/TF complex, and conversion of factor X to factor Xa. Thrombin generation is subsequently augmented by intrinsic (factors XI, IX, and VIII-dependent) activities. Though both the factor VIIa/TF and intrinsic tenase (factors IXa/VIIIa) complexes produce factor Xa, the relative contributions of these activities are

dictated, in part, by the local TF concentration and cell surface supporting enzyme/cofactor complex assembly (discussed below). Cellular and plasma-dependent mechanisms culminate in prothrombinase complex (factors Xa, Va, and prothrombin) assembly, and production of the enzyme thrombin. Most current antithrombotic agents target one or more of the active enzymes generated during the clotting cascade, including factors Xa, IXa, and thrombin.

Proteolytic conversion of circulating, soluble fibrinogen to an insoluble fibrin meshwork involves cleavage of N-terminal peptides from the fibrinogen substrate, subsequent end-to-end polymerization of fibrin monomers to protofibrils, and lateral aggregation of protofibrils to fibers. This sequence of events has been extensively studied and reviewed.[5-7] Fibrin's remarkable biophysical characteristics make it extraordinarily suited to provide structural support to the clot; individual fibers can be strained greater than 330% without rupturing.[8] As such, fibrin is an effective therapeutic target for both minimizing bleeding in hemostatic disorders[9], and dissolving intravascular thrombi in myocardial infarction, ischemic stroke, and deep vein thrombosis.[10-12]

Determinants of fibrin network characteristics have been almost entirely studied in purified systems or in platelet-poor plasma. These studies have shown the conditions under which fibrinogen is converted to fibrin determine the fiber thickness, branching, and network density of the resulting clot. These conditions include the local pH, ionic strength, and concentrations of calcium, polyphosphate, fibrin(ogen)-binding proteins, thrombin, and fibrinogen present during fibrin formation.[13-18] Of these, the influences of the thrombin and fibrinogen concentrations on fibrin structure have been best characterized. At a constant fibrinogen concentration, low thrombin concentrations produce coarse, unbranched networks of thick fibrin fibers, whereas high thrombin concentrations produce dense, highly-branched

networks of thin fibers [16, 17] Similarly, at a constant thrombin concentration, increasing the fibrinogen concentration produces denser, highly-branched fibrin networks.[14, 17, 18] The structural composition of a fibrin clot is important because the structure determines its biochemical and mechanical properties (reviewed in [19]). In general, coarse networks have a lower elastic modulus and increased susceptibility to fibrinolysis, whereas dense networks have a higher elastic modulus and are relatively resistant to fibrinolysis.[18, 20, 21]

1.4 Pathophysiologic mechanisms in thrombosis and bleeding

Abnormal levels of pro- and anti-coagulant proteins[22-40], thrombin generation[34-38, 40, 41], clotting factor activity[42], resistance to inactivation[43, 44], markers of vascular cell damage or activation[45, 46], and fibrinolysis inhibitors[47, 48] have each been correlated with thrombosis or bleeding. That no one of these abnormalities predicts coagulopathy *a priori* suggests coagulopathies are multifactorial in origin. Traditionally invoked to explain pathophysiologic mechanisms leading to thrombosis/thromboembolism, Virchow's Triad postulates concerted roles for these abnormalities in blood composition, vessel wall components, and blood flow/shear in promoting development of arterial and venous thrombosis (Figure 1.1).[49]

Arterial thrombosis is typically associated with atherosclerotic plaque rupture, release/exposure of subendothelial cells and procoagulant material (TF, collagen) from within the plaque, activation and aggregation of platelets, and fibrin deposition, which produces an occlusive platelet-rich intravascular thrombus ("white clot", Figure 1.2). In contrast, venous thrombosis/thromboembolism is typically associated with plasma hypercoagulability and thought to be triggered by expression of procoagulant activity on

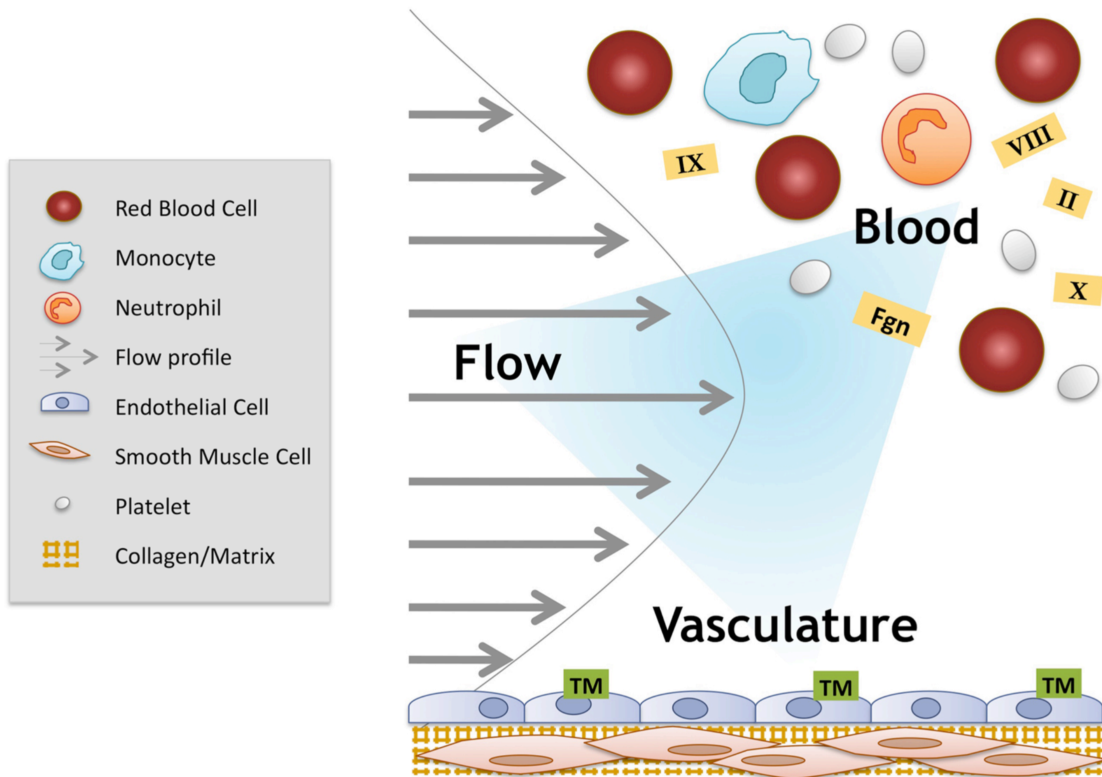

Figure 1.1 Virchow's Triad suggests interplay between abnormalities in blood components, the vasculature, and blood flow contribute to the development of both arterial and venous thrombosis. Abbreviations: IX, factor IX; VIII, factor VIII; II, prothrombin; Fgn, fibrinogen; TM, thrombomodulin

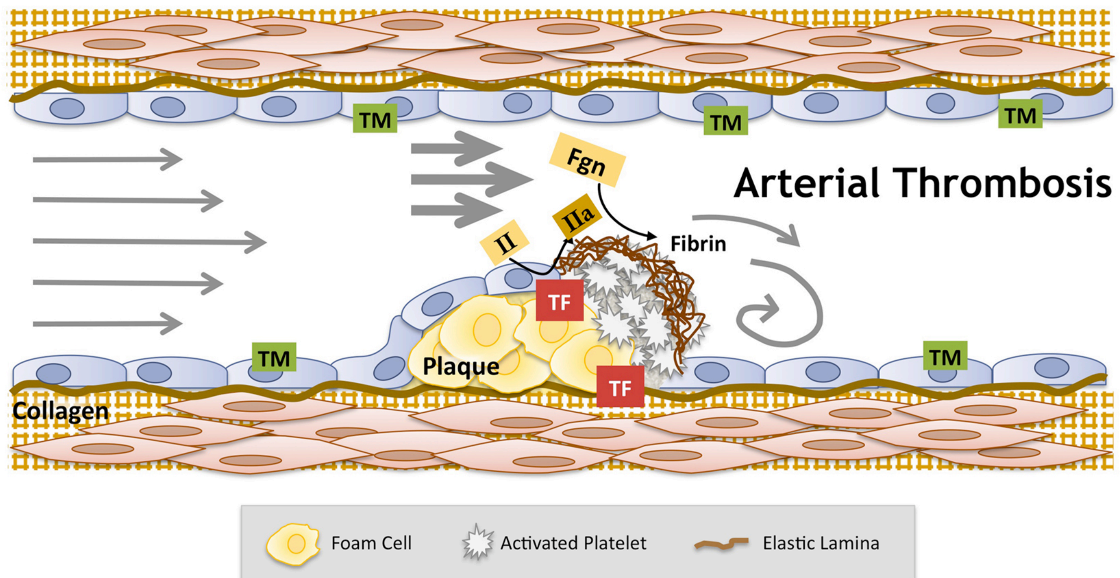

Figure 1.2 Arterial thrombosis involves the formation of platelet-rich ‘white clots’ that form following rupture of atherosclerotic plaques and exposure of procoagulant material such as collagen and tissue factor and/or endothelial breach, in a high shear environment. Abbreviations: TM, thrombomodulin; II, prothrombin; IIa, thrombin; Fgn, fibrinogen; TF, tissue factor

intact endothelium from inflammation or stasis resulting from prolonged immobility. Venous clots are called "red clots," with regions showing substantial erythrocyte incorporation (Figure 1.3). Given the tissue-specific bleeding (predominantly joints and muscles) observed in humans and mice with hemophilia[50-52], it may be instructive to consider the principles of Virchow's Triad when interrogating mechanisms operant in hemostatic disorders, as well. In these patients, bleeding results from transection of the full thickness of the vessel wall and extravasation of blood into the extravascular space. Hemophiliacs rarely bleed into tissues with high subendothelial/extravascular TF expression, suggesting relative contributions from blood composition and tissue-specific procoagulant activity dictate hemostatic potential at an injury site.

Predicting thrombotic or hemorrhagic events and devising targeted approaches for minimizing these events requires thorough consideration of mechanisms that are common and unique to each of these pathologies. *This dissertation chapter focuses on the procoagulant contributions of blood, vasculature, and flow/shear to hemostasis and thrombosis.*

1.5 Role of plasma procoagulant activity in thrombosis and bleeding.

Likely due to the ease of obtaining blood for *ex vivo* experiments, blood composition (circulating blood cells and plasma proteins) is the best-characterized facet of Virchow's Triad. Techniques to evaluate the isolated effects of abnormal plasma clotting factor levels in purified assays have given way to technologies that measure the global hemostatic potential of blood and plasma. These newer techniques permit translation of changes in individual factor levels to net changes in the resulting pattern of thrombin generation.

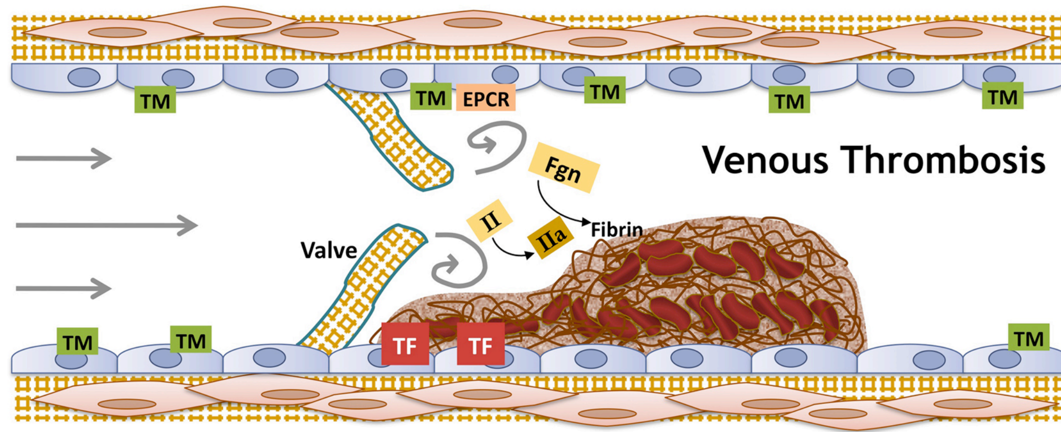

Figure 1.3 Venous thrombosis involves the formation of fibrin-rich “red clots” that result from exposure of procoagulant activity on intact endothelium plus plasma hypercoagulability, in a low shear environment. Venous thrombi are thought to initiate behind valve pockets, where turbulent blood flow differently regulates endothelial cell phenotype. Abbreviations: TM, thrombomodulin; EPCR, endothelial protein C receptor; II, prothrombin; IIa, thrombin; TF, tissue factor; Fgn, fibrinogen

Deficiencies in either factor VIII or IX (hemophilia A or B, respectively) are well-characterized hypocoagulabilities in which patients produce a primary platelet plug in response to vascular injury, but typically re-bleed hours to days following injury. *In vitro* studies show hemophilic plasmas exhibit reduced thrombin generation, with a prolonged onset and lower generation rate and peak thrombin level.[34, 35, 37, 40] This abnormal thrombin generation pattern causes deficiencies in fibrin production, fibrin network density and permeability, and susceptibility of the fibrin network to fibrinolysis.[31, 34, 37, 38, 40, 53-55] Both replacement and bypassing strategies used to treat and prevent bleeding in hemophilia improve thrombin generation parameters and accelerate and stabilize the resulting fibrin clot.[34, 38, 54, 56]

The role(s) of elevated plasma factor levels (hypercoagulabilities) on clotting are less well understood. Elevated prothrombin levels, typically associated with the G20210A mutation, increase risk of venous thrombosis.[22] Hyperprothrombinemia increases the rate and peak of thrombin generation *in vitro* with reconstituted systems and patient plasmas.[57-61] The high thrombin generation rates promote formation of fibrin clots composed of a fine network of thin fibrin fibers[59] and increased activation of TAFI[62]. Although still to be demonstrated *in vivo*, these data suggest a direct connection between elevated prothrombin, thrombin generation, and clot stability.

Abnormalities in fibrin(ogen) formation and function are implicated in both bleeding and thrombotic disorders. To date, over 500 fibrinogen gene mutations have been identified, resulting in expression of low (afibrinogenemia) or abnormally-functioning (dysfibrinogenemia) fibrinogen chains. In addition, post-translational modification (e.g. oxidation, nitration, homocysteinylation, and glycation) of circulating fibrinogen has been

implicated as an "acquired dysfibrinogenemia" in prothrombotic disorders, including acute coronary syndrome, diabetes, hyperhomocysteinaemia, and thrombosis associated with acute cigarette smoke exposure, presumably because it alters fibrinogen cleavage, incorporation of fibrin into the fibrin network, and/or fibrin network stability (reviewed in [63]). Hyperfibrinogenemia is a well-established risk factor for both arterial and venous thrombosis[25-30, 39], however its etiologic role has been controversial. Transgenic mice that over-express murine fibrinogen show elevated fibrin degradation products (D-dimer) and spontaneous fibrin deposition in the spleen, but only marginal shortening of the time to 75% occlusion following 20% ferric chloride ($FeCl_3$) application to the carotid artery.[64-66] Using a different hyperfibrinogenemia model in which wild type mice were infused with human fibrinogen, we showed that hyperfibrinogenemia shortens the time to occlusion following application of 10% and 5% $FeCl_3$ to the carotid artery and saphenous vein, respectively.[18] Furthermore, hyperfibrinogenemia increases thrombus fibrin content, promotes faster fibrin formation, and increases fibrin network density, strength, and stability.[18] Importantly, hyperfibrinogenemia increases resistance to pharmacologically-induced thrombolysis *in vivo*, demonstrating a direct, etiologic link between hyperfibrinogenemia, thrombosis, and thrombolysis in acute settings.[18]

1.6 Role of cellular procoagulant activity in thrombosis and bleeding.

Vascular cells contribute at least two essential functions during coagulation. First, cellular adhesion molecules including P-selectin glycoprotein ligand-1 (PSGL-1) exposed during vascular injury recruit circulating leukocytes and platelets to sites of vascular damage.(Reviewed in [67]) Second, accumulation of these cells, as well as exposure of

active cellular TF (normally sequestered from blood in healthy humans) provides a procoagulant signal of vascular breach and a surface upon which to assemble procoagulant complexes. Recent evidence suggests extravascular TF exists in complex with factor VII[68], such that vascular injury exposes the fully-formed factor VIIa/TF complex, enabling immediate activation of factor X to factor Xa. Subsequent assembly of the prothrombinase complex (factors Xa, Va, and prothrombin) takes place on the negatively-charged lipid (phosphatidylserine) surface provided by TF-bearing cells and activated platelets. Cells also provide a site for factor XI(a) binding and activation of factor IX, as well as assembly of the intrinsic tenase complex (factors IXa, VIIIa, and X) to augment thrombin generation.

We and others have directly compared the ability of intravascular and extravascular cells to support procoagulant activity. Whereas quiescent intravascular cells have little to no TF activity, extravascular cells (e.g., fibroblasts and smooth muscle cells) express TF and are primed to trigger coagulation upon contact with blood. Since procoagulant activity (thrombin generation) dictates fibrin network formation and network properties, the highly procoagulant extravascular cells support the rapid production of a dense fibrin network that is relatively resistant to fibrinolysis.[69-71] Such networks stabilize the primary platelet plug at a site of vascular injury. The ability of extravascular procoagulant activity to trigger clotting in blood escaping from a vessel can be implicated in tissue-specific hemostasis. Vascular breach into a site of high TF activity would not require an intact intrinsic pathway; thrombin generation could take place entirely via the TF-driven extrinsic pathway. In contrast, vessel disruption into tissues with lower extravascular TF expression would necessitate intrinsic pathway activity to augment thrombin generation and promote stable fibrin formation. Indeed, hemophilia patients frequently bleed into muscles and joints, which

has been related to physical stress placed on these tissues.[50-52] However, hemophilia patients do not bleed into other tissues that endure high physical activity such as the heart, suggesting that attributing bleeding solely to physical stress oversimplifies the pathology. Cardiomyocytes express high levels of TF, which can promote substantial thrombin generation independently of intrinsic factors VIII and IX, suggesting tissue-specific extrinsic activity can compensate for reduced intrinsic activity. This scenario illustrates the required integration of two components of Virchow's Triad – blood and tissues – in maintaining hemostasis.

Extravascular TF expression is implicated in arterial thrombosis resulting from atherosclerotic plaque rupture. The amount of TF found in atherosclerotic plaques in humans is positively correlated with the thrombogenicity of the lesions after plaque rupture.[72] The importance of TF in the progression of arterial thrombosis is underscored by observations that treatment of human plaques with TFPI significantly reduces adhesion of both fibrinogen and platelets to the plaque, indicating the thrombotic potential of atherosclerotic lesions may be decreased by inhibiting TF-dependent procoagulant activity.[73]

Inappropriate expression of intravascular procoagulant activity is hypothesized to trigger venous thromboembolism. Pro-inflammatory mediators including bacterial lipopolysaccharide, and the cytokines interleukin-1β and tumor necrosis factor-α (TNFα), induce procoagulant activity in cultured endothelial cells via *de novo* TF synthesis and decreased thrombomodulin expression in a time- and dose-dependent manner.[18, 70, 74, 75] TNFα-stimulated endothelial cells support robust fibrin formation.[18, 70] Laser-induced injury of cultured endothelial cells has also been shown to induce procoagulant activity and fibrin formation, although the time course of those experiments suggests TF activity is

released from intracellular stores and is not synthesized *de novo*.[76] *In vivo*, endothelial cells residing in different vascular beds (e.g., aorta *versus* pulmonary microcirculation) have different phenotypes. Even within a given vessel, endothelial cells vary in their expression of coagulation proteins. Brooks et al. elegantly showed increased expression of thrombomodulin and endothelial cell protein C receptor, and decreased expression of von Willebrand factor in the valve sinus relative to the immediately adjacent vein lumen in the saphenous vein harvested during cardiac bypass surgery.[77] Although these studies convincingly show vascular bed-specific expression of anticoagulant proteins on endothelial cells, evidence for endothelial cell TF expression *in vivo* remains controversial.

Vascular bed-specific function is best illustrated clinically by the specific presentations of thrombosis in either arterial or venous circulation, but rarely both. For example, risk factors strongly associated with arterial thrombosis only modestly increase risk of venous thrombosis/thromboembolism; compared with control subjects, the risk of VTE is 1.51 for hypertension, 1.42 for diabetes mellitus, 1.18 for smoking and 1.16 for hypercholesterolemia.[78] In addition, meta-analysis suggests history of unprovoked venous thrombosis only slightly increases (~1.5-fold) risk of arterial cardiovascular events over long-term followup.[79] Since any abnormality in the blood is present systemically, these events reflect contributions from unique vascular bed-specific pro- or anti-coagulant activity and/or shear rates (discussed below). Vascular bed-specific activities are illustrated by animal models of coagulation dysfunction showing bleeding and/or fibrin deposition in specific tissues. For example, mice with partial TFPI deficiency and decreased TM function demonstrate fibrin deposition in the brain and liver, but not other tissues.[80] These observations suggest critical functions of anticoagulant proteins in specific tissues.

Neither “cell” nor “plasma protein,” cell-derived microparticles express cell-specific markers and retain procoagulant properties derived from their parent cell, and can circulate and evade normal cell regulatory mechanisms. Although circulating microparticles derived from leukocytes, platelets, erythrocytes, endothelium, megakayocytes, and tumors have been identified in numerous studies (Figure 1.4), their precise role(s) in hemostasis and thrombosis is poorly understood. Scott’s syndrome patients with a defect in membrane regulation that reduces the number of circulating platelet-derived microparticles exhibit a bleeding defect[81]; however, it is difficult to separate the role(s) of reduced expression of phosphatidylserine on platelets and reduced production of phosphatidylserine-bearing microparticles. Healthy humans have few circulating leukocyte-derived microparticles; however, the numbers of leukocyte-derived microparticles rise in certain diseases, including cancer[82, 83], diabetes[84], and sickle cell disease[85]. Microparticles derived directly from tumors have also been specifically associated with venous thromboembolism in cancer patients, and are thought to contribute to the thrombotic presentation.[86, 87] Expression of procoagulant activity on microparticles (TF and/or phosphatidylserine) has been implicated in microparticle function *in vitro* and *in vivo*. One prospective study demonstrated a sharp rise in microparticle TF activity immediately prior to venous thrombosis in two patients[83], suggesting a causative role of microparticles in the thrombotic event. Murine studies support this observation; human monocyte-derived microparticles promote thrombus formation[88, 89] and increase thrombus weight[90] in intravascular thrombosis models. Additional theories regarding the mechanistic role(s) of microparticles in thrombosis invoke their expression of cellular adhesion molecules (from their parent cell), and ability to activate

endothelium and leukocytes.

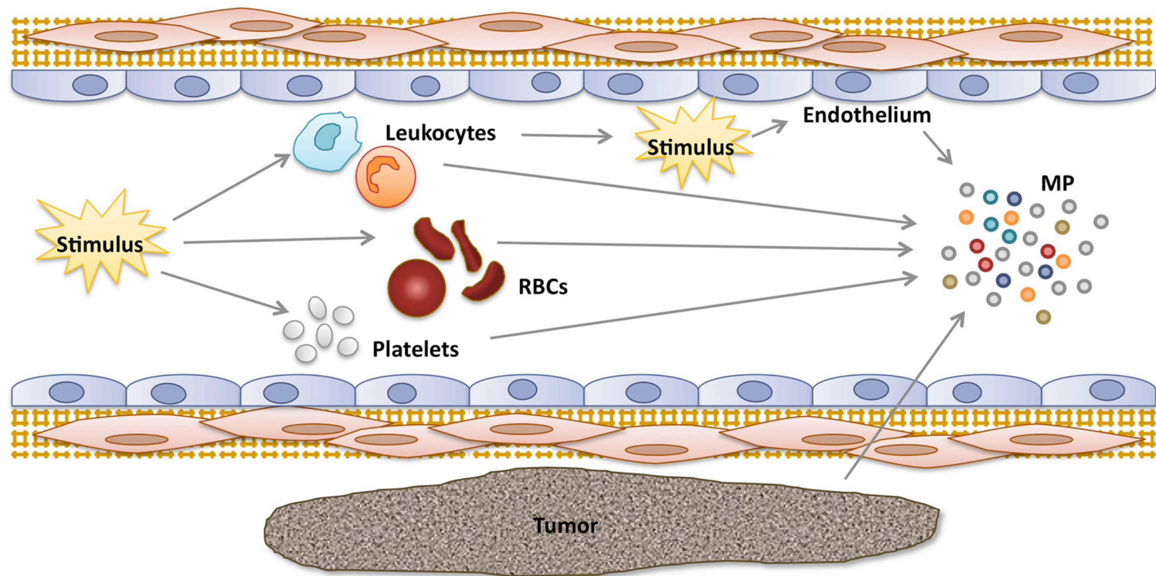

Figure 1.4 Circulating microparticles are derived from a variety of cell types including leukocytes, platelets, megakaryocytes, red blood cells, endothelial cells, and tumors. Microparticles carry cell-specific markers and functional properties of their parent cell.

1.7 Role of flow/shear in thrombosis and bleeding.

The contributions of blood flow/shear to clotting are perhaps the least interrogated aspect of Virchow's Triad. Biological models for examining platelets and endothelial cells in their native shear environments are technically challenging; reconstituting a sufficient model of the vasculature in fluidics devices to visualize clot formation has been difficult. Most frequently studied is the role of shear in platelet deposition at sites of vascular damage. Typical venous and arterial shear rates are 10-100 s^{-1} and 500-1500 s^{-1}, respectively, although shear may reach 70,000 s^{-1} to 250,000 s^{-1} in severely stenotic vessels.[91, 92] Platelet deposition rates increase with shear rates in a vWF-dependent mechanism.[93, 94] Shear-dependent expression of platelet P-selectin and formation of monocyte-platelet aggregates suggest that both platelets and leukocytes are activated when they traverse stenotic regions, priming these cells for deposition on dysfunctioning or ruptured vasculature.[95] Moreover, soluble agonist-independent accumulation of platelets at a stenotic region is attributed to flow (rheology)-dependent aggregation.[96] Both systemic and pulmonary hypertension increases shear stress and mechanical damage to arteries, increasing circulating levels of both endothelial- and platelet-derived microparticles.[97] Accumulation of platelets and procoagulant microparticles is expected to not only increase thrombus mass and decrease lumen size at a developing thrombus (further increasing the local shear rate), but also provide additional surface for thrombin generation and subsequent fibrin deposition, further stabilizing the growing thrombus.

Stasis induced by immobility (hospitalization or long-haul air travel) also alters local shear rates, which can subsequently modulate the endothelial cell phenotype. The flow-responsive Kruppel-like transcription factors (KLF) have been implicated in shear-dependent

endothelial cell function.[98-100] In general, laminar shear stress upregulates KLF expression, inducing expression of anti-inflammatory and antithrombotic proteins, including thrombomodulin; whereas, stasis- or proinflammatory cytokine-induced loss of KLF expression leads to enhanced expression of vascular cell adhesion molecule-1 and TF.[101, 102] As such, KLF regulation represents an intriguing therapeutic approach for novel antithrombotics aimed at combating stasis-induced thrombosis.

Interestingly, flow/shear also dramatically modulates the kinetics of both fibrin monomer formation and polymerization.[103, 104] At a constant shear rate, initiation of clotting is a function of the size of the exposed region of TF.[103] Once initiated, shear dictates both influx of procoagulant reactants including prothrombin and fibrinogen, and efflux of reaction products including thrombin and fibrin. In *in vitro* assays, increasing shear from 10 to 100 s^{-1} depletes local fibrin monomer concentrations, limiting lateral aggregation and protofibril extension. However, increasing thrombin generation or decreasing shear increases local monomer concentrations, permitting protofibril and subsequent fibrin fiber formation.[104] Although fibrin networks formed under static conditions show an isotropic distribution of fibers with relatively uniform diameters, fibrin produced in flowing blood is oriented along flow vectors[104-106], with a prominent network of thick fibers as well as fiber "aggregates" in which multiple individual fibers are coalesced into bundles.[106] Since the fibrin network's ability to withstand both biochemical dissolution and mechanical disruption is a function of its fiber thickness and branching[19], these studies indicate networks produced under flow would demonstrate significantly different stability than isotropic networks formed under stasis.[106]. In fact, a recent study found that mechanical stress creates fibrin networks that are resistant to fibrinolysis[107], underscoring the importance of the effects of

flow/shear on thrombus formation, structure and function.

1.8 Multiple "hits" in thrombosis and bleeding

Virchow's proposition that multiple "hits" culminate in abnormal coagulation suggests simultaneous consideration of soluble, cellular, and physical biomarkers is required to delineate the pathophysiology of these disorders. For example, the prognostic importance of hyperfibrinogenemia appears to be independent of, but additive to, myocardial damage (assessed by troponin-T levels) in patients with unstable coronary artery disease.[30] However, few diagnostic algorithms simultaneously consider markers of vascular damage and plasma hypercoagulability when determining thrombosis risk. Acevedo et al.[27] showed that elevated levels of both homocysteine (a trigger of endothelial damage) and fibrinogen contributed to an increased hazard ratio in patients in a high-risk thrombosis cardiology clinic. These studies suggest dysfunction in components of the blood and vessel wall simultaneously create an environment conducive to thrombosis.

1.9 Conclusions

Understanding the interplay between the components of Virchow's triad is necessary to effectively diagnose and treat bleeding and thrombotic disorders. Increasing awareness of the complexities of these presentations, along with increasingly sophisticated technologies to analyze soluble, cellular, and physical dysfunctions in concert will shed new light on these pathologies and identify novel therapeutic targets. With such targets, we can achieve the long-sought grail for coagulation disorders: antithrombotics with no bleeding risk and hemostatic agents with no thrombotic risk.

1.10 Focus of this dissertation

There remains much to learn about the three arms of Virchow's triad and their separate and combined role(s) in both bleeding and thrombotic disorders. However, this dissertation is focused primarily on plasma *hyper*coagulability and thrombosis. Specifically, this thesis will address 1) the effect of elevated plasma coagulation factors on thrombin generation, 2) the role of elevated fibrinogen (hyperfibrinogenemia) on thrombosis and thrombolysis and 3) the role of elevated factor VIII on thrombus formation and stability.

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Chapter 2

Effects of tissue factor, thrombomodulin and elevated clotting factor levels on thrombin generation in the calibrated automated thrombogram

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2.1 Abstract

Background: Elevated procoagulant levels have been correlated with increased thrombin generation *in vitro* and with increased venous thromboembolism (VTE) risk in epidemiological studies. Thrombin generation tests are increasingly being employed as a high throughput method to provide a global measure of procoagulant activity in plasma samples. ***Objectives:*** Distinguish the effects of assay conditions [tissue factor (TF), thrombomodulin, platelets/lipids] and factor levels on thrombin generation parameters, and determine the conditions and parameters with the highest sensitivity and specificity for detecting elevated factor levels. ***Methods***: Thrombin generation was measured using calibrated automated thrombography (CAT) in corn trypsin inhibitor (CTI)-treated platelet-free plasma (PFP) and platelet-rich plasma (PRP). Statistical analysis was performed using logarithms of observed values with analysis of variance that accounted for experiment and treatment. ***Results***: The relative sensitivity of lag time (LT), time to peak (TTP), peak height and endogenous thrombin potential (ETP) to elevated factors XI, IX, VIII, X, and prothrombin was as follows: PFP initiated with 1 pM TF > PFP initiated with 5 pM TF > PRP initiated with 1 pM TF. For all conditions, inclusion of thrombomodulin prolonged the LT and decreased the peak and ETP; however, addition of thrombomodulin did not increase the ability of CAT to detect elevated levels of individual procoagulant factors. ***Conclusions***: CAT conditions differentially affected the sensitivity of thrombin generation to elevated factor levels. Monitoring the peak height and/or ETP following initiation of clotting in PFP with 1 pM TF was most likely to detect hypercoagulability due to increased procoagulant factor levels.

2.2 Introduction

Thrombin activates platelets, converts fibrinogen to fibrin, and initiates wound healing. Elevated procoagulant levels are correlated with abnormal thrombin generation *in vitro*[1-5] and increased risk of VTE.[6-12] For example, prothrombin concentrations >115% of normal are associated with a 2.1-fold increased VTE risk, and factor VIII concentrations >200% are associated with markedly elevated VTE risk (OR=10.8).[6, 7] Tests that assess the ability of plasma to generate thrombin are thought to provide a more global measure of hemostasis and thrombosis than standard clotting assays. Such tests may be useful for determining risk of primary and recurrent VTE, and therefore, duration of antithrombotic therapy.

The most commonly used assay for continuous thrombin measurement is calibrated automated thrombography (CAT).[13] CAT is based on monitoring cleavage of a slow-reacting fluorogenic substrate and comparing it to a known thrombin concentration added to non-clotting samples.[13] Studies have demonstrated CAT's ability to detect reduced thrombin generation in clotting factor deficiency.[14-17] CAT appears most sensitive to hypocoagulability when initiated by low (1 pM) TF.[16]

Given these findings, it is logical to hypothesize that CAT can be used to detect *hyper*coagulability due to elevated factor levels. Using high (184–429 pM) TF and no added phospholipids, Siegemund *et al*. observed increased ETP in platelet-poor plasma with high concentrations of factors XI and IX, but not VIII.[4] In contrast, in PRP assays in which clotting was initiated with low (0.5 pM) TF, Regnault *et al*. observed increased ETP with elevated factor VIII.[3] Several studies have correlated recurrent VTE risk with elevated peak height and/or ETP.[18-24] However, another study correlated abnormal thrombin

generation with first, but not recurrent VTE.[25] It has been proposed that differences in findings from these studies stem, in part, from variations in assay conditions.[25-27] Notably, these studies differed in the source and concentration of TF and phospholipids, and use (or not) of contact pathway inhibitors (CTI) and protein C pathway sensitizing agents [activated protein C (APC), thrombomodulin] because the experimental conditions providing the highest sensitivity and specificity for detecting hypercoagulability have not been defined.

The goals of this study were to: 1) distinguish the effects of assay conditions (added TF, thrombomodulin, platelets/phospholipids) and elevated factor levels on thrombin generation parameters, and 2) determine the conditions and parameters with the highest sensitivity and specificity for detecting elevated factor levels. We simulated "hypercoagulability" by spiking plasma with factors XI, IX, VIII, X, V, prothrombin, or fibrinogen to 200% and 400% of normal, and measured the effect of these factors on thrombin generation parameters in different experimental conditions. We report that the conditions under which CAT was performed differentially affected its sensitivity. Changes in the peak height and/or ETP following initiation of clotting in PFP with 1 pM TF was most likely to detect hypercoagulability due to increased factor levels.

2.3 Materials and Methods

All proteins used in this study were of the highest quality and specific activity available. CTI and factors XI, V, and prothrombin were purchased from Haematologic Technologies, Inc (Essex Junction, VT). Factor X and plasminogen-, von Willebrand factor- and fibronectin-depleted fibrinogen were purchased from Enzyme Research Laboratories (South Bend, IN); factor X was further purified as described.[5] Factor VIII (Hemofil M, Baxter, Deerfield, IL) and factor IX were the generous gifts of Dr. Dougald M. Monroe (UNC at Chapel Hill). Briefly, factor IX was prepared from prothrombin complex concentrations by Q Sepharose chromatography, heparin affinity, metal chelate chromatography using copper, and pseudo-affinity calcium elution from HiTrap Q. Factor IX could be activated completely by factor XIa and gave the expected molar concentration when the activated material was titrated with antithrombin in the presence of heparin. Protein purity was verified by both non-reducing and reducing 12% SDS-PAGE prior to use. Analysis of factors XI, IX, X, V, prothrombin, and fibrinogen: 1) revealed only bands of the expected molecular weight for each zymogen and cofactor, and 2) indicated no activated protein species (data not shown). The factor VIII preparation (Hemophil M) also contained albumin, however, control experiments showed no effect of this albumin concentration on thrombin generation (data not shown). The specific activity of the factor V preparation was 51.3 U/mg, consisted with that expected for factor V (50-60 U/mg), and ~40X lower than that expected for factor Va (2000 U/mg), indicating no factor Va contamination in the preparation. Rabbit lung thrombomodulin was from American Diagnostica, Inc (Stamford, CT). Fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC), TF/Lipid Reagents (PFP-Low, PFP-High), and thrombin calibrator (α2-macroglobulin/thrombin) were generously provided by Diagnostica

Stago (Parsippany, NJ). Lipids were composed of phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine at a ratio of 20:60:20, respectively. Blood was collected according to protocols approved by the UNC Institutional Review Board.

Plasma preparations

For PFP, blood was collected from 18 healthy individuals [20-45 years old, 50% female, 50% non-Caucasian, with normal partial thromboplastin times (PTT)] through a 21-gauge needle into a syringe and immediately transferred to sodium citrate/CTI [0.105 M (3.2%, pH 6.5) and 18.3 μg/mL, respectively] to minimize contact activation.[28, 29] PFP was prepared by sequential centrifugation (150xg, 15 minutes, 13,000xg, 15 minutes), aliquoted, and snap-frozen in liquid nitrogen within two hours of phlebotomy. PFPs were then thawed, pooled, and refrozen for subsequent assays. Pooled PFP was analyzed for levels of factors XI, IX, VIII, X, V, VII, protein C, free protein S, prothrombin, and fibrinogen at UNC Hospitals McLendon Clinical Laboratory; all were in the normal reference range.

For PRP, blood was collected from six healthy individuals (21-50 years old, 67% female, normal PTTs) who had not taken aspirin for two weeks or ibuprofen for three days prior to phlebotomy. Blood was drawn as for PFP. PRP was prepared by centrifugation (150xg, 15 minutes) within one hour. The platelet count was measured on a Z1 Series Coulter Counter (Beckman Coulter, Fullerton, CA), normalized by dilution with autologous PFP to 225,000/μL, and used at 150,000/μL (final).

Thrombin generation assay

Factors XI, IX, VIII, X, V, prothrombin and fibrinogen were added to normal, pooled PFP and individual PRPs to 200% or 400% of normal (final). Thrombin generation was measured by CAT.[13] For PFP experiments, 20 μL of TF/phospholipids were mixed with 80 μL PFP in 96-well round-bottom microtiter plates (Becton Dickinson, Falcon™). The plate was inserted into a Fluoroskan Ascent® fluorometer (ThermoLabsystem, Helsinki, Finland) and warmed to 37°C for 5 minutes. Reactions were initiated by automatically dispensing 20 μL of 2.5 mM fluorogenic substrate in 0.1 M $CaCl_2$ to each well. Reactions were performed in duplicate or triplicate in each experiment. The final TF, phospholipid, fluorogenic substrate, and $CaCl_2$ concentrations were 1 or 5 pM, 4 μM, 416 μM and 16 mM, respectively. Experiments with PRP were performed under identical conditions, but without added phospholipids. Reactions were calibrated against wells containing 20 μL of α2-macroglobulin/thrombin complex and 80 μL PFP or PRP.[13] Thrombin generation was monitored at 37°C with excitation and emission filters at 390 nm and 460 nm, respectively, every 20 seconds for 120 minutes. Experiments with PFP and 1 pM TF, PFP and 5 pM TF, and PRP and 1 pM TF were performed 10, 5, and 6 times, respectively.

Thrombin parameters were calculated using Thrombinoscope software version 3.0.0.29 (Thrombinoscope BV, Maastricht, Netherlands), which defines the lag time (LT) as the first time point after the thrombin concentration exceeded one-sixth peak height, the TTP as the time to the peak height, the peak height as the maximum thrombin concentration produced, and the ETP as the time integral of thrombin formation (the area under the thrombin generation curve).

Statistical methods

Statistical analysis was performed for logarithms of parameters using analysis of variance (ANOVA) that accounted for experiment date (PFP experiments), or donor (PRP experiments) and elevated factors. Each condition (factor level) was handled separately; factor concentration was not considered a covariate. An ANOVA model was first fit to each log-transformed parameter with covariates of date or donor, factor level, an indicator variable for platelets, and the interaction between factor level and platelet indicator. We compared PFP initiated with 1 pM TF to: 1) PFP initiated with 5 pM TF, and 2) PRP initiated with 1 pM TF. Hypothesis testing was performed using a contrast matrix corresponding to a 1 degree of freedom F-test for each parameter. In a separate analysis, for each log-transformed parameter, a separate ANOVA model was fit with covariates of date or donor and factor level. To determine the effect of thrombomodulin in the presence of elevated factors, ANOVA models were applied to the log of the ratio of the parameter value with and without thrombomodulin. Differences in least square means between conditions and the control were evaluated by t-tests. Multiple comparisons were handled with Bonferroni corrections; significance was $p<0.0033$ (0.05/15) unless otherwise indicated. The percent coefficient of variation (%CV) was calculated as the ratio of the standard deviation to the mean multiplied by one hundred for inter-assay variability (PFP) and inter-individual variability (PRP).

2.4 Results

Thrombin generation in normal PFP and PRP

We first characterized the effects of TF on thrombin generation in normal PFP and PRP. Thrombin generation in PFP required addition of both TF and phospholipids; omission of either prevented thrombin generation for >120 minutes (data not shown), indicating that the PFP was not contaminated with detectable endogenous TF activity, procoagulant lipids, or contact activation. Similarly, thrombin generation in PRP required addition of TF (data not shown), indicating that PRP from these healthy subjects did not contain detectable TF activity or contact activation.

Table 2.1 shows baseline thrombin generation characteristics for PFP and PRP. Reactions initiated by 1 and 5 pM TF are indicated: $PFP_{(1\ pM\ TF)}$, $PFP_{(5\ pM\ TF)}$, and $PRP_{(1\ pM\ TF)}$, respectively. Compared to $PFP_{(1\ pM\ TF)}$, $PFP_{(5\ pM\ TF)}$ demonstrated shortened LT and TTP and increased peak height and ETP. The inter-assay variability (%CV) of PFP (Table 2.2) and inter-individual variability (%CV) in PRP (Table 2.3) were consistent with prior studies.[13, 26, 30] Thrombin generation in $PFP_{(1\ pM\ TF)}$ demonstrated a significantly shorter LT and TTP, increased peak height and decreased ETP than $PRP_{(1\ pM\ TF)}$, consistent with previous studies.[13, 26, 30]

Effect of thrombomodulin on thrombin generation in normal PFP and PRP

Thrombin generation in normal PFP is decreased in the presence of thrombomodulin or APC[3, 13, 22, 24, 31, 32]; however, patients with a protein C pathway deficiency (factor V Leiden, protein C/S deficiency) have decreased thrombomodulin/APC sensitivity.[22, 24] Previous studies have recommended that CAT assays include APC or thrombomodulin to

Table 2.1 Baseline characteristics (±SD) of thrombin generation in PFP and PRP, in the absence and presence of 5 pM thrombomodulin (TM).

	$PFP_{(1\ pM\ TF)}$		$PFP_{(5\ pM\ TF)}$		$PRP_{(1\ pM\ TF)}$	
	-TM	+TM	-TM	+TM	-TM	+TM
LT (min)	4.5 ±0.4	5.3 ±0.5*	1.9 ±0.2^	2.4 ±0.2*	9.7 ±2.9^	13.1 ±4.4*
TTP (min)	9.1 ±0.6	8.6 ±0.6*	4.4 ±0.3^	4.5 ±0.2	20.4 ±2.9^	22.0 ±4.8
Peak height	197.0 ±30.2	35.9 ±16.8*	344.4 ±10.4^	296.4 ±22.2*	132.2 ±37.6^	52.6 ±15.6*
ETP	1569.1 ±168.8	197.9 ±90.2*	1754.7 ±176.2^	1303.9 ±128.7*	1893.6 ±455.3^	602.1 ±94.9*

$^*p<0.0001$ *vs.* "-TM"

^$p<0.0001$ *vs.* "$PFP_{(1\ pM\ TF)}$-TM"

Table 2.2 Inter-assay Variability (%CV) of Thrombin Generation in PFP

	$PFP_{(1\ pM\ TF)}$		$PFP_{(5\ pM\ TF)}$	
	-TM	+TM	-TM	+TM
LT	8.38	9.15	10.83	9.75
TTP	6.41	6.96	5.81	5.25
Peak height	15.35	46.81	3.03	7.49
ETP	10.76	45.55	10.04	9.87

Table 2.3 Inter-individual Variability (%CV) of Thrombin Generation in PRP

	$PRP_{(1\ pM\ TF)}$	
	-TM	+TM
LT	29.72	33.89
TTP	14.36	21.94
Peak height	28.39	29.65
ETP	24.04	15.76

increase ability to discriminate between healthy subjects and VTE patients, as well as predict VTE recurrence.[19, 23, 24, 31] We first compared thrombin generation in the presence and absence of thrombomodulin in $PFP_{(1\ pM\ TF)}$ and $PFP_{(5\ pM\ TF)}$ (Figs 2.1a-2.1b, Table 2.1). Thrombomodulin significantly prolonged the LT and reduced the peak height and ETP in both $PFP_{(1\ pM\ TF)}$ and $PFP_{(5\ pM\ TF)}$. Thrombomodulin significantly shortened the TTP in $PFP_{(1\ pM\ TF)}$, but not $PFP_{(5\ pM\ TF)}$. Increased TF decreased thrombomodulin sensitivity; that is, 5 nM thrombomodulin reduced the ETP ~87±1.9% and ~28±3.1% for $PFP_{(1\ pM\ TF)}$ and $PFP_{(5\ pM\ TF)}$, respectively. Thrombomodulin significantly increased the inter-assay %CV of the peak height and ETP in $PFP_{(1\ pM\ TF)}$, but had less effect in $PFP_{(5\ pM\ TF)}$ (Table 2.2).

We then examined the impact of thrombomodulin on thrombin generation in $PRP_{(1\ pM\ TF)}$. As in $PFP_{(1\ pM\ TF)}$, thrombomodulin prolonged the LT and reduced the peak height and ETP in $PRP_{(1\ pM\ TF)}$ (Fig 1c, Table I). In contrast to $PFP_{(1\ pM\ TF)}$, thrombomodulin slightly prolonged the TTP in $PRP_{(1\ pM\ TF)}$, likely because the TTP in PRP is limited by the time to platelet activation. These findings are somewhat different, although not altogether inconsistent with previous findings that thrombomodulin decreased ETP, but did not change LT or TTP in PRP.[13] The differences in response to thrombomodulin in these studies may relate to the thrombomodulin source (human *vs*. rabbit lung), although another study found similar effects of thrombomodulin from different sources on ETP.[24] The inter-individual variability increased for LT and TTP, decreased for ETP, and did not change for peak height in the presence of thrombomodulin (Table 2.3). Platelets decreased CAT sensitivity to thrombomodulin; that is, 5 nM thrombomodulin reduced the ETP ~66±4.8% in $PRP_{(1\ pM\ TF)}$, compared to ~87±1.9% in $PFP_{(1\ pM\ TF)}$ (Fig 2.1c, Table 2.1). The reduced thrombomodulin

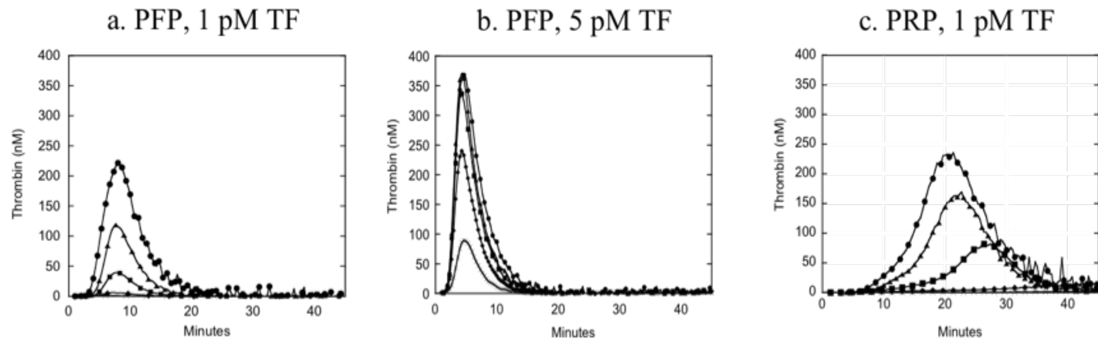

Figure 2.1 Thrombomodulin decreases thrombin generation in $PFP_{(1\ pM\ TF)}$, $PFP_{(5\ pM\ TF)}$ and $PRP_{(1\ pM\ TF)}$. A, B) Thrombin generation in PFP was initiated by addition of 4 μM phospholipids and A) 1 pM TF or B) 5 pM TF. C) Thrombin generation in PRP was initiated by addition of 1 pM TF. Curves show increasing thrombomodulin: 0 (circles), 2.5 (triangles), 5 (squares), 10 (diamonds), and 20 (crosses) nM, representative of 3 separate experiments.

sensitivity of PRP *versus* PFP likely reflects contributions of platelet-derived factor V(a) to thrombin generation[33, 34], and is consistent with previous studies.[13]

Effect of procoagulant factors on thrombin generation in PFP and PRP

Finally, we compared the ability to detect hypercoagulability under each of these conditions (1 and 5 pM TF, in the presence and absence of thrombomodulin, in PFP and PRP). We measured thrombin generation in PFP and PRP spiked with factors XI, X, IX, VIII, V, prothrombin, or fibrinogen. We initiated clotting in PFP with 1 or 5 pM TF and 4 μM phospholipids, and in PRP with 1 pM TF (no phospholipids), in the presence and absence of thrombomodulin.

LT: The LT in $PFP_{(1\ pM\ TF)}$ and $PFP_{(5\ pM\ TF)}$ were qualitatively similar, although changes in $PFP_{(5\ pM\ TF)}$ were smaller than changes in $PFP_{(1\ pM\ TF)}$ (Fig 2.2a, 2.2b). In both the absence and presence of thrombomodulin, intrinsic factors XI, IX, and VIII did not significantly alter the LT, although effects were slightly greater in the presence of thrombomodulin. Factor X significantly shortened the LT in both conditions. Interestingly, factor V significantly shortened the LT in $PFP_{(1\ pM\ TF)}$ but not $PFP_{(5\ pM\ TF)}$. Both prothrombin and fibrinogen significantly increased the LT, irrespective of the TF concentration or presence of TM. The opposing effects of factor X and prothrombin on LT in PFP may relate to differences in their plasma concentrations. Whereas 200% factor X increases the plasma level from 8 to 16 μg/mL, 200% prothrombin increases the plasma level from 100 to 200 μg/mL. This high concentration of prothrombin or the prothrombin activation product fragment 1.2 may competitively inhibit binding of other Gla proteins to the lipid surface, thereby prolonging the time to complex assembly and enzyme generation. Indeed, elevated prothrombin did not

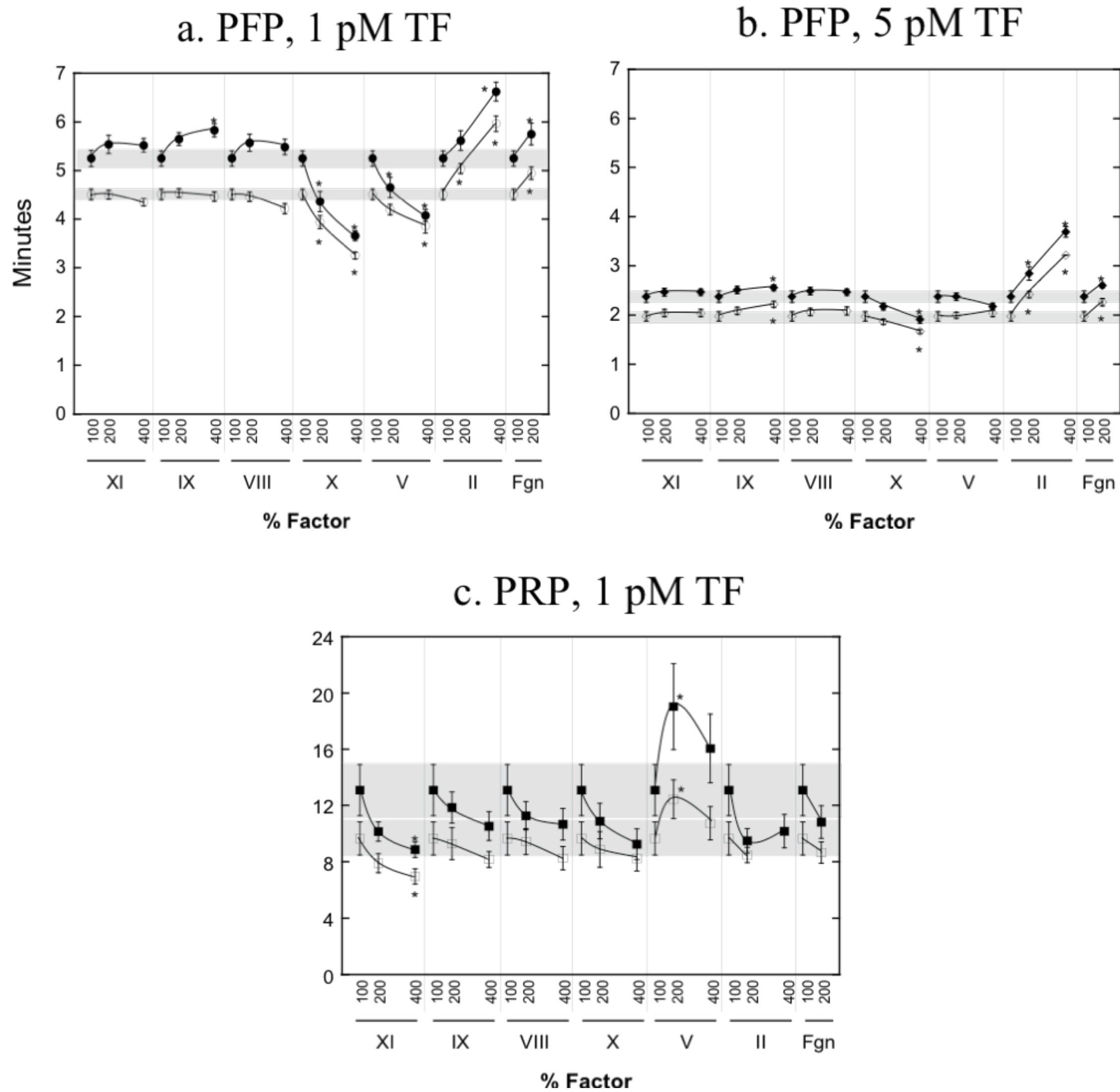

Figure 2.2 Procoagulant factors and thrombomodulin differently influence LT in PFP and PRP. PFP or PRP was spiked with factors XI, IX, VIII, X, V, prothrombin, or fibrinogen to 200% or 400% of normal. Clotting was initiated by addition of 1 pM TF and 4 μM phospholipids (circles), 5 pM TF and 4 μM phospholipids (diamonds), or 1 pM TF (no lipids, squares) for A) $PFP_{(1\ pM\ TF)}$, B) $PFP_{(5\ pM\ TF)}$, and C) $PRP_{(1\ pM\ TF)}$, respectively, in the absence (open symbols) and presence (closed symbols) of thrombomodulin, as described in Methods. Note y-axis scaling in panel C. The shaded gray box encompasses the 100% value and its standard error of the mean (SEM). Data show means ±SEM. *$p<0.0033$ *versus* 100%.

prolong the LT in assays performed in the presence of increased (300 μM) lipid concentrations (data not shown).

Procoagulant factors produced unique effects on the LT in $PRP_{(1\ pM\ TF)}$ (Fig 2.2c). In both the presence and absence of thrombomodulin, factors XI, IX, VIII, X, prothrombin, and fibrinogen non-significantly shortened the LT in $PRP_{(1\ pM\ TF)}$; only 400% factor XI significantly shortened the LT. These findings suggest that in contrast to PFP, the LT in PRP is predominated by the time required for platelet activation and supercedes the time necessary to form procoagulant complexes on exposed lipid. Factor V exhibited a biphasic effect on LT in $PRP_{(1\ pM\ TF)}$, significantly increasing the LT at 200%, with no effect at 400%. These interesting observations were unique to experiments with platelets and may reflect differences in the procoagulant activity of plasma and platelet-derived factor V(a).[34]

TTP: Factors XI, IX, and VIII generally shortened the TTP in $PFP_{(1\ pM\ TF)}$, but did not affect the TTP in $PFP_{(5\ pM\ TF)}$ (Fig 2.3a, 2.3b). Factor X shortened the TTP in both $PFP_{(1\ pM\ TF)}$ and $PFP_{(5\ pM\ TF)}$. Factor V and fibrinogen produced little to no effect on TTP in either condition. With the exception of prothrombin, the factors produced qualitatively similar effects in $PRP_{(1\ pM\ TF)}$ as in $PFP_{(1\ pM\ TF)}$, with a slight decrease in the TTP with factors XI, IX, VIII, X, and fibrinogen (Fig 2.3c). Effects were similar in the presence and absence of thrombomodulin.

Interestingly, as seen in the LT, whereas prothrombin prolonged the TTP in PFP, it shortened the TTP in PRP. Again, prothrombin's effect may arise in PFP because of prothrombin binding to the constitutively exposed lipid surface of the synthetic lipids, whereas the lipid surface is protected from prothrombin binding on unactivated platelets until

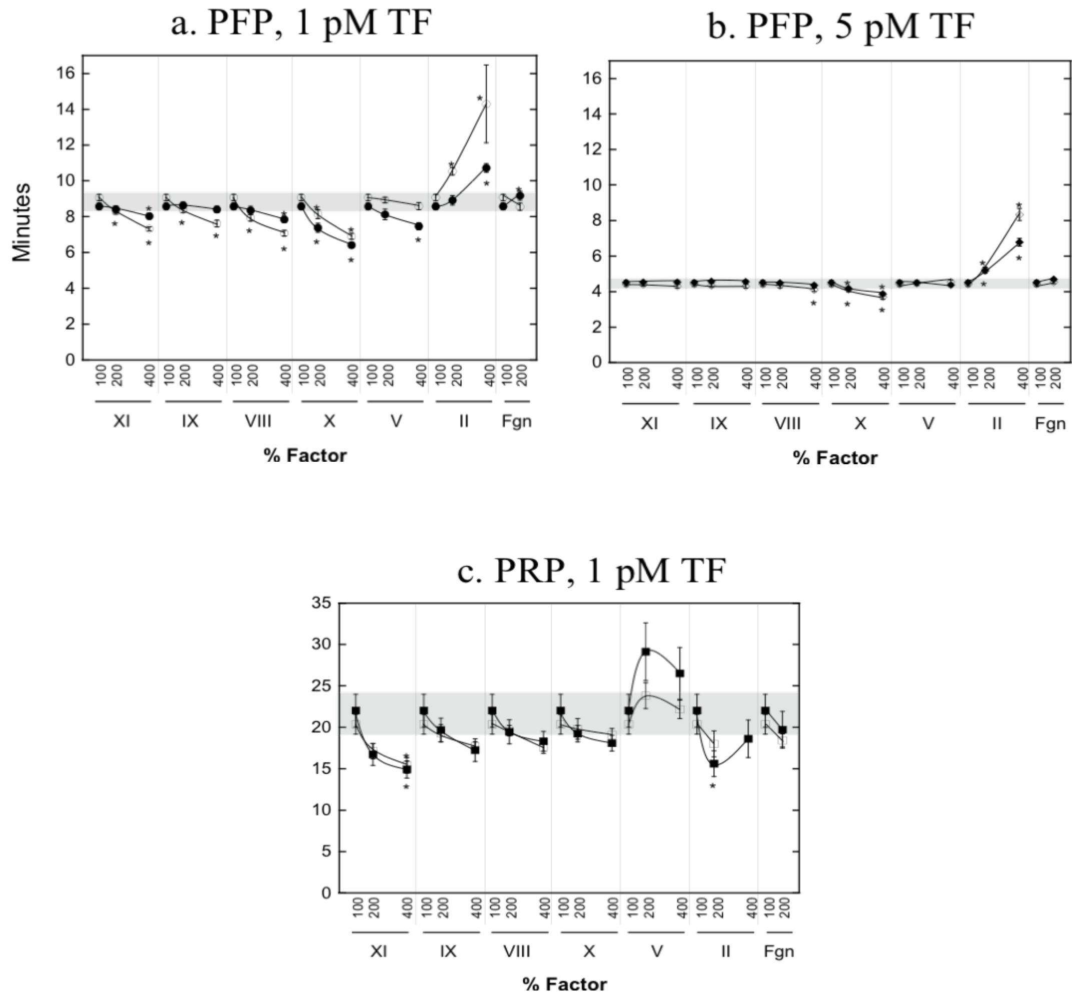

Figure 2.3. Procoagulant factors and thrombomodulin differently influence TTP in PFP and PRP. Reactions were performed as in Fig 2.2. A) $PFP_{(1\ pM\ TF)}$, B) $PFP_{(5\ pM\ TF)}$, C) $PRP_{(1\ pM\ TF)}$. The shaded gray box encompasses the 100% value ±SEM. Data show means ±SEM for the absence (open symbols) and presence (closed symbols) of thrombomodulin. Note y-axis scaling in panel C. *$p<0.0033$ *versus* 100%

the time of activation. Platelet activation permits simultaneous formation of both tenase and prothrombinase, preventing inhibition by high prothrombin concentrations.

Peak height: In contrast to effects on LT and TTP, factors exhibited similar effects on peak height in PFP and PRP; however the magnitude of the effects differed substantially (Fig 2.4a – 2.4c). For all conditions, factors XI, IX, VIII, X, prothrombin, and fibrinogen increased the peak height, whereas factor V had little effect on peak height. Changes were largest in $PFP_{(1\ pM\ TF)}$, followed by $PFP_{(5\ pM\ TF)}$ and $PRP_{(1\ pM\ TF)}$, respectively, but similar in the presence and absence of thrombomodulin for all three conditions.

ETP: As with peak height, factors similarly affected ETP in PFP and PRP; however, the magnitude of the effects differed substantially with the different conditions (Fig 2.5a – 2.5c). Factors IX, VIII, X, prothrombin, and fibrinogen increased the ETP in $PFP_{(1\ pM\ TF)}$, $PFP_{(5\ pM\ TF)}$, and $PRP_{(1\ pM\ TF)}$. Factor XI increased ETP only in reactions initiated with 1 pM TF, in the presence of thrombomodulin. Factor V had little effect on ETP in any condition.

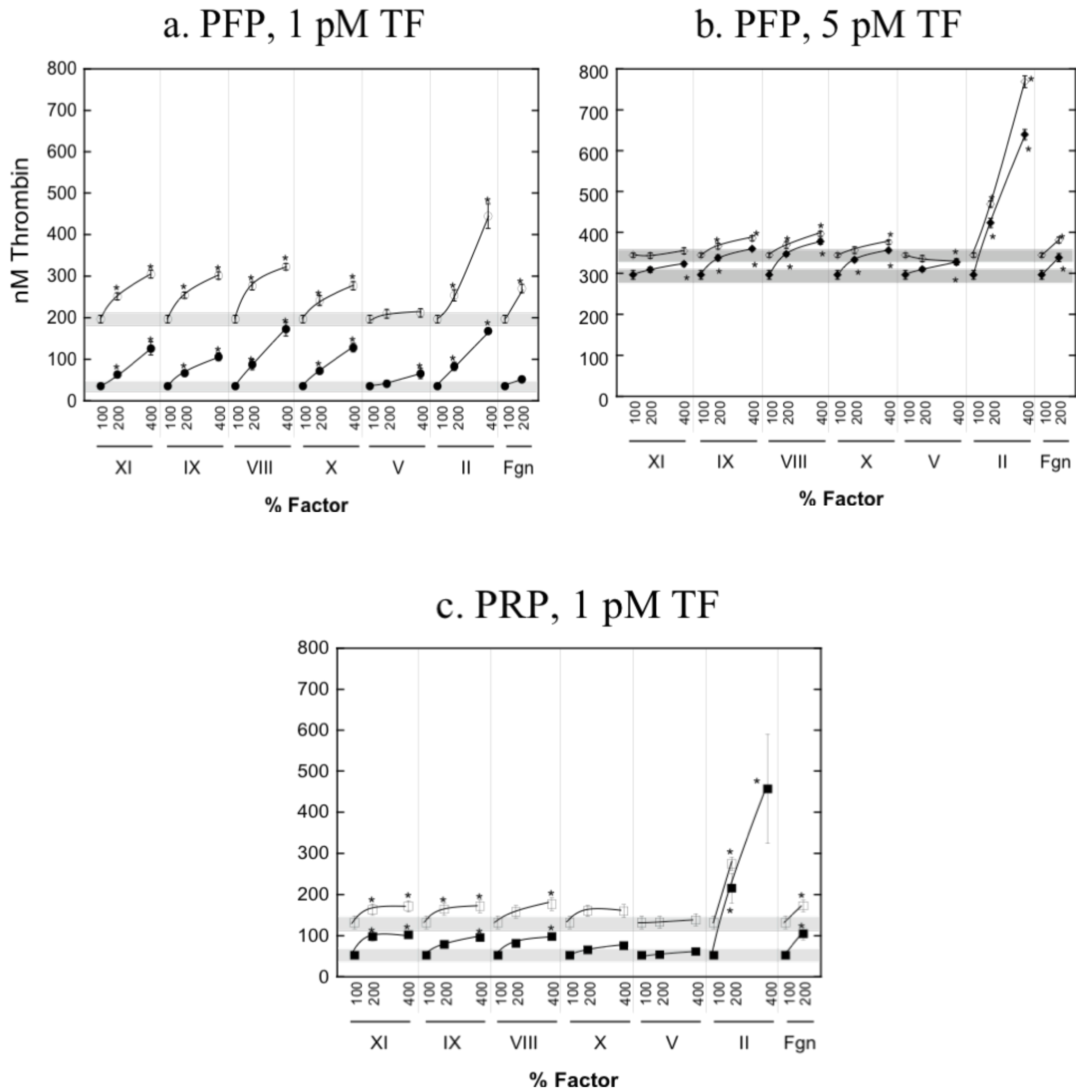

Figure 2.4. Procoagulant factors and thrombomodulin differently influence peak height in PFP and PRP. Reactions were performed as in Fig 2.2 A) $PFP_{(1\ pM\ TF)}$, B) $PFP_{(5\ pM\ TF)}$, C) $PRP_{(1\ pM\ TF)}$. The shaded gray box encompasses the 100% value ±SEM. Data show means ±SEM for the absence (open symbols) and presence (closed symbols) of thrombomodulin. Note the y-axis scaling. *$p<0.0033$ *versus* 100%

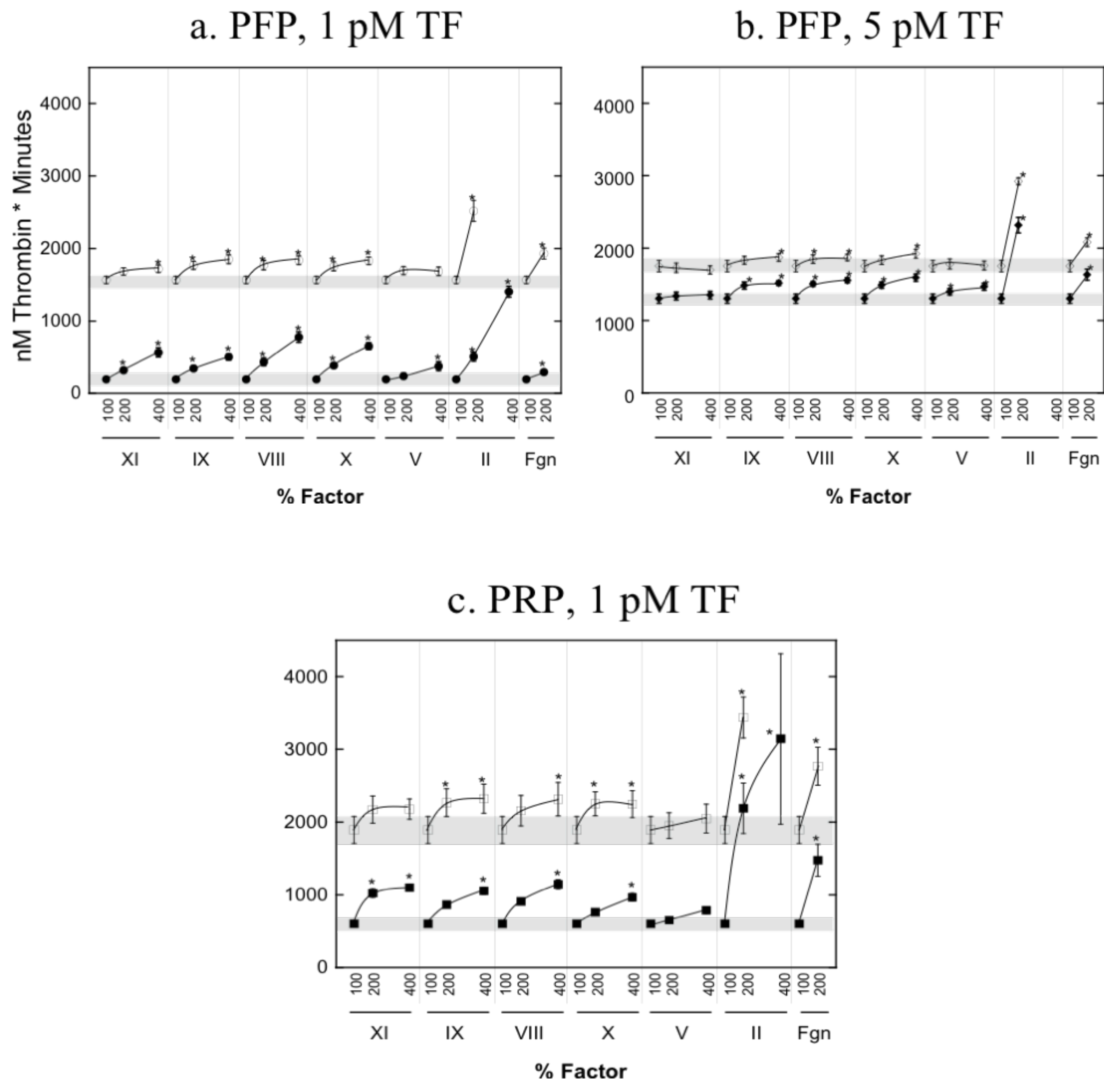

Figure 2.5. Procoagulant factors and thrombomodulin differently influence ETP in PFP and PRP. Reactions were performed as in Fig 2.2 A) $PFP_{(1\ pM\ TF)}$, B) $PFP_{(5\ pM\ TF)}$, C) $PRP_{(1\ pM\ TF)}$. The shaded gray box encompasses the 100% value ±SEM. Data show means ±SEM for the absence (open symbols) and presence (closed symbols) of thrombomodulin. Note the y-axis scaling. *$p<0.0033$ *versus* 100%

2.5 Discussion

Using a range of TF and phospholipid concentrations in the presence and absence of protein C pathway inhibitors, previous studies have correlated VTE risk with elevated peak height[18], ETP[20, 22-25] or both[19, 21]. Other studies have focused on the LT, rate, and peak as an indicator of circulating TF and prothrombotic disease.[19, 35, 36] Although differences in findings between studies have been attributed to differences in assay conditions, few studies have contrasted different assay conditions within a single design, and even fewer under *hyper*coagulable conditions. It has not been explicitly shown that all conditions provide equal sensitivity *and* specificity to detect hypercoagulability. Given limited plasma sample sizes and limited availability of samples from large-scale epidemiologic studies of hypercoagulability, empirical evidence for the use of specific conditions and parameters is essential for designing prospective studies of plasma hypercoagulability. Our study design permitted the *direct* comparison of conditions and parameters to identify those most sensitive and specific to factor-induced hypercoagulability. To our knowledge, this work is the first comprehensive, systematic comparison of these conditions in a single study.

Our analysis shows that in PFP, factors XI, IX, VIII, X, prothrombin, and fibrinogen significantly increased peak height and ETP, though increases were proportionally larger for peak height than ETP. Whereas the baseline peak height of $PFP_{(5\ pM\ TF)}$ was 74% higher than that of $PFP_{(1\ pM\ TF)}$ (344 nM *vs*. 197 nM, respectively, Table 2.1), the apparent "maximum peak height" in $PFP_{(5\ pM\ TF)}$ plus elevated factors XI, IX, VIII, X, or V up to 400% was only 23% higher than that in $PFP_{(1\ pM\ TF)}$ plus elevated factors (396 nM *vs*. 322 nM, respectively). These findings suggest that the maximum observable difference between peak height in

normal and "hypercoagulable" PFP was reduced in assays initiated with higher TF (15% for $PFP_{(5\ pM\ TF)}$ *vs*. 63% for $PFP_{(1\ pM\ TF)}$). Similarly, the maximum ETP in $PFP_{(5\ pM\ TF)}$ with elevated factors XI, IX, VIII, X, or V (1925 nM*min) was only 3.8% higher than that in $PFP_{(1\ pM\ TF)}$ (1854 nM*min). Thus, the maximum observable difference in ETP between PFP and "hypercoagulable" PFP was reduced in $PFP_{(5\ pM\ TF)}$ (9.7%) *versus* $PFP_{(1\ pM\ TF)}$ (18%). This difference is even smaller than that seen for peak height. The exception to these results is in PFP containing elevated prothrombin. Prothrombin (200%) significantly increased peak height and ETP in both in both low (1 pM) (1.29-fold and 1.60-fold, respectively) and high (5 pM) (1.36-fold and 1.66-fold, respectively) TF. These findings suggest that the mechanism limiting thrombin generation in this system results, at least in part, from the prothrombin concentration and indicates that peak height and ETP are exquisitely sensitive to hyperprothrombinemia.

It is widely accepted that PRP may provide greater physiologic relevance in assays of this nature, however studies of PFP persist, in part because of logistical limitations associated with the use of fresh PRP. Our study demonstrates that CAT assays of PFP and PRP show different trends in LT and TTP, but provide qualitatively similar data on peak height and ETP. Thus, studies comparing the presence and absence of platelets are likely to provide consistent information on peak height and ETP, but inconsistent findings on LT and TTP. Differences in response of LT or TTP in PFP and PRP may, however, provide important mechanistic information on the role of platelets and platelet abnormalities in thrombosis.

Epidemiologic studies have correlated factors XI, IX, VIII, X, prothrombin, and fibrinogen, but not factor V, with increased VTE risk.[6-12] In this regard, CAT appears specific to hypercoagulability due to these factors. It is important to note, however, that the

magnitude of change in thrombin generation did not correlate with the degree of thrombotic risk associated with each factor. For example, elevated prothrombin produced a large linear increase in peak height. However, thrombotic risk associated with elevated prothrombin is relatively small (OR ~2.1).[7] Discrepancies between the thrombin generation measurements and clinical risk in the case of elevated prothrombin may result from difficulties in measuring (pro)thrombin concentrations in excess of the antithrombin level present in the plasma. Additionally, although 200% fibrinogen increased the peak height and ETP, its role in VTE risk is controversial. It is unclear whether the effects of fibrinogen on thrombin generation parameters resulted from changes in thrombin generation, itself, or in the ability of fibrinogen ("antithrombin I") to bind thrombin and preserve its proteolytic activity towards small molecular substrates. Thus, CAT may demonstrate disproportionately high peak height and ETP for patients with lower overall risk, reducing the specificity and predictive value of this technique in certain patients.

As a means of distinguishing effects of procoagulant factors on CAT, we noted that whereas several clotting factors significantly increased peak height and ETP, these factors had different and sometimes unique effects on other parameters. For example, in $PFP_{(1\ pM\ TF)}$, elevated levels of factors IX, X, and prothrombin significantly increased peak height and ETP. However, factor IX did not affect the LT, factor X significantly decreased the LT, and prothrombin prolonged the LT in low but not high lipid concentrations (data not shown). Thus, analysis of multiple experimental conditions (*e.g.*, varied lipid concentrations) and/or parameters may be helpful in discerning factors contributing to abnormal thrombin generation in certain PFPs. Indeed, Tripodi *et al*. recently suggested that use of three abnormal thrombin generation parameters (LT, peak height, and ETP) improves the

identification of patients at risk of recurrent VTE *versus* analysis based on a single parameter.[37]

Comparisons of findings between centers have been difficult, as inter-center variability is high partly due to the use of "in house" reagents and protocols. Using a particularly elegant study design, Dargaud *et al*. (2007) showed that the use of different TF and phospholipid sources produces large variability in CAT, but standardizing conditions significantly reduces center-to-center variability.[27] We used commercially-available reagents from the CAT manufacturer, which may offer consistency in results and enable the continued evaluation of these conditions in future studies.

This study has several limitations. First, although thrombomodulin may be helpful in characterizing plasmas with proteins C or S deficiency or factor V Leiden, the physiological thrombomodulin concentration has not been established because it is primarily a cell-associated protein. Thus, the concentration of soluble thrombomodulin that provides the most clinically-useful information has not been determined. Our data suggested that thrombomodulin increased the inter-assay %CV and dampened the effects of 200% fibrinogen at low TF, but did not significantly impact the ability to detect hypercoagulability from the other factors tested. Thus, its general use for detecting protein C pathway abnormalities is compatible with assays used to detect elevated levels of clotting factors. Second, although epidemiological studies have suggested elevated levels of certain clotting factors independently increase VTE risk[6-12], the factor levels we tested were generally higher than those reported in these studies. Of note, however, prothrombin levels as high as 500% of normal have been reported in patients with type 2 diabetes.[38] CAT's ability to detect elevated prothrombin suggests abnormal thrombin generation in these patients would

be readily detected by this technique. Third, our study design compared frozen/thawed, pooled PFP with individual PRP, which may emphasize differences in %CV between PFP and PRP. Fourth, as opposed to tests of individual factor levels, CAT offers the advantage of testing global hemostatic potential. Effects of multiple abnormal coagulation factor levels on thrombin generation may be additive, synergistic, or reflect only the effects of the most limiting factor. Additional studies are warranted to fully appreciate the effects of multiple factor abnormalities on thrombin generation measured with this technique. Finally, it is not clear whether CAT recapitulates the *in vivo* pathologic effects of procoagulant factors; other mechanisms besides thrombin generation may contribute to thrombotic risk.

In sum, we have shown that CAT's ability to detect elevated factors varies between factors and depends on the assay conditions. The largest changes in thrombin generation in response to elevated factors XI, IX, VIII, X, and prothrombin were seen in peak height and ETP in $PFP_{(1\ pM\ TF)}$. Smaller changes were observed in $PFP_{(5\ pM\ TF)}$ and $PRP_{(1\ pM\ TF)}$. Therefore, monitoring the peak height and/or ETP following initiation of clotting in PFP with 1 pM TF is most likely to detect hypercoagulability due to increased procoagulant factor levels.

Our findings support efforts to standardize reagents (TF and lipid concentrations)[27] to reliably achieve the assay conditions necessary for maximal sensitivity. Our data also confirm effects previously seen in normal plasma, but importantly, extend these findings to hypercoagulable situations. Identification of conditions that best identify hypercoagulability and predict VTE warrants further investigation. *In vivo*, VTE risk likely depends on a combination of increased procoagulant and decreased anticoagulant activities, and/or other pathologic mechanisms. In a clinical setting, the concerted use of several different assay

conditions may be necessary to identify patients with distinct clinical phenotypes, not unlike the use of both aPTT and PT to diagnose factor deficiencies.

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Chapter 3

Causal relationship between hyperfibrinogenemia, thrombosis, and resistance to thrombolysis

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3.1 Abstract

Epidemiologic studies have correlated elevated plasma fibrinogen (hyperfibrinogenemia) with risk of cardiovascular disease and arterial and venous thrombosis. However, it is unknown whether hyperfibrinogenemia is merely a biomarker of the pro-inflammatory disease state or is a causative mechanism in the etiology. We raised plasma fibrinogen levels in mice via intravenous infusion and induced thrombosis by ferric chloride application to the carotid artery (high shear) or saphenous vein (lower shear); hyperfibrinogenemia significantly shortened the time to occlusion in both models. Using immunohistochemistry, turbidity, confocal microscopy, and elastometry of clots produced in cell and tissue factor-initiated models of thrombosis, we show that hyperfibrinogenemia increased thrombus fibrin content, promoted faster fibrin formation, and increased fibrin network density, strength, and stability. Hyperfibrinogenemia also increased thrombus resistance to tenecteplase-induced thrombolysis *in vivo*. These data indicate hyperfibrinogenemia directly promotes thrombosis and thrombolysis resistance, and does so via enhanced fibrin formation and stability. These findings strongly suggest a causative role for hyperfibrinogenemia in acute thrombosis and have significant implications for thrombolytic therapy. Plasma fibrinogen levels may be used to identify patients at risk for thrombosis and inform thrombolytic administration for treating acute thrombosis/thromboembolism.

3.2 Introduction

Elevated plasma fibrinogen is associated with risk of cardiovascular disease and arterial and venous thrombosis.[1-9] Several studies have detected dose effects, with increased risk of death or thrombosis in subjects with the highest plasma fibrinogen concentrations.[6-9] The Framingham[7] and Fragmin During Instability in Coronary Artery Disease (FRISC)[8] studies positively correlated fibrinogen levels with risk of cardiovascular disease and incidence of death and/or myocardial infarction, respectively. The Leiden Thrombophilia Study (LETS) showed individuals with elevated fibrinogen levels (4.0-4.9 *versus* <3.0 mg/mL, 130-160% of normal) have an adjusted odds ratio for venous thrombosis of 1.6, while individuals with ≥5 mg/mL fibrinogen (≥170% of normal) have a 4-fold higher thrombotic risk, even after adjusting for C-reactive protein levels.[9] These epidemiologic studies suggest elevated fibrinogen is an independent risk factor for both arterial and venous thrombosis, and therefore a potential diagnostic and therapeutic target for predicting and reducing thrombosis.

Importantly, however, epidemiological studies have not and cannot show a causal relationship between fibrinogen and disease etiology.[2, 10, 11] Fibrinogen levels increase with age, inflammatory processes, hematocrit, hypertension, glucose intolerance, cigarette smoking, and adiposity, and high fibrinogen levels increase plasma viscosity, a demonstrated risk factor for coronary heart disease.[5, 6, 12] These potential confounders have not permitted distinction between fibrinogen's role as a biomarker of inflammation or coincident co-morbidity and a direct, causative role in the etiology of cardiovascular disease.

Prior studies using animal models to clarify the role of hyperfibrinogenemia in thrombosis[13-17] have been equivocal and controversial. Transgenic mice over-expressing

murine fibrinogen (~45% higher than wildtype) demonstrate elevated D-dimer and spontaneous fibrin deposition in the spleen, suggesting hyperfibrinogenemia is mildly prothrombotic.[15] However, these mice demonstrate only marginal shortening of the time to 75% occlusion following 20% $FeCl_3$ application to the carotid artery, indicating hyperfibrinogenemia is not important in arterial thrombosis.[15] In contrast, rabbits treated with turpentine to elevate fibrinogen prior to stasis- or mechanical injury-induced venous thrombosis demonstrate a positive correlation between thrombus size, weight, and fibrin content.[16] However, because turpentine also increases factor VIII, another thrombosis risk factor, the specific prothrombotic contribution of elevated fibrinogen is difficult to discern. One recent study in which the human gamma prime (γ’) chain of fibrinogen was expressed in transgenic mice suggested fibrinogen’s thrombin-binding properties are antithrombotic[17], further questioning a pathologic mechanism relating hyperfibrinogenemia to disease.

The aim of the current study was to determine whether elevated fibrinogen directly contributes to thrombosis and identify the operant mechanism(s). We used *in vivo* models to assess fibrinogen’s effects on thrombus formation and stability, and cell and tissue factor (TF)-based *ex vivo* and *in vitro* methods to identify biochemical and biomechanical mechanisms by which fibrinogen modulates fibrin formation, structure, and function. Our data indicate hyperfibrinogenemia directly and independently shortened the time to occlusion (TTO) and increased thrombus resistance to thrombolysis. These effects were mediated through enhanced fibrin formation and increased fibrin network density and mechanical and fibrinolytic stability. Together, these findings strongly suggest a causative role for hyperfibrinogenemia in the pathology of thrombosis. Information on plasma fibrinogen levels may be used to identify patients at risk for thrombosis and inform thrombolytic

administration for treating arterial and venous thrombosis.

3.3 Materials and Methods

Proteins and materials

Dulbecco's Modified Eagle's Medium with high glucose/2 mM L-glutamine, 0.05% trypsin and ethylenediamine tetraacetic acid (EDTA), and phosphate-buffered saline (10 mM phosphate pH 7.1, 150 mM NaCl, PBS) were from Gibco (Grand Island, NY). Thrombin fluorogenic substrate (Z-Gly-Gly-Arg-AMC) and calibrator (α2-macroglobulin/thrombin) were from Diagnostica Stago (Parsippany, NJ). Factor Xa chromogenic substrate (Pefachrome FXa) was from Pentapharm (Basel, Switzerland). Mouse anti-human TF antibody (HTF-1) was the kind gift of Dr. Ronald Bach (University of Minnesota). Tissue-type plasminogen activator (tPA) and goat anti-mouse and anti-rabbit peroxidase-conjugated antibodies were from Calbiochem (La Jolla, CA). Monoclonal anti-fibrin(ogen) antibody (59D8) was the generous gift of Drs. Marschall Runge [University of North Carolina (UNC) Department of Medicine] and Charles Esmon (Oklahoma College of Medicine). Biotinylated secondary antibodies were from Vector Laboratories (Burlingame, CA). Target Retrieval Solution was from Dako (Carpinteria, CA). Non-immune mouse IgG antibody (MOPC-1), bovine serum albumin (BSA), and adenosine diphosphate (ADP) were from Sigma-Aldrich (Saint Louis, MO). Recombinant human tumor necrosis factor α (TNFα) was from Millipore (Temecula, CA). Corn trypsin inhibitor (CTI) and factor X were from Haematologic Technologies Inc (Essex Junction, VT). Fibronectin-, plasminogen- and von Willebrand factor (vWF)-depleted fibrinogen was from Enzyme Research Laboratories (South Bend, IN). Fibrinogen was further depleted of factor XIII by immunoaffinity chromatography. The AlexaFluor-488 protein labeling kit was from Invitrogen (Carlsbad, CA). AlexaFluor-488-labeled fibrinogen (~8 mole fluorophore/mole fibrinogen) was prepared as described.[18]

Thrombin receptor activation peptide (Serine-Phenylalanine-Leucine-Leucine-Arginine-Asparagine, TRAP) was from Bachem (Torrance, CA). Collagen was from Chrono-Log (Havertown, PA). Tenecteplase (TNKase) was the generous gift of Genentech (San Francisco, CA). Contact-inhibited normal pooled plasma (NPP) was prepared from whole blood from 40 healthy subjects (50% female, 68% non-Caucasian) in a protocol approved by the UNC Institutional Review Board.[19] The fibrinogen concentration in human NPP (3 mg/mL) was determined by enzyme-linked immunosorbent assay.

Murine Thrombosis and Thrombolysis Models

Procedures were approved by the UNC Institutional Animal Care and Use Committee. Mice (6-8 week old male C57BL/6, Charles River Laboratories, Raleigh, NC) were anesthetized with 1.5-2% isofluorane in 2% oxygen, and the left saphenous vein was exposed under a SZX12 dissecting microscope (Olympus, Tokyo, Japan) using a catheter constructed of pulled PE-10 tubing (Braintree Scientific, Braintree, MA) with a 3.0-mil (0.076 mm diameter) cleaning wire (Hamilton, Reno, NV) placed into the lumen as a stylet, as described.[20] Human fibrinogen or vehicle [20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid pH 7.4, 150 mM NaCl (HBS) or HBS/BSA] was administered through the cannula on a per weight basis [blood volume (mL) is 7% of body weight (g), plasma is 50% of blood volume] to achieve 135 or 170% of normal (endogenous murine fibrinogen plus infused human fibrinogen) in murine circulation 5 minutes prior to injury. Final plasma fibrinogen levels were measured as described[21] in mice not subject to ferric chloride ($FeCl_3$) injury. Nanobead diffusion experiments showed these fibrinogen concentrations did not alter plasma viscosity (R. Spero, unpublished observation September 2010).

For carotid artery thrombosis, the right common carotid artery was exposed after midline cervical incision. A Doppler transonic flow probe (Transonic Systems, Ithaca, NY) was applied and connected to a flow meter (model T206, Transonic Systems, Ithaca, NY) supplying a data acquisition system (PowerLab 4/30 model ML866, AD Instruments, Australia). The carotid artery was dried and 10% $FeCl_3$ (0.62 M $FeCl_3$ on 0.5x0.5 mm filter paper) placed on the artery for 3 minutes, removed, and tissues washed 3 times with warm saline. Following injury, blood flow was continuously monitored. For saphenous vein thrombosis, the saphenous vein of the right leg was dissected and exposed, 5% $FeCl_3$ (0.31 M $FeCl_3$ on 0.5x2 mm filter paper) placed on the vein for 3 minutes, removed, and tissues washed 3 times with warm saline. Blood flow was monitored auditorily by Doppler ultrasonic flow probe. In both models, the TTO was the time between $FeCl_3$ administration and lack of flow for 60 consecutive seconds. Experiments were stopped at 45 minutes if no occlusion occurred. Occluded vessels were excised and fixed in 10% formalin.

Thrombolysis was assessed in mice subject to $FeCl_3$ carotid artery thrombosis. After 5 consecutive minutes of blood flow below 0.1 mL/min, mice were infused with TNKase (0.5–5 mg/kg) through the saphenous vein intravenous catheter while continuously monitoring carotid blood flow.

H&E Staining and Immunohistochemistry

Fixed tissues were dehydrated and paraffin-embedded, and consecutive, 5 µm sections cut and mounted with vectamount (UNC Lineberger Comprehensive Cancer Center Animal Histopathology Core). Slides were stained with hematoxylin and eosin to visualize the thrombus, and imaged with a Retiga 400R camera (Q imaging) linked to an Olympus Bx 61

microscope with a 20x U Plan FL N, 0.5 NA objective lens. A computer equipped with Velocity software (v5.5) was used to operate the system. Images were analyzed with Adobe Photoshop CS (v8.0). For immunohistochemistry, antigen retrieval was performed in Target Retrieval Solution in a 95° C water bath. Slides were stained with anti-fibrin antibody (59D8, 1:1000) that detects both human and mouse fibrin(ogen) ([15, 22], Figure 3.1) for 1 hour at room temperature in a humidity-controlled chamber and developed using avidin-biotin complex (Dako). Negative controls were stained simultaneously in the absence of primary antibody. Staining intensity of thrombi from at least four representative sections per mouse were analyzed by three independent, blinded observers on a scale of 0-3.

Platelet Aggregation

Human platelet-rich plasma (PRP) obtained by centrifugation (150xg, 15 minutes) of citrated blood was adjusted to 250×10^3 platelets/mL with autologous platelet-poor plasma (PPP). Aggregation was triggered by TRAP (50 µg/mL, final), collagen (2 µg/mL, final), or ADP (2.5 µM, final). Light transmission was recorded on a Chrono-Log Optical Aggregometer 470.

Cell culture

Primary human saphenous vein endothelial cells (HSVEC, PromoCell, Heidelberg, Germany) and human smooth muscle cells (SMC, Lonza Walkersville, Walkersville, MD), were cultured as directed to 80-95% confluence in 5% CO_2 at 37°C. Cells were used between passages 3-6 to reduce phenotypic drift.

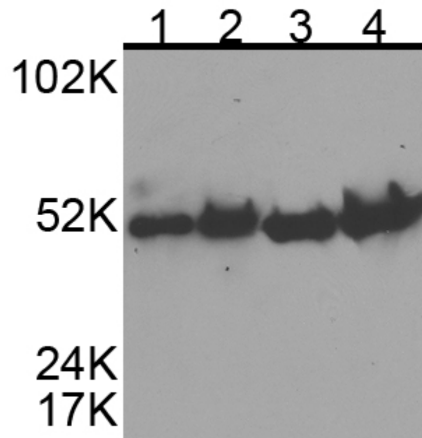

Figure 3.1. 59D8 recognizes both human and mouse fibrin. Human or mouse fibrinogen (0.5 mg/mL, final) was clotted with human thrombin (5 nM, final) in the presence of 10 mM $CaCl_2$. Clots were then dissolved in 12.5 mM EDTA and 40 mM dithiothreitol in 8 M urea for 1 hour at 60°C. Human (lanes 1 and 2) or mouse (lanes 3 and 4) fibrin [3.7 (lanes 1 and 3) or 7 (lanes 2 and 4) µg] samples were separated by SDS-PAGE on a 4-12% Tris-Glycine gel under reducing conditions. Samples were electro-transferred to nitrocellulose, blocked overnight in TBS/1% Tween-20 and then incubated for one hour with the primary antibody against fibrin(ogen), 59D8 (1:250 dilution).

Cellular activity assays

TF activity was measured by chromogenic substrate cleavage on a SpectraMax Plus340 plate reader (Molecular Devices, Silicon Valley, CA) in the presence and absence of inhibitory anti-TF antibody (HTF-1, 10 μg/mL) or isotype control (MOPC-21, 10 μg/mL), as described.[19] Thrombin was measured by calibrated automated thrombography using a Fluoroskan Ascent fluorometer (ThermoLabsystem, Waltham, MA) as described.[19] Thrombin generation was calculated using Thrombinoscope software Version 3.0.0.29 (Thrombinoscope BV, Maastricht, Netherlands). The thrombin generation rate was calculated by dividing peak height by the difference from time to peak to lag time. The endogenous thrombin potential is not reported because thrombin generation curves did not always return to baseline.

Phospholipid vesicles

Phosphatidylcholine (egg), phosphatidylethanolamine (soy), and phosphatidylserine (porcine brain) were from Avanti Polar Lipids (Alabaster, AL). Large unilamellar vesicles (41% phosphatidylcholine /44% phosphatidylethanolamine/15% phosphatidylserine) were made as described.[23] Briefly, lipids were combined, dried under nitrogen gas, and resuspended in cyclohexane. Resuspended lipids were lyophilized, resuspended in HBS containing 1 mM EDTA, and extruded through a 0.2 μm filter ten times.

Clot formation and lysis by turbidity

For human plasma experiments, recalcified (16 mM, final), lipidated (4 μM, final) NPP was spiked with fibrinogen to 4.5, 6, or 7.5 mg/mL (150%, 200%, and 250% of normal,

respectively), final, and immediately added to washed cell monolayers (67.7% plasma, final). For mouse plasma experiments, recalcified (16 mM, final) murine PPP containing 2.4 ± 0.2 mg/mL fibrinogen (100%) was spiked with human fibrinogen to approximately 180% and 270% of normal, diluted 1:3 in HBS, and clotted with TF (Innovin 1:30,000 final). Fibrinolysis assays included tPA (250 ng/mL, final) or TNKase (concentrations indicated) at the reaction start. Clotting and lysis were detected by turbidity at 405 nm in a SpectraMax Plus340 plate reader.[18, 19]

Laser scanning confocal microscopy (LSCM)

Clots were formed over washed cells in Lab-Tek II Chamber #1.5 coverglasses (Nalge Nunc International, Rochester, NY) with addition of AlexaFluor-488–labeled fibrinogen as described.[18, 19] Clotting proceeded until a constant final turbidity was reached in separate, parallel reactions. Clots were imaged on a Zeiss LSM5 Pascal laser scanning confocal microscope (Carl Zeiss, Inc) linked to a Zeiss Axiovert 200M microscope equipped with a Zeiss 63x 1.4 NA oil immersion plan apo-chromatic lens, as described. The 488 nm line of a medium power multi-line argon ion laser was used for excitation and a 505-530-nm band-pass filter for emission. A computer equipped with Carl Zeiss software (v1.5) was used to operate the system. Optical sectioning was achieved by closing the pinhole in the front of the detector to one airy unit. The zoom factor was 1. Thirty optical sections (1024x1024 pixels) in 3 randomly-chosen locations were collected at 0.36-μm intervals in the z-axis at the cell surface. Image volumes were 146 x 146 x 10 μm. Single images were collected in 15.47 seconds. Optical resolution was ~0.14 um in the xy-plane and ~0.5 um on the z-axis. The sectioning interval in z was smaller than the calculated z- axis optical section resolution to

achieve Nyquest sampling in z based on the Zeiss software calculation. Images were deconvolved using 3-dimensional deconvolution algorithms in AutoQuant's Autodeblur (Version x1.4.1; Media Cybernetics Inc., Bethesda, MD). Fibrin network density was analyzed using ImageJ (1.37V; National Institutes of Health) by placing random grids of 2 pixel crosses on individual slices (121-144 crosses/slice) and counting fibers intersecting the middle of the crosses divided by the total number of crosses, less crosses in the volume occupied by the cells, as described.[18, 19]

Clot viscoelastometry

Human PRP and PPP from blood drawn into 3.2% sodium citrate/18.3 μg/mL CTI was recalcified (16 mM, final) and spiked with fibrinogen or BSA in HBS. PRP and PPP had $>300x10^3$ and $<8x10^3$ platelets/mL, respectively. The baseline PRP and PPP fibrinogen concentration was estimated in accord with NPP determinations. PRP was clotted with TF (1 pM, final), which corresponds to the TF activity of TNF-α-stimulated HSVEC (TNFα-HSVEC). PPP was clotted with 1 pM TF and 4 μM phospholipid. Lysis assays were performed in the presence of tPA (500 ng/mL, final). Clot elastic modulus (CEM) was measured by Hemodyne HAS™.[24]

Statistical Methods

For TTO and immunohistochemistry, normal and hyperfibrinogenemic conditions were compared by Mann-Whitney test. For CEM, normal and hyperfibrinogenemic conditions were compared by unpaired Student's t tests. For clotting assays, fibrin density, and fibrinolysis parameters, significant differences between groups were identified by one-way

analysis of variance and analyzed by Dunnett's post-hoc test using 3 mg/mL fibrinogen as the index group (on unstimulated HSVEC, SMC or TNFα-HSVEC as indicated) to limit Type I error. Statistical analyses were performed using Kaleidagraph v4.1 (Synergy Software, Reading, PA).

3.4 Results

Hyperfibrinogenemia shortens the time to vessel occlusion after $FeCl_3$ injury.

To determine the contribution of elevated fibrinogen to intravascular thrombus formation, we utilized two murine thrombosis models based on $FeCl_3$ application to the carotid artery (high shear) or saphenous vein (lower shear) following intravenous infusion of human fibrinogen. This infusion-based approach enabled us to precisely control the plasma fibrinogen level during thrombus formation. Control experiments and published studies demonstrate human fibrinogen is incorporated into murine clots, supports murine platelet aggregation[25], and has appropriate half-life[26] in mouse circulation. The endogenous fibrinogen concentration in mice was 2.4±0.2 mg/mL (100%) and levels were raised to 3.2±0.2 or 4.0±0.1 mg/mL, final (135 or 170% of normal, respectively), consistent with levels associated with thrombosis in humans.[7, 9]

Consistent with transgenic hyperfibrinogenemic mice[13, 15] and a prior study in which human fibrinogen was injected into BALB/c mice[26], elevated fibrinogen did not trigger spontaneous thrombosis (Figure 3.2). $FeCl_3$ application to the carotid artery or saphenous vein of HBS-infused mice produced occlusive thrombi in 9.3 and 17 minutes (median values), respectively, confirming the prothrombotic effect of vascular disruption. Infusion of control protein (BSA) did not further shorten the TTO in either vessel (data not shown). Mice infused with fibrinogen to 3.2±0.2 mg/mL (135%) demonstrated a non-significant trend to shorter TTO in the carotid artery model (data not shown); we did not test this fibrinogen concentration in the saphenous vein model. Interestingly, compared to control (HBS or BSA infusion), fibrinogen infusion to 4.0±0.1 mg/mL final (170%) prior to $FeCl_3$ injury

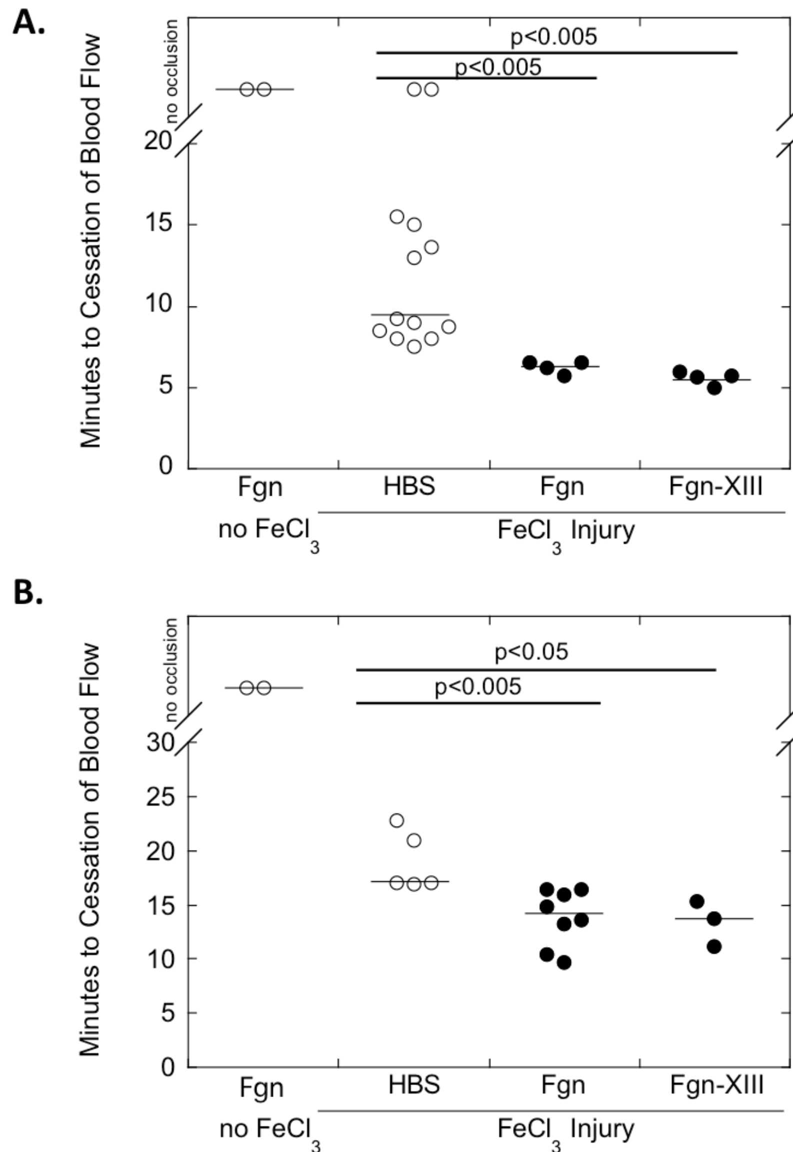

Figure 3.2 Elevated fibrinogen shortens the time to vessel occlusion following $FeCl_3$ injury. Wildtype C57Bl/6 mice were infused with HBS or fibrinogen (plasminogen-, fibronectin-, and vWF-depleted or plasminogen-, fibronectin-, vWF-, and factor XIII-depleted) to 170% of normal. Thrombosis was induced by $FeCl_3$ application to the carotid artery (A) or saphenous vein (B), and the TTO was determined by flow probe or Doppler, respectively. In vessels that did not occlude, the TTO was recorded as 45 minutes. Each point represents a separate mouse. Lines show median values.

significantly ($p<0.005$) shortened the TTO in both carotid artery and saphenous vein models (6.4 and 14.2 minutes (medians), respectively, Figure 3.2). The shortened TTOs were not due to factor XIII in the fibrinogen preparation; infusion of factor XIII-depleted fibrinogen also significantly ($p<0.05$) shortened the TTO *versus* control mice (Figure 3.2). These data demonstrate a direct contribution of hyperfibrinogenemia to thrombus formation following vascular injury.

Elevated fibrinogen increases the fibrin(ogen) content of thrombi.

To probe the mechanism for the shortened TTO, we first examined the morphology of thrombi formed in the murine carotid artery and saphenous vein. H&E staining showed extensive, occlusive thrombi containing distinct regions of proteinaceous material and erythrocytes in injured vessels from both control and fibrinogen-injected mice (Figures 3.3A, 3.3B). Immunohistochemistry of thrombi in carotid artery and saphenous vein thrombi from control (HBS or BSA-infused) mice demonstrated weak fibrin staining concentrated primarily at the luminal edge of proteinaceous regions. No staining was detected in the absence of primary antibody, confirming that the secondary antibody did not bind mouse tissue non-specifically (Figure 3.3A, 3.3B). Staining was slightly more intense in thrombi from saphenous vein than carotid artery, consistent with higher fibrin production in lower shear conditions.[27] Both carotid artery and saphenous vein thrombi from mice infused with human fibrinogen demonstrated more intense fibrin staining at the periphery of proteinaceous regions and intense, diffuse staining in regions containing erythrocytes (Figures 3.3C, 3.3D). These findings suggest hyperfibrinogenemia increased thrombus fibrin content in both high and low shear vessels.

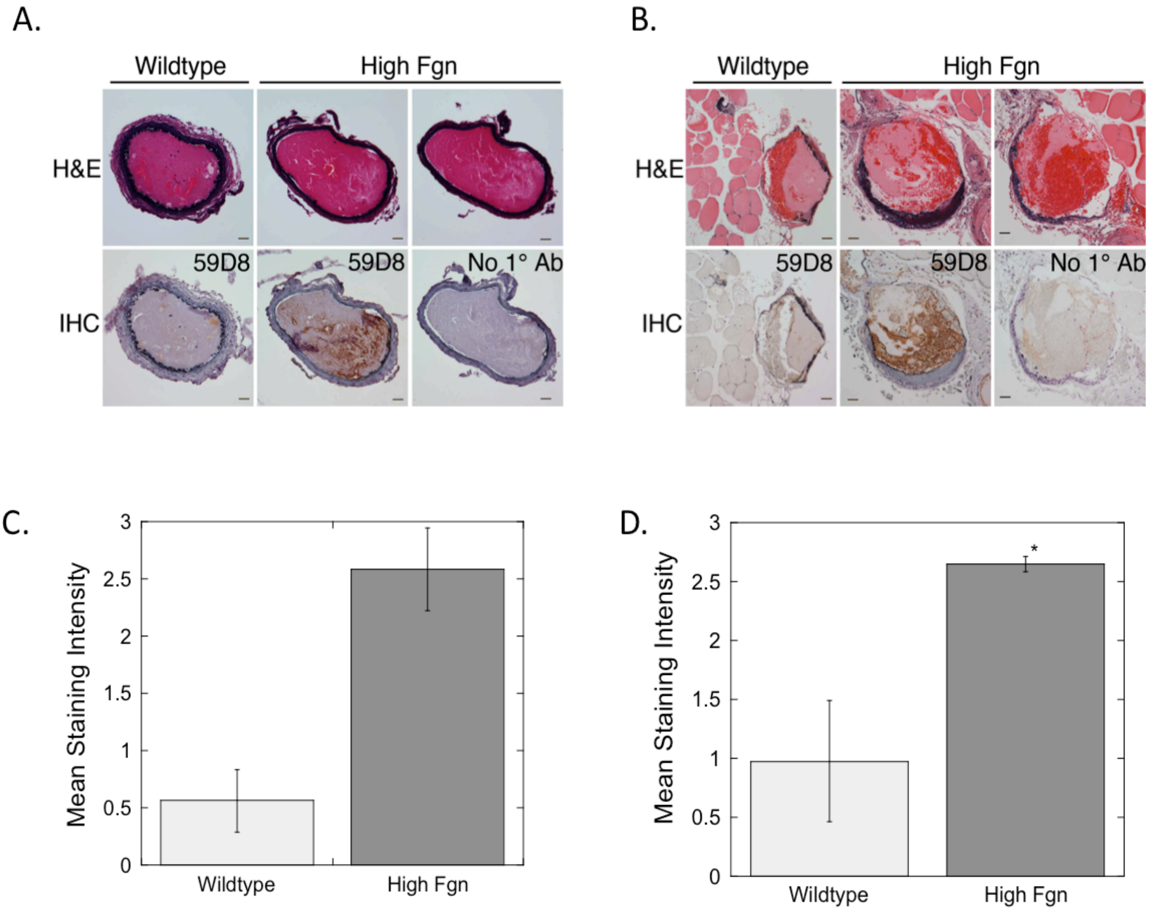

Figure 3.3. Elevated fibrinogen increases fibrin(ogen) incorporation into thrombi following $FeCl_3$ injury. Representative sections through thrombi following $FeCl_3$ injury to the carotid artery (A) or saphenous vein (B). H&E staining shows regions of protein and packed erythrocytes. Immunohistochemistry (IHC) for fibrin (59D8) on corresponding sections shows darker staining for fibrin(ogen) at the thrombi margins and in thrombi from hyperfibrinogenemic (170% fibrinogen) mice. "No 1° Ab" indicates antibody 59D8 was omitted from IHC as a negative control. Staining intensity (on a scale of 0-3) was normalized to assign the value of '3' to the most intensely stained section in each vessel, separately. Mean staining intensity from immunohistochemistry images from three separate carotid arteries (C) or four separate saphenous veins (D) for each condition was determined as described in Methods and compared to control (wildtype) carotid artery ($p=0.1$) and saphenous vein (*, $p<0.05$), respectively.

***In vitro* platelet aggregation is not increased by elevated fibrinogen levels**.

We then tested whether hyperfibrinogenemia increased platelet aggregation, a process dependent on fibrinogen binding to platelet αIIbb3. The endogenous fibrinogen concentration of human plasma was 3 mg/mL (100%), and fibrinogen levels were raised with additional human fibrinogen as indicated. As shown in Figure 3.4 and Table 3.1, elevated fibrinogen did not increase, and even slightly decreased, platelet aggregation induced by TRAP, collagen, or ADP, consistent with a mechanism where high fibrinogen promotes full occupancy of platelet αIIbβ3 and inhibits inter-platelet bridging.[28] These data suggest the mechanism by which hyperfibrinogenemia shortened the TTO was not via enhanced platelet aggregation.

Both cellular procoagulant activity (PCA) and elevated fibrinogen increase fibrin formation.

We next determined the effect of fibrinogen level on fibrin formation in models of vasculature by incubating cultured cell monolayers with recalcified NPP spiked with fibrinogen. We used unstimulated HSVEC to model unperturbed endothelium and SMC to model $FeCl_3$-injured vessels. We also used TNFα-HSVEC (100 ng/mL TNFα for 6 hours) to model intact cytokine-stimulated endothelium thought to promote clotting in venous thrombosis (Figure 3.5). While quiescent endothelial cells *in vivo* do not express significant TF, cultured unstimulated endothelial cells exhibit low TF activity.[19] However, the net PCA of cultured HSVEC was significantly lower than cultured SMC or TNFα-HSVEC (Figure 3.5, Table 3.2).

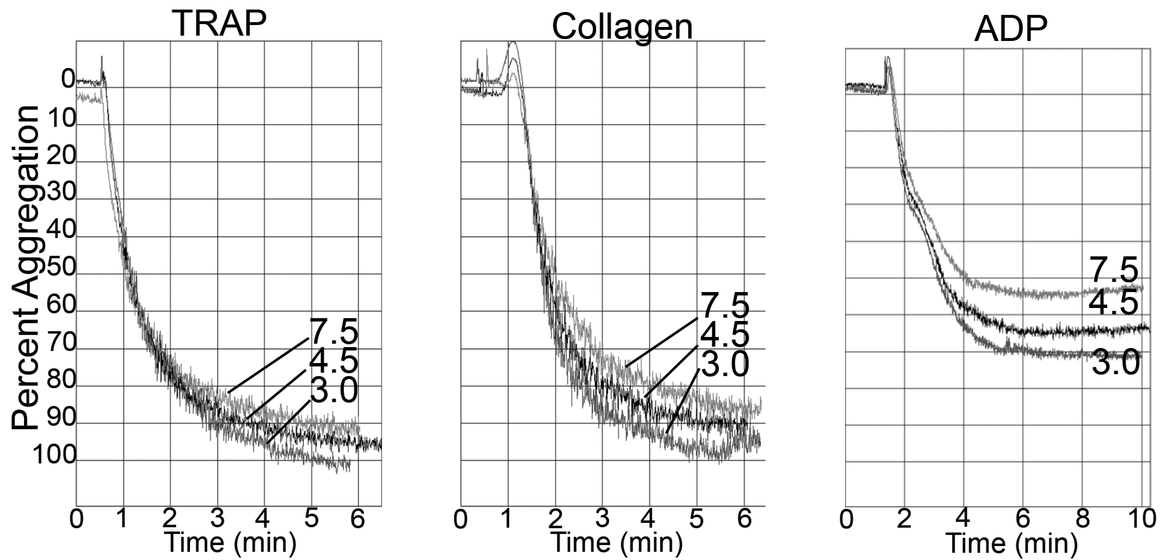

Figure 3.4 Fibrinogen does not increase platelet aggregation. Platelet aggregation in human PRP obtained from citrated whole blood spiked with human fibrinogen (final concentrations indicated in the figure) was triggered by addition of 50 µg/mL TRAP (A), 2 µg/mL collagen (B), or 2.5 µM ADP (C) and monitored by turbidity as described in Methods. Graphs shown are from one experiment, representative of 4-6 experiments with each agonist.

Table 3.1. *In vitro* aggregation of human platelets is not enhanced in the presence of elevated fibrinogen.

	TRAP			Collagen			ADP		
Fibrinogen (mg/mL)	**3**	**4.5**	**7.5**	**3**	**4.5**	**7.5**	**3**	**4.5**	**7.5**
Lagtime (s)	13.4±3.8	11.9±2.3	10.7±2.7	78.1±22	67.7±16	62.0±20	19±1.2	21.3±1.5	21.3±2.2
Rate (%/s)	77.9±8.8	70.4±8.8	61.3±15	90.1±25	77.1±21	*60.3±23	54.5±11	51.3±8.4	45.8±5.9
Maximum Amplitude (%)	92.3±13	88.0±12	74.9±20	79.6±17	74.0±18	68.6±22	81.0±6.6	74.5±12	68.5±11

Differences between groups were identified by a one-way analysis of variance and analyzed by unpaired Student's t tests using 3 mg/mL fibrinogen as the index group. *$P < 0.05$ vs. 3 mg/mL fibrinogen. Data show mean±SD

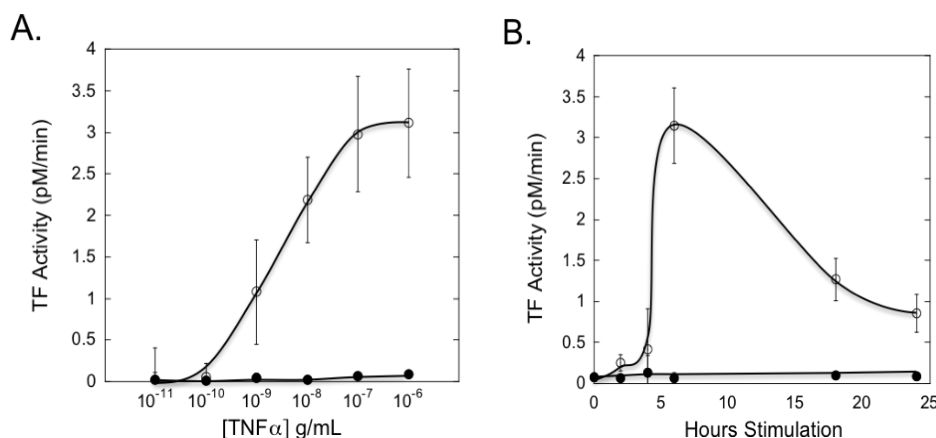

Figure 3.5 TNFα upregulates TF activity on HSVEC in a dose- and time-dependent manner. HSVEC were incubated with A) TNFα (0-10 μg/mL) for 6 hours, or B) 100 ng/mL for 0-24 hrs. Factor Xa generation (±SD) was measured by incubating cells with factors VIIa and X in the presence of $CaCl_2$ and absence (open circles) or presence (closed circles) of anti-TF inhibitory antibody (HTF-1), and measuring factor Xa by chromogenic substrate in 3 separate experiments. Data were converted to TF activity by comparison with a standard curve.

Table 3.2 TF activity and thrombin generation supported by HSVEC, SMC, and TNFα-HSVEC

	HSVEC	SMC	TNFα-HSVEC[A]
TF Activity			
-HTF1 (pM/min)	0.073±0.012	*12.08±0.443	*3.141±0.459
+HTF1 (pM/min)	0.075±0.007	0.973±0.021	0.066±0.066
Thrombin Generation			
Lag Time (min)	41.9±12.1	*2.7±0.2	*6.1±1.7
Rate (nM/min)	11.5±5.0	*77.1±16.6	21.0±1.7
Time to Peak (minutes)	48.0±11.6	*6.8±0.6	*11.6±1.3
Peak Height (nM)	54.1±21.6	*274.5±48.6	*113.9±10.9

[A]HSVEC treated with 100 ng/mL TNFα for 6 hours
*P < 0.05 vs. unstimulated HSVEC

These experiments demonstrated unique effects of cellular PCA and fibrinogen level on fibrin formation (Figures 3.6A-C). Relative to unstimulated HSVEC, increased PCA of SMC and TNFα-HSVEC (Figure 3.5, Table 3.2) significantly ($p<0.002$) shortened the fibrin formation onset (Figure 3.6D), consistent with the premise that exposure of procoagulant cells to blood triggers clotting. Elevated fibrinogen trended toward a shortened clotting onset in reactions induced by unstimulated HSVEC, but did not reach statistical significance due to large variability. In addition, fibrinogen did not further shorten the onset in reactions triggered by the more procoagulant SMC or TNFα-HSVEC. Elevated fibrinogen did, however, significantly ($p<0.001$) increase peak turbidity (Figure 3.6E), indicating increased fibrin(ogen) incorporation into clots. Interestingly, relative to NPP clots produced by unstimulated HSVEC, both cellular PCA [SMC ($p=0.08$) and TNFα-HSVEC ($p<0.02$)] and hyperfibrinogenemia ($p<0.001$) increased the fibrin formation rate (Figure 3.6F). We observed a similar increase in the rate and final turbidity of TF-initiated clotting of murine plasma spiked with human fibrinogen (Figure 3.6G). This increase in the rate and amount of fibrin production is consistent with the shortened TTO observed in the murine thrombosis model (Figure 3.2).

Both cellular PCA and elevated fibrinogen increase fibrin network density.

We and others have previously correlated fibrin formation parameters with fibrin network structure.[18, 19, 29, 30] To assess the impact of elevated fibrinogen on fibrin structure, we used LSCM to examine NPP clots produced by unstimulated HSVEC, SMC, and TNFα-HSVEC with increasing fibrinogen concentrations. Clots produced by SMC and TNFα-HSVEC were composed of more densely-packed fibers ($p<0.05$) than those produced by

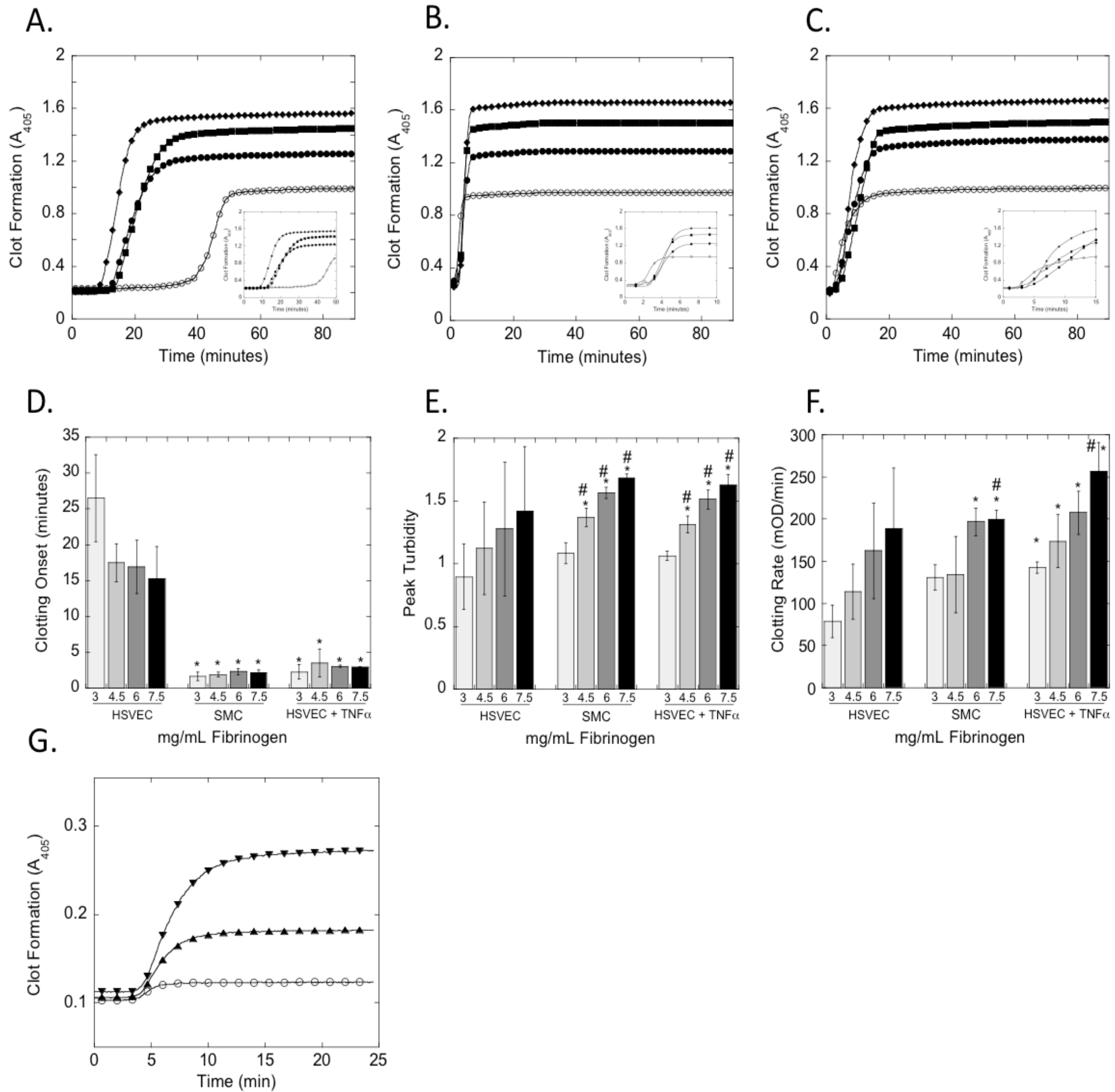

Figure 3.6 Both cellular PCA and elevated fibrinogen promote fibrin formation. A-F) Recalcified (16 mM, final) human NPP spiked with fibrinogen or HBS was added to confluent cell monolayers. Fibrin polymerization was measured by turbidity at 405 nm. Panels A-C show polymerization curves representative of four independent experiments with human NPP and unstimulated HSVEC (A), SMC (B), and TNFα-HSVEC (C). Insets expand the x-axis (time) for each panel. Symbols are: 3 (open circles), 4.5 (closed circles), 6 (closed squares), and 7.5 (closed diamonds) mg/mL fibrinogen, final. Panels D-F show the onset, final turbidity, and fibrin formation rate (mean±SD) of all four experiments with human NPP, respectively. *: $p<0.05$ *versus* 3 mg/mL fibrinogen on HSVEC, #: $p<0.05$ *versus* 3 mg/mL within each cell type. G) Recalcified murine PPP was spiked with human fibrinogen or HBS, diluted 1:3 in HBS, and clotting was initiated with TF addition (Innovin 1:30,000 final) and monitored by turbidity at 405 nm. Symbols are: 2.4 (open circles), 4.4 (closed triangles), or 6.4 (closed inverted triangles) mg/mL fibrinogen, final. Polymerization curves are from a single experiment representative of four independent experiments.

unstimulated HSVEC (Figure 3.7), consistent with observations that high thrombin generation promotes formation of dense networks of thin fibers.[18, 19, 29] Notably, fibrin network density also correlated positively and significantly ($p<0.001$) with fibrinogen concentration in both human (Figures 3.7A, B) and murine (Figure 3.7C) plasmas, suggesting elevated fibrinogen levels promote formation of abnormally dense fibrin networks.

Elevated fibrinogen increases clot mechanical strength.

Fibrin network structure determines clot viscoelasticity[24, 30], suggesting elevated fibrinogen alters clot mechanical properties. To assess the impact of fibrinogen level on clot elasticity, we measured the CEM in clots forming in human PRP or PPP spiked with fibrinogen to 6 mg/mL, final (200%) or BSA (control). Because these assays cannot be initiated by cell monolayers, we initiated reactions with 4 μM phospholipid and 0 (to model unstimulated endothelium) or 1 (to model vascular disruption) pM TF. No CEM developed in the absence of added TF reflecting the requirement for a procoagulant stimulus (i.e., vascular disruption) to initiate clotting, even in hyperfibrinogenemic conditions. Initiation of clotting by TF showed a significant ($p<0.05$) fibrinogen-dependent increase in peak CEM in both PRP and PPP (Figure 3.8A) suggesting elevated fibrinogen increases the structural integrity (mechanical strength) of the clot.

Elevated fibrinogen increases plasma clot resistance to fibrinolysis.

Fibrin network density also determines a clot's resistance to fibrinolysis.[18, 19, 31] To evaluate the effect of elevated fibrinogen on clot resistance to fibrinolysis, we utilized both turbidimetric and mechanical (elastometry) lysis assays. We first initiated clotting by

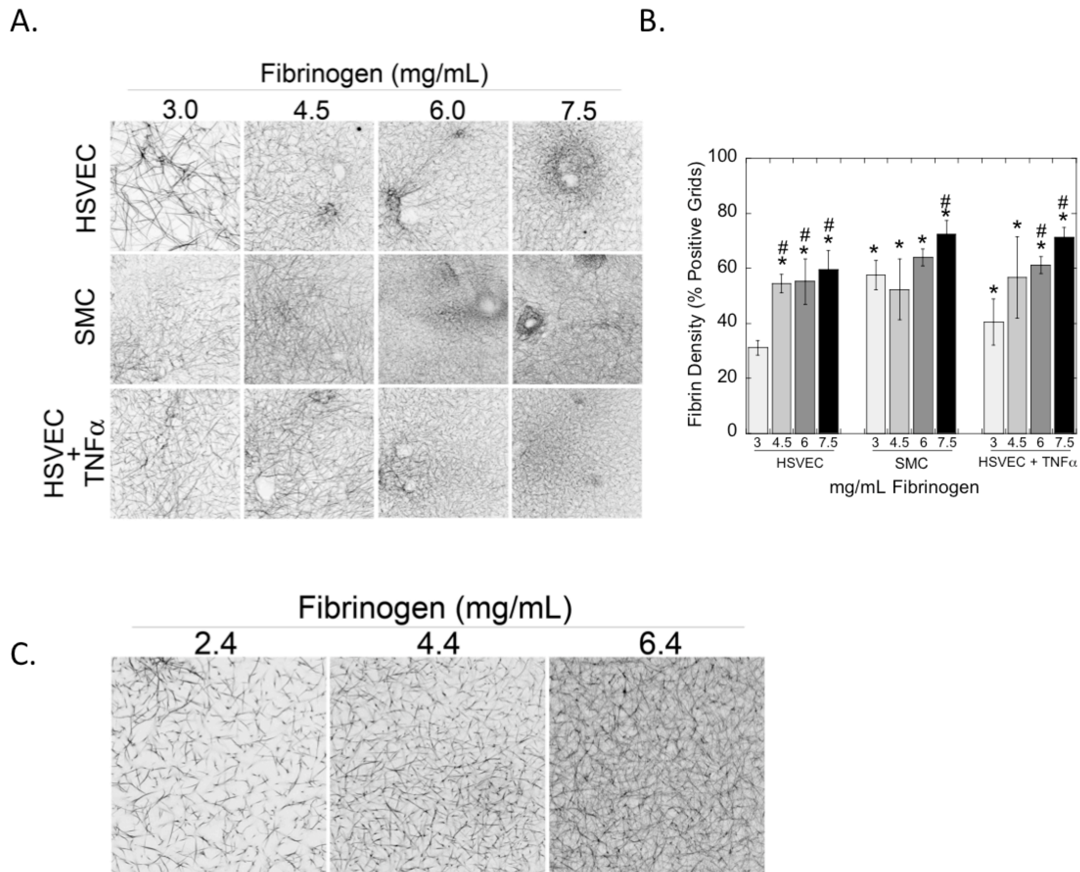

Figure 3.7 Both cellular PCA and the fibrinogen level modulate fibrin network density. A and B) Clots were formed by incubating unstimulated HSVEC, SMC and TNFα-stimulated HSVEC monolayers with recalcified human NPP spiked with human fibrinogen or BSA, as indicated, and imaged by LSCM as described.[18, 19] A) Representative micrographs (146x146 μm, xy) show three-dimensional projections from 10 μm stacks at the cell surface (n≥3). Darker areas show increased fibrin density. B) Fibrin network density (mean±SD) of clots was determined as described in Methods. *: $p<0.05$ *versus* 3 mg/mL fibrinogen on HSVEC, #: $p<0.05$ *versus* 3 mg/mL within each cell type. C) Clots were formed by addition of TF (1:30,000 Innovin) to recalcified murine PPP spiked with human fibrinogen or HBS.

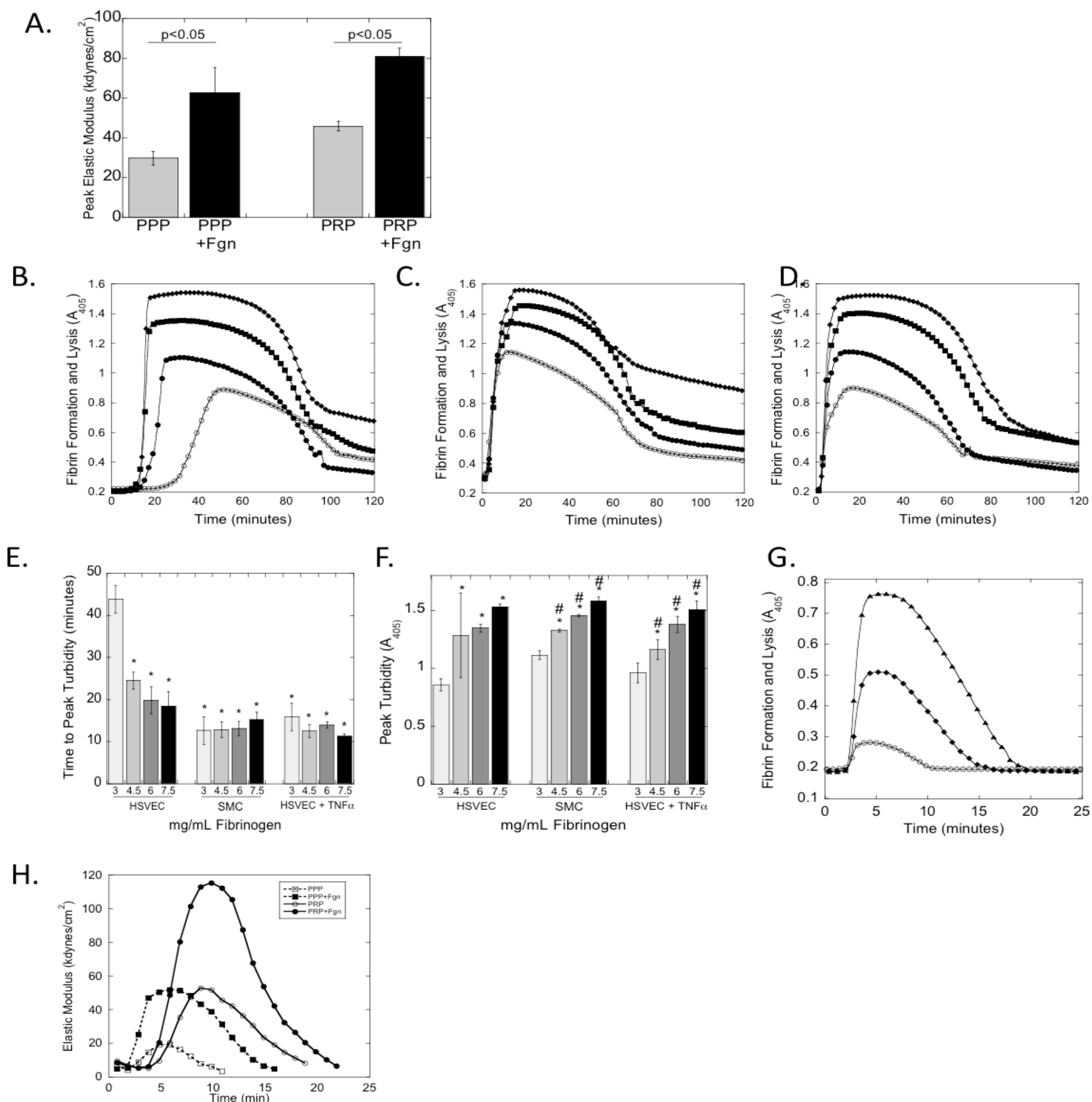

Figure 3.8 Elevated fibrinogen increases clot stability. A) Human PRP and PPP prepared from CTI-inhibited whole blood was spiked with fibrinogen (to 6 mg/mL final, 200%) or BSA, recalcified, and clotted with TF (Methods). Bars show peak CEM (mean±SD). B-D) Recalcified human NPP spiked with fibrinogen or control was added to confluent cell monolayers. Fibrin polymerization was initiated in the presence of tPA; clotting and lysis were measured by turbidity at 405 nm. Panels B-D show representative turbidity curves with human NPP and unstimulated HSVEC (B), SMC (C), and TNFα-HSVEC (D). Symbols are: 3 (open circles), 4.5 (closed circles), 6 (closed squares), and 7.5 (closed diamonds) mg/mL fibrinogen, final. E) Time to peak turbidity and F) peak turbidity (mean±SD, n=4), respectively. *: $p<0.05$ vs 3 mg/mL fibrinogen on HSVEC, #: $p<0.05$ vs 3 mg/mL within each cell type. G) Recalcified murine PPP was spiked with human fibrinogen or HBS to achieve 2.4 (open circles), 4.4 (closed triangles), or 6.4 (closed inverted triangles) mg/mL fibrinogen, final, diluted 1:3 in HBS, and clotting was initiated with TF (Innovin 1:30,000 final) and monitored by turbidity. Figure shows representative polymerization curves (n=2). H) Representative elastometry curves (n=3) of human PRP and PPP prepared from CTI-inhibited whole blood, spiked with human fibrinogen (to 6 mg/mL, final) or BSA, recalcified and clotted with TF in the presence of tPA (Methods). The longer initiation phase of PRP clots *versus* PPP clots reflects the time to platelet activation.[32]

Table 3.3 Elevated fibrinogen increased clot elastic modulus during lysis.

	Peak CEM	Area Under Curve	Half-lysis
PPP	1	1	1
PPP+Fibrinogen	*2.7±0.6	*3.9±1.5	*1.3±0.1
PRP	1	1	1
PRP+Fibrinogen	*2.2±0.6	*2.6±1.3	1.3±0.5

Data are presented as fold increase over PPP or PRP with no additional fibrinogen. *$P < 0.05$ vs. no additional fibrinogen

incubating human NPP spiked with fibrinogen or HBS with HSVEC, SMC, and TNFα-HSVEC in the presence of tPA and monitored clotting and lysis by turbidity (Figures 3.8B-D). Compared to unstimulated HSVEC, SMC and TNFα-HSVEC significantly ($p<0.05$) shortened the time to peak turbidity of NPP clots (Figure 3.8E). In contrast, elevated fibrinogen significantly ($p<0.05$) shortened the time to peak turbidity only on unstimulated HSVEC, but increased peak turbidity on all cell types. We observed a similar pattern in mouse PPP spiked with human fibrinogen (Figure 3.8G). These data indicate cellular PCA triggers fibrin formation, but fibrinogen concentration dictates fibrin incorporation into the clot and its resistance to lysis.

We then evaluated the effects of elevated fibrinogen on mechanical stability during tPA-mediated lysis of human PRP and PPP clots. Compared to control, addition of fibrinogen [to 6 mg/mL, final (200%)] increased peak CEM, area under the lysis curve, and half lysis time (Figure 3.8H, Table 3.3). Because fibrinogen produced similar effects in both PRP and PPP, these assays support findings that elevated fibrinogen promotes resistance of clots to fibrinolytic and mechanical disruption by a fibrin- (not platelet-) dependent mechanism.

Elevated fibrinogen increases resistance to thrombolysis *in vivo*

Finally, because *in vitro* experiments suggested elevated fibrinogen increased clot stability, we assessed thrombolysis *in vivo* in mice with normal and elevated fibrinogen. Due to the small size of the saphenous vein and its proximity to the saphenous artery, it was not feasible to continuously monitor blood flow during thrombolysis in the saphenous vein. Therefore, thrombolysis was only performed in the carotid artery model. Following occlusive thrombus formation, we initiated lysis via bolus infusion of the tPA analog

TNKase. TNKase's increased fibrin specificity and enhanced resistance to inhibition by plasminogen activator inhibitor-1[33] provides a longer plasma half-life and facilitates dosing regimens (single IV bolus *versus* continuous infusion required for tPA/alteplase). Lysis of murine PPP clots was TNKase-dose-dependent (Figure 3.9A). Importantly, human and murine fibrin are similarly cleaved by murine plasmin[34], and TNKase-mediated lysis was similarly dose-dependent with regard to fibrinogen [human (Figure 3.9B) or mouse (Figure 3.9C)] spiked into murine PPP.

TNKase infusion produced dose-dependent lysis of *in vivo* thrombi; 0.5, 2.5 and 5 mg/kg TNKase produced no (2/2 mice), partial (30-70% in 2/2 mice), or complete (5/5 mice) return of flow, respectively, in vehicle-infused mice within 1 hour of infusion (Figures 3.10A-F). Mice infused to 3.2±0.2 mg/mL final plasma fibrinogen (135%) showed only partial (0-30%) return of flow in 3/3 mice with subsequent re-occlusion following 5 mg/kg TNKase infusion (Figures 3.10G, H). In contrast, mice infused to 4.0±0.1 mg/mL final plasma fibrinogen (170%) did not re-acquire flow for up to 120 minutes (3/3 mice), even at the highest TNKase dose or with repeat dosing (Figures 3.10I, J). Together with *in vitro* lysis data (Figure 3.8), these *in vivo* data demonstrate hyperfibrinogenemia promotes resistance to thrombolysis.

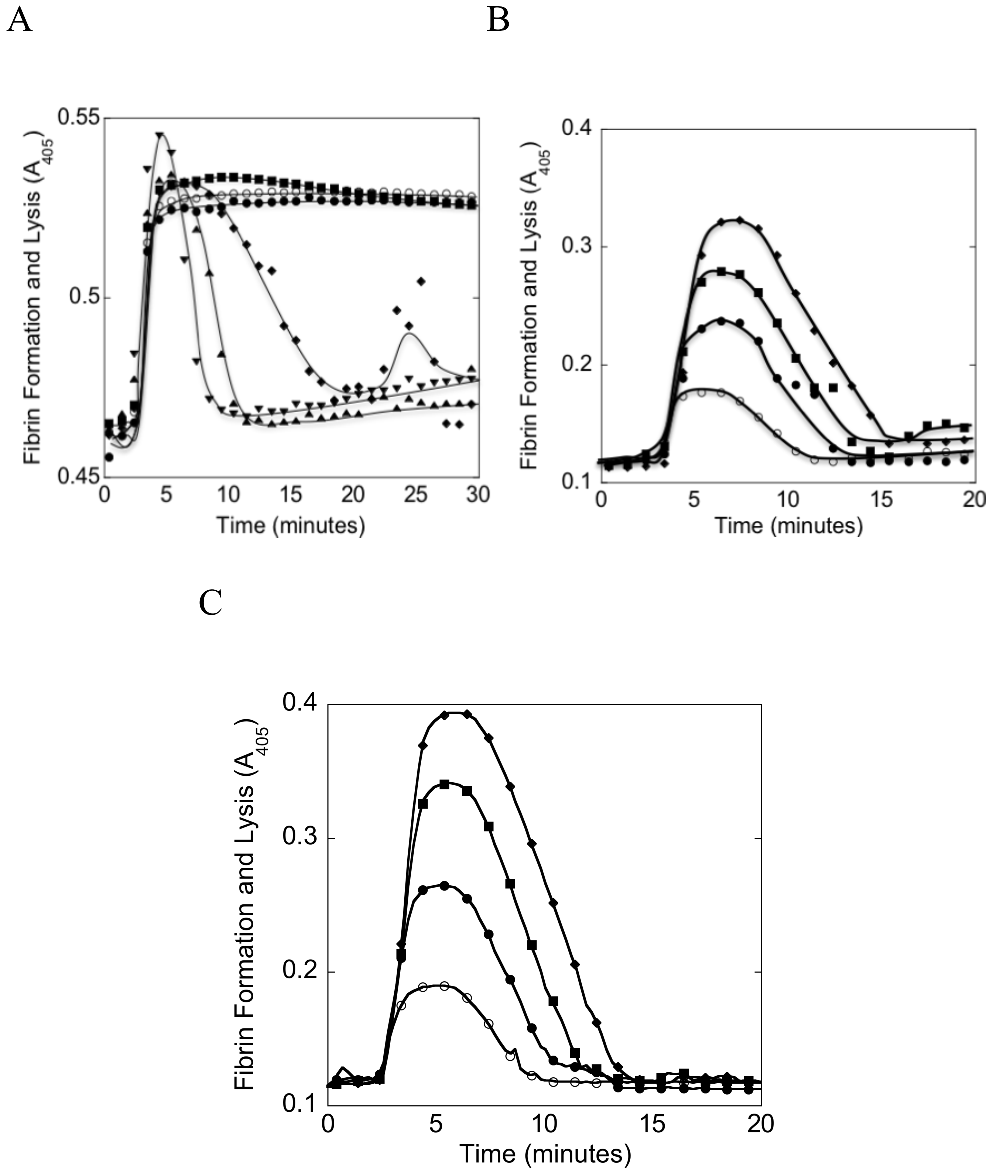

Figure 3.9 Fibrinolysis of murine plasma clots is dose-dependent with regard to the TNKase and mouse or human fibrinogen concentrations. A) Clotting was initiated with TF (1:30,000 Innovin) to recalcified murine PPP diluted 1:2 in the presence of 0 (open circles), 1 (closed circles), 2 (closed squares), 4 (closed diamonds), 8 (closed triangles), or 16 (closed inverted triangles) µg/mL TNKase. Clot formation and lysis were followed by turbidity. Curves are from a single experiment representative of two independent experiments. B, C) Recalcified murine PPP was spiked with human (B) or mouse (C) fibrinogen to achieve 2.4 (open circles), 3.4 (closed circles), 4.4 (closed squares), or 5.4 (closed diamonds) mg/mL fibrinogen, final, diluted 1:2, and clotting was initiated with TF in the presence of 8 µg/mL TNKase and monitored by turbidity. Polymerization curves were normalized to the starting turbidity from a single experiment representative of two independent experiments.

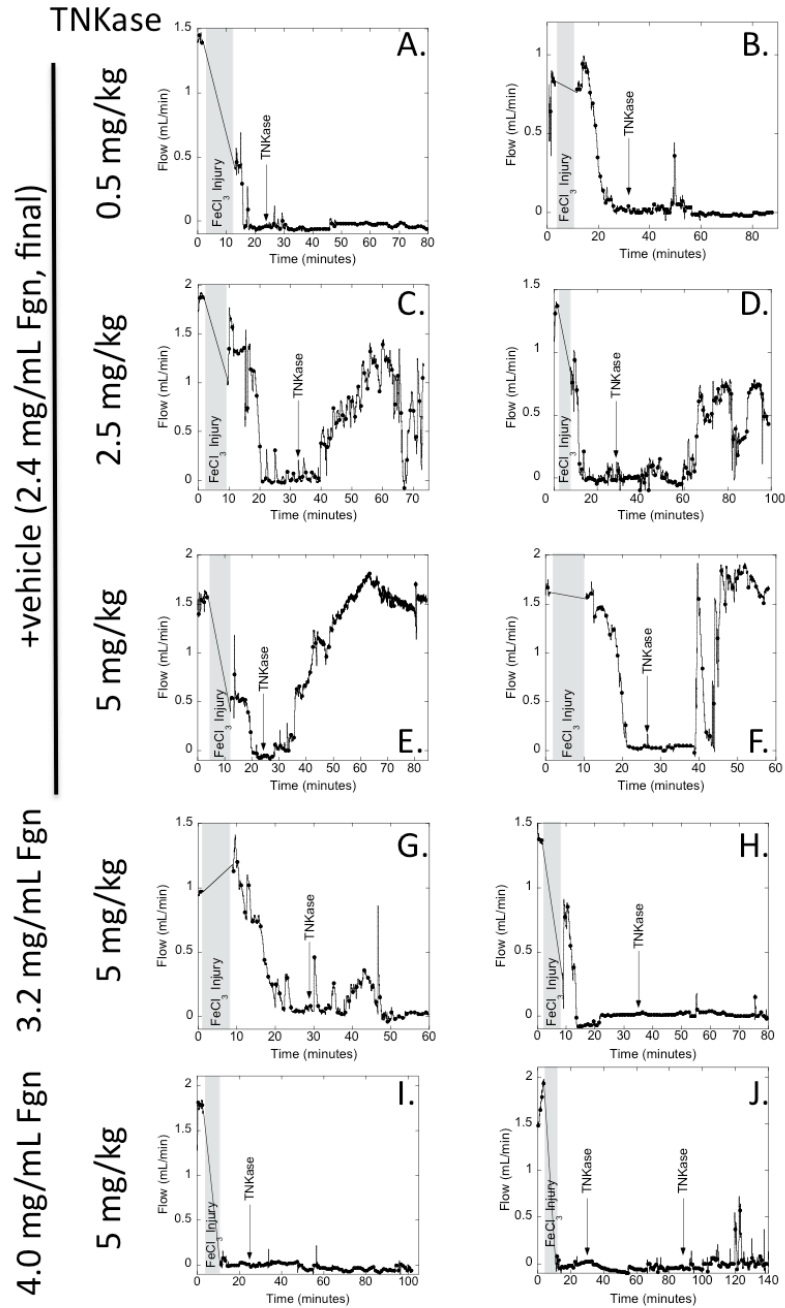

Figure 3.10 Hyperfibrinogenemia increases resistance to thrombolysis *in vivo*. Thrombosis was triggered in the carotid artery of wildtype mice infused with fibrinogen (plasminogen-, fibronectin-, vWF-, and factor XIII-depleted, concentrations indicated in the figure) or vehicle control. Following stable occlusion for 5 minutes, mice were infused with TNKase (concentrations indicated in the figure). Blood flow was monitored by flow probe throughout the experiment. Shaded gray area represents the time of $FeCl_3$ treatment plus time to reacquire flow information. Each panel shows data from an individual mouse, representative of at least 2 mice for each condition.

3.5 Discussion

Although epidemiological studies have implicated elevated plasma fibrinogen as an independent risk factor for both arterial and venous thrombosis, it remains highly controversial whether fibrinogen is merely a biomarker of a coincident inflammatory state or is also a causative mechanism in the etiology. We addressed this question *in vivo* by acutely elevating fibrinogen in mice and examining their susceptibility to thrombosis, and used *ex vivo* and *in vitro* experiments to elucidate biochemical and biophysical mechanisms by which fibrinogen contributes to thrombus formation and stability. We also utilized a murine thrombolysis model to test thrombus stability *in vivo*. Our findings show elevated fibrinogen levels: 1) specifically and independently shortened the TTO in both high (arterial) and lower (venous) shear injury models, 2) increased thrombus fibrin content, 3) promoted faster fibrin formation, higher network density, and increased clot strength and stability in TF-initiated *in vitro* models that recapitulate *in vivo* thrombosis, and 4) increased thrombus resistance to lysis *in vivo*. Together, these findings show hyperfibrinogenemia independently promotes thrombosis, identify biochemical and biomechanical mechanisms by which elevated fibrinogen levels contribute to thrombus formation, and demonstrate a critical role for plasma fibrinogen levels in resistance to thrombolytic therapy.

Our study is the first to show elevated fibrinogen significantly and independently shortens the TTO after acute injury. These findings differ somewhat from observations that transgenic hyperfibrinogenemic mice demonstrate a non-significant trend to shorter time to 75% occlusion in a $FeCl_3$-carotid model.[15] Differences in the two studies include the method of elevating fibrinogen levels (infusion *versus* transgenic expression) and final fibrinogen levels (170% *versus* 145%[15]). In our study, mice infused with lower fibrinogen

levels (to 135%) showed a TTO of 9 minutes (median) after carotid artery injury, which was not significantly different from control mice (data not shown). Additionally, Kerlin et al.[15] utilized mice with elevated murine fibrinogen, whereas the mice in our study had a mixture of human and mouse fibrinogen. Differences between these two molecules may make human fibrinogen more prothrombotic. Finally, the higher $FeCl_3$ concentrations (20%) used in the earlier study[15] may have dampened differences[35] between normal and hyperfibrinogenemic mice. Our findings also differ somewhat from observations that human fibrinogen g' chain expression in mice paradoxically *decreases* thrombus weight following electrolytic femoral injury.[17] However, the total fibrinogen levels achieved in human g'-expressing mice were lower than in controls, so any role of hyperfibrinogenemia was not examined in that study. Our *in vivo* observations are supported by *ex vivo* and *in vitro* cell-based experiments that recapitulate aspects of the murine thrombosis models. Notably, these findings are consistent with studies demonstrating fibrin network density, mechanical stability, and resistance to lysis are positively correlated with fibrinogen concentration in clots triggered by exogenous thrombin[24, 30, 36, 37], but extend these findings to a more physiologically-relevant model in which clotting was initiated by TF-bearing primary cells. The vascular cells used in the current study model both arterial (atherosclerotic) disease where plaque rupture exposes blood to subendothelial cells (e.g. SMC) and venous disease where clots are thought to arise on intact but inflamed endothelium (e.g. TNFα-HSVEC).[19] To our knowledge, these experiments are the first to address the role of elevated fibrinogen in a cellular, TF-based system. Together, these *in vivo* and *in vitro* observations link hyperfibrinogenemia to thrombotic disease and suggest specific mechanisms by which elevated fibrinogen is pathogenic.

Our study correlating abnormal fibrin quality with thrombosis in hyperfibrinogenemia informs several patient cohort studies. Of note, both hyperfibrinogenemia and denser, fibrinolysis-resistant clots were observed in plasma from patients with coronary artery disease, uniting observations of elevated fibrinogen, abnormal clot quality and cardiovascular disease in a single patient cohort.[36] Abnormal fibrin network structure and/or function (high mechanical strength and/or increased resistance to lysis) have also been detected in plasma clots from patients with diabetes[38], ischemic stroke[39], pulmonary hypertension[40], myocardial infarction (MI)[36, 41], venous thromboembolism (VTE)[42, 43], and healthy relatives of patients with premature coronary artery disease[44]. Overall, these studies suggest abnormal fibrin quality is a pathologic mechanism operant not only in hyperfibrinogenemia, but also in other prothrombotic pathologies.

A particularly interesting outcome of our study is the observation that hyperfibrinogenemia did not cause spontaneous thrombosis *in vivo*. This observation is consistent with reports that injecting human fibrinogen into mice does not cause spontaneous fibrin deposition[26] and importantly, with findings that while hyperfibrinogenemia is a thrombosis risk factor[1-9], it does not cause thrombosis *ipso facto*. Virchow proposed multiple "hits" from abnormalities in plasma composition, vascular cell function, and blood flow are required to trigger thrombosis. This paradigm suggests an initiating trigger, likely exposure of cellular PCA during plaque rupture or vasculitis, is required to initiate thrombus formation. Indeed, markers of vascular disruption including circulating leukocyte and endothelial-derived microparticles are elevated 1.3-fold in VTE patients.[45] Consistent with this hypothesis, the prognostic importance of elevated fibrinogen levels appears to be independent of, and additive to, myocardial damage (troponin-T levels) in patients with

unstable CAD.[8] To our knowledge, few diagnostic algorithms simultaneously consider markers of tissue damage and plasma hypercoagulability when assessing thrombosis risk. Of note is one study[4] that examined independent and combined effects of elevated fibrinogen and homocysteine (a potential initiator of endothelial damage) on mortality of patients from a high-risk cardiology clinic; elevated levels of both homocysteine and fibrinogen contributed to an increased hazard ratio, consistent with our findings that endothelial dysfunction (TF expression) and hyperfibrinogenemia independently promoted thrombus formation. Reduced blood flow (stasis), the third component of Virchow's Triad, is thought to explain differences in arterial and venous thrombosis. The shear rates between arteries and veins differ significantly; in humans large arteries like the carotid have wall shear rates of 300-800 s^{-1} while venous shear rates are in the range of 20-200 s^{-1} (reviewed in [46]). Interestingly, hyperfibrinogenemia has been correlated with both arterial and venous thrombosis, suggesting hyperfibrinogenemia contributes to thrombus formation independently of the shear rate. We addressed the role of shear by applying the $FeCl_3$ injury model to both high (artery) and lower (vein) shear vessels. Thrombi from saphenous veins appeared more fibrin-rich than thrombi from carotid arteries (Figure 3.3), consistent with observations that lower shear promotes fibrin deposition.[27] Notably, however, hyperfibrinogenemic mice demonstrated shortened TTOs and increased fibrin content in thrombi in both high and lower shear vessels (Figures 3.2, 3.3). These findings do not diminish a role for stasis in thrombosis, but rather suggest the contributions of shear are eclipsed in the setting of hyperfibrinogenemia and potentially other pathologies as well.

Thrombolytic therapy has met limited success. The GUSTO-I trial demonstrated complete coronary artery perfusion determines 30-day survival after MI; however, tPA

therapy achieved perfusion in only 54% of patients.[47] Similarly, in acute stroke, proximal arterial patency following tPA infusion correlated with positive long-term outcome, but was achieved in only 27% of patients.[48] Interestingly, although prior studies associated thrombus platelet content with decreased tPA efficacy[49], our study shows that within a given thrombosis model increased fibrin content also decreases thrombolytic efficacy. This novel finding suggests the plasma fibrinogen level present during thrombus formation is an independent predictor not only of thrombotic risk, but also of the potential efficacy of thrombolysis. Of note is the experiment in which we saw no return of flow in the occluded carotid artery in spite of two doses of TNKase (Figure 3.10J); post-mortem dissection revealed diffuse bleeding from microvasculature within the neck and abdominal cavity. Given the risk and devastating consequences of intracerebral hemorrhage with thrombolytic therapy, our data suggest screening patients for fibrinogen levels may inform risk/benefit analysis prior to initiating thrombolysis. Confirming a role for fibrinogen level in thrombolysis will require a study of patients whom received thrombolytic therapy and for whom both the fibrinogen level and degree of reperfusion are known. Our data provide strong justification for such an investigation.

This study has potential limitations. First, we used human fibrinogen to increase circulating levels in the mouse. However, published studies[17, 25, 26] as well as our data demonstrate human fibrinogen is stable in murine circulation and incorporated into murine clots. Moreover, elevating either human or mouse fibrinogen in mouse plasma increased peak turbidity and prolonged tPA- or TNKase-mediated fibrinolysis similar to that seen with tPA- or TNKase-mediated lysis of human fibrinogen in human clots (Figures 3.8, 3.9). Second, the $FeCl_3$ model may not fully recapitulate thrombosis/thromboembolism; it will be

interesting to examine the effects of hyperfibrinogenemia in other models (stasis- or electrolytic-based) in future studies. Finally, we evaluated the immediate effects of fibrinogen on thrombosis, however, elevated fibrinogen may have additional prothrombotic effects *in vivo*. Previous studies[50] demonstrated fibrin induction of TF in human vascular EC, suggesting prolonged exposure of vasculature to hyperfibrinogenemia may feedback on additional cellular mechanisms.

In conclusion, our results show hyperfibrinogenemia independently promotes thrombus formation and stability via increased fibrin network density and resistance to dissolution. These findings establish hyperfibrinogenemia in the etiology of both arterial and venous thrombosis/thromboembolism, and suggest fibrin is a potential therapeutic target in the management of these pathologies. Furthermore, our study establishes a model for future investigations of plasma hypercoagulability and vascular dysfunction; modulating plasma composition via intravenous procoagulant infusion will allow examination of the specific roles of additional plasma proteins in thrombosis and thrombolysis.

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Chapter 4

Extent of Vascular Injury Determines Contribution of Elevated Factor VIII to Thrombogenesis in Mice

4.1 Abstract

Studies have correlated elevated plasma factor VIII (FVIII) with thrombosis; however, it is unclear whether elevated FVIII is a proinflammatory biomarker, causative agent, or both. We raised FVIII levels in mice and measured the time to vessel occlusion (TTO) following ferric chloride-induced injury. Compared to control (saline-infused) mice, elevated FVIII had no effect after longer (3-minute) carotid artery injury, but shortened the TTO after shorter (2-minute) injury ($p<0.008$). Following injury, circulating thrombin-antithrombin (TAT) complexes were lower after short versus long injury ($p<0.04$), suggesting short treatment produced less coagulation activation. TAT levels in FVIII-infused mice were higher than controls after short, but not longer injury. Accordingly, elevated FVIII had no effect on *in vitro* thrombin generation or platelet aggregation triggered by high TF, but increased thrombin generation rate and peak (2.4- and 1.5-fold, respectively), and accelerated platelet aggregation (up to 1.6-fold) when initiated by low TF. Compared to control mice, elevated FVIII stabilized thrombi (fewer emboli) after short injury, but had no effect after longer injury. TTO and emboli correlated with TATs. These results demonstrate dependence of FVIII activity on extent of vascular injury. We propose elevated plasma FVIII is an etiologic, prothrombotic agent following minimal but not extensive vascular damage.

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4.2 Introduction

Elevated factor VIII (FVIII) levels have been consistently and positively associated with primary and recurrent venous thromboembolism (VTE) [Odds Ratio (OR) 2.0 – 10.8].[1-8] For example, the Leiden Thrombophilia (LITE) Study[6] showed FVIII concentrations over 1.5 U/mL (150%) led to an OR greater than 5, and Kyrle *et al.*[4] showed relative risk of VTE recurrence was 6.6 in patients with FVIII levels over 2.34 U/mL (234% of normal). FVIII concentrations over 2 U/mL (200%) have been associated with an OR of VTE recurrence as high as 10.8.[3]

In contrast to VTE, the role of FVIII in arterial thrombosis is controversial. Using broad definitions of coronary heart disease (CHD) encompassing atherosclerosis, angina, transient ischemic attack, acute and non-acute myocardial infarction (MI) and death, several studies have associated elevated FVIII with CHD and/or stroke, with OR ranging from 1.2 – 2.65.[9-12] However, associations between FVIII activity and CHD[13] or ischemic heart disease[12] were lost following multivariate adjustment for diabetes and von Willebrand factor (vWF) levels, respectively. Importantly, since FVIII is increased in diseases that induce an acute phase response, including myocardial infarction[14], surgery[15], and sepsis[16], it is unclear whether FVIII's association with either venous or arterial thrombosis simply reflects an ongoing prothrombotic inflammatory process, or is a direct, causative mechanism in the thrombosis etiology and therefore a therapeutic target, or both.

Studies examining the role of elevated FVIII in murine thrombosis models have also shown discord. Mice infused with recombinant human FVIII to 250% of normal demonstrate FVIII accumulation in thrombi and significantly enhanced thrombus size after photochemical (Rose Bengal)-induced carotid injury.[17] In addition, inhibiting FVIII activity with a

monoclonal antibody blocks thrombus formation in a murine inferior vena cava (IVC) stenosis model and in baboons implanted with arteriovenous shunts.[18, 19] In contrast, elevated FVIII (20% murine FVIII plus 280% infused human FVIII) does not promote microvascular or vena cava occlusion in vWF-deficient mice.[20, 21] Together, these studies suggest a role for FVIII in arterial and venous thrombosis, but that additional modifiers modulate its relative impact.

In the current study, we used murine models of thrombosis in the vein and artery to assess the role of elevated FVIII in thrombus formation and stability, and used *ex vivo* and *in vitro* methods to identify the operant biochemical mechanisms. Elevated FVIII augmented thrombus formation and stability after mild, but not severe ferric chloride ($FeCl_3$) injury. FVIII's effects were manifest via its ability to increase thrombin generation and accelerate platelet aggregation initiated by low, but not high TF activity. These results demonstrate dependence of plasma FVIII activity on the extent of vascular injury. We propose elevated plasma FVIII is an etiologic, prothrombotic agent in situations where there is moderate, but not extensive, vascular damage.

4.3 Materials and Methods

Proteins and materials

Dulbecco's modified Eagle's Medium with high glucose/2 mM L-glutamine, 0.05% trypsin/ethylenediamine tetraacetic acid, and phosphate-buffered saline (10 mM phosphate pH 7.1, 150 mM NaCl) were from Gibco (Grand Island, NY). Human monoclonal purified FVIII (Hemofil M) was from Baxter (Deerfield, IL). FVIII-deficient (Hemophilia A, <1% FVIII activity) platelet-poor plasma (PPP) was from HRF Inc (Raleigh, NC). Kontact (aPTT reagent) was from Thermo Fisher Scientific (Waltham, MA). Thrombin fluorogenic substrate (Z-Gly-Gly-Arg-AMC) and calibrator (α2-macroglobulin/thrombin) were from Diagnostica Stago (Parsippany, NJ). Factor Xa chromogenic substrate (Pefachrome FXa) and Pefabloc FG (Glycine-Proline-Arginine-Proline) was from Pentapharm (Basel, Switzerland). Thrombin receptor activation peptide (Serine-Phenylalanine-Leucine-Leucine-Arginine-Asparagine, TRAP) was from Bachem (Torrance, CA). Collagen was from Chrono-Log (Havertown, PA). Innovin was from Siemens (Plainsfield, IN). Mouse anti-human tissue factor (TF) antibody (HTF-1) was a kind gift from Dr. Ronald Bach (University of Minnesota). Non-immune mouse IgG antibody (MOPC-1), adenosine diphosphate (ADP), $FeCl_3$ and bovine serum albumin (BSA) were from Sigma-Aldrich (Saint Louis, MO). Goat anti-mouse and anti-rabbit peroxidase-conjugated antibodies were from Calbiochem (La Jolla, CA). Monoclonal anti-fibrin antibody (59D8) was the generous gift of Drs. Marschall Runge [University of North Carolina (UNC) Department of Medicine] and Charles Esmon (Oklahoma College of Medicine). Biotinylated secondary antibodies were from Vector Laboratories (Burlingame, CA). Target Retrieval Solution was from Dako (Carpinteria, CA). Tenecteplase was the generous gift of Genentech (San Francisco, CA). Corn trypsin

inhibitor and factor X were from Haematologic Technologies Inc (Essex Junction, VT). Contact-inhibited normal pooled plasma (NPP) was prepared from whole blood from 40 healthy subjects (50% female, 68% non-Caucasian) in a protocol approved by the UNC Institutional Review Board, as previously described.[22]

Murine Thrombosis and Thrombolysis Models

Procedures were approved by the UNC Institutional Animal Care and Use Committee. Mice (6-8 week old male C57BL/6, Charles River Laboratories, Raleigh, NC) were anesthetized with 1.5-2% isofluorane in 2% oxygen, and the left saphenous vein was exposed under a SZX12 dissecting microscope (Olympus, Tokyo, Japan) and catheterized as described.[22, 23] FVIII or vehicle (saline or saline/BSA) was administered through the catheter on a per weight basis [blood volume (mL) is 7% of body weight (g)] less than 5 minutes prior to injury. Published studies demonstrate human FVIII binds murine vWF[17], has comparable cofactor activity as murine FVIII[17], promotes coagulation after tail clipping and vessel injury in hemophilic mice[17, 24-27], and is inactivated by murine activated protein C (Steve Pipe, personal communication). In addition, previous studies and our findings (data not shown) show FVIII has sufficient half-life in murine circulation[28, 29] for these experiments. The endogenous FVIII concentration in mice (1 U/mL, 100%)[30] was raised by infusing human FVIII to 285% (total murine plus human FVIII) of normal, consistent with levels associated with thrombosis in humans.[1-12]

The saphenous vein and carotid artery thrombosis models were performed as described.[22] Briefly, the right saphenous vein was exposed, treated with 5% $FeCl_3$ (0.31 M $FeCl_3$ on 0.5x2 mm filter paper) for 3 minutes, and washed with warm saline. Blood flow

was monitored auditorily by Doppler ultrasonic flow probe. On separate mice, the right common carotid artery was exposed after midline cervical incision, dried, treated with 10% $FeCl_3$ (0.62 M on 0.5x0.5 mm filter paper) for 3 or 2 minutes, and washed with warm saline. Following injury, blood flow was monitored via Doppler transonic flow probe. In both models, the time to occlusion (TTO) was the time between $FeCl_3$ administration and lack of flow for 60 consecutive seconds. Experiments were stopped at 45 minutes if no occlusion occurred. Embolization was defined as a rapid increase in blood flow of at least 0.2 mL/min in the 5 minutes after stable occlusion occurred. Either 10 minutes after stable occlusion or after 45 minutes without stable occlusion, blood was drawn from the IVC and processed to platelet-poor plasma (PPP) by centrifugation at 5,000xg for 10 minutes.

Thrombolysis was assessed in mice subject to 3-minute $FeCl_3$ carotid artery thrombosis as described.[22] Briefly, after 5 consecutive minutes of blood flow below 0.1 mL/min, mice were infused with tenecteplase (5 mg/kg) through the saphenous vein catheter while continuously monitoring blood flow.

FVIII Activity Assay

Factor VIII activity was measured in PPP from FVIII-infused mice not subject to thrombosis. Five and 30 minutes after FVIII infusion, blood was drawn from the IVC and processed to PPP. Murine PPP was mixed with human FVIII-deficient PPP (5% murine PPP and 95% FVIII-deficient PPP), and clotting was initiated with Kontact (40% total volume) and calcium (10 mM, final). The clot formation rate was measured in a SpectraMax Plus 340 plate reader (Molecular Devices, Silicon Valley, CA)[31] and compared to a standard curve created by mixing mouse PPP spiked with FVIII (to 150-300%) with FVIII-deficient plasma.

Five and 30 minutes after FVIII infusion, FVIII activity was 285% and 220%, respectively (endogenous murine FVIII plus infused human FVIII).

Measurement of circulating thrombin-antithrombin (TAT) complexes

Citrated blood samples were drawn from the IVC of mice subject to the $FeCl_3$ carotid artery thrombosis model 10 minutes after stable occlusion. TAT levels were measured by ELISA (Enzygnost TAT micro ELISA, Siemens, Deerfield, IL)

Cell culture

Primary human aortic smooth muscle cells (SMC, Lonza Walkersville, Walkersville, MD) were cultured as directed in 5% CO_2 at 37°C, and used between passages 3-6 to reduce phenotypic drift.

Phospholipid vesicles

Phosphatidylcholine (egg), phosphatidylethanolamine (soy), and phosphatidylserine (porcine brain) were from Avanti Polar Lipids (Alabaster, AL). Large unilamellar vesicles (41% phosphatidylcholine/44% phosphatidylethanolamine/15% phosphatidylserine) were made by extrusion, as described.[32]

TF Activity

TF activity was measured by chromogenic substrate cleavage on a SpectraMax Plus340 plate reader in the presence and absence of inhibitory anti-TF antibody or isotype control (10 μg/mL HTF-1 or MOPC-1, respectively), as described.[22, 33]

Thrombin generation

Thrombin generation was measured by calibrated automated thrombography using a Fluoroskan Ascent fluorometer (ThermoLabsystem) as described.[33] Briefly, recalcified (16 mM, final), lipidated (100 μM, final) NPP was spiked with FVIII to 2, 3, or 4 U/mL (200%, 300%, and 400% of normal, respectively), final, and immediately added to washed SMC monolayers (67.7% plasma, final). Thrombin generation parameters (lag time and peak) were calculated using Thrombinoscope software Version 3.0.0.29 (Thrombinoscope BV, Maastricht, Netherlands). The thrombin generation rate was calculated by dividing peak height by the difference from time to peak and lag time.

H&E Staining and Immunohistochemistry

Fixed tissues were dehydrated and paraffin-embedded, and consecutive, five-micron sections cut and mounted at the UNC Lineberger Comprehensive Cancer Center Animal Histopathology Core. Hematoxylin and eosin (H&E) staining was used to visualize the thrombus. Immunohistochemistry (IHC) for fibrin(ogen) was performed with monoclonal antibody 59D8, as described.[22] Negative controls were stained simultaneously in the absence of primary antibody.

Platelet Aggregation

Platelet aggregation was performed as described on a Chrono-Log Optical Aggregometer 470 (Havertown, PA).[22] Aggregation was triggered by TRAP (50 μg/mL, final), collagen (2 μg/mL, final), ADP (2.5 μM, final) or TF [1:30,000 (high TF) or 1:200,000 (low TF)].

For experiments triggered with TF, Pefabloc FG (5 mg/mL, final) was included to inhibit fibrin polymerization.

Statistical Methods

For TTO, conditions were compared pairwise by a log-rank test due to censored observations at 45 minutes (no stable occlusion). For TATs and embolization analysis, conditions were compared by Mann-Whitney tests to account for both unequal variance and non-normality of outcomes. For *in vitro* thrombin generation assays, significant differences between groups were identified by a one-way analysis of variance (ANOVA) with homogeneity tests and residual plots to check statistical assumptions. To analyze the effect of FVIII concentration on thrombin generation parameters (lag time, rate and peak height), a one-sided Dunnett's post-hoc test using 1 U/mL FVIII (100% FVIII) as the index group (on SMC of varying confluency, as indicated) was used, with a controlled type-I error rate no larger than 0.05 in the multiple testing between experimental and index groups. For platelet aggregation data, normalized lag times where ratios were calculated between 200%, 300%, and 400% to 100% FVIII were tested by a one-sided t-test with a null hypothesis no smaller than 1. Bivariate correlation analyses between TTO, TAT, and number of emboli were presented in Pearson's coefficient (denoted as R) that indicates the degree of linear dependence. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago IL). P-values less than 0.05 were considered significant

4.4 Results

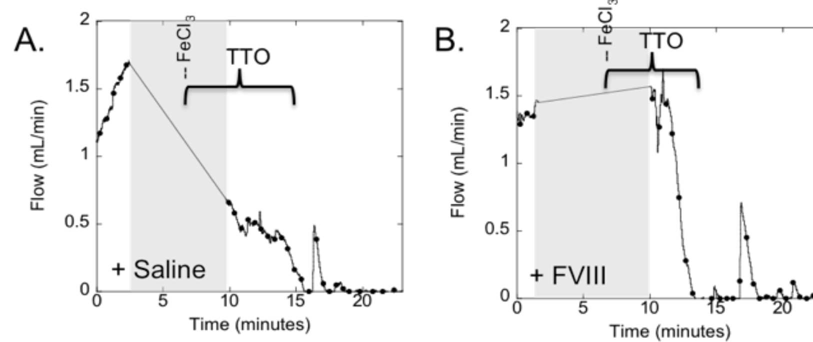
Elevated FVIII shortens the TTO in a saphenous vein thrombosis model.

$FeCl_3$ application to the saphenous vein of control (saline-infused) mice for 3 minutes produced occlusive thrombi in 20.3 (6.0) minutes [median (range)]. Consistent with epidemiologic studies showing a significant (up to 10.8-fold) increased risk of VTE in patients with elevated FVIII[1-8], 285% FVIII significantly shortened the TTO [12.7 (2.8) minutes, median (range), $p<0.001$] in the saphenous vein. Infusing non-specific protein (BSA, equivalent w/w) did not shorten the TTO versus control mice. Additionally, the shortened TTO was not due to vWF in the FVIII preparation; Hemofil M contains only trace human vWF (0.083 μg vWF/U FVIII).[34] These findings indicate FVIII's effect was specific and confirm a direct, pathogenic contribution of elevated FVIII in a murine model of thrombosis in a vein.

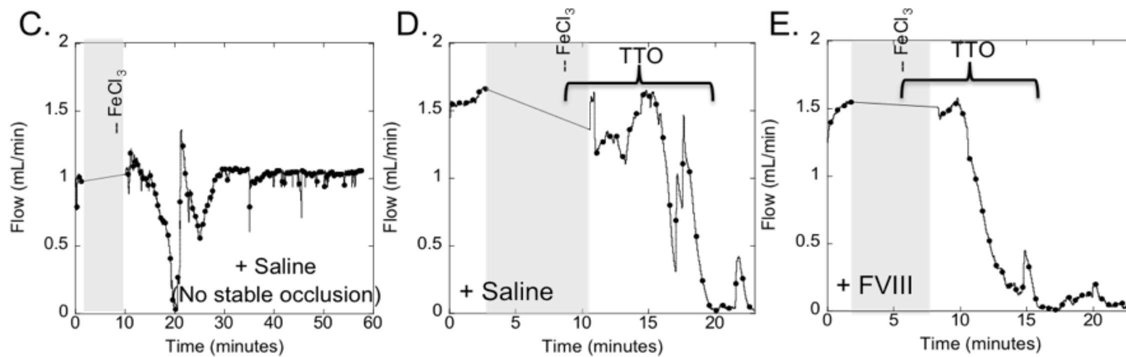
Elevated FVIII shortens the TTO following short (2-minute), but not longer (3-minute), $FeCl_3$ treatment to the carotid artery.

In contrast to VTE, the role of FVIII in arterial thrombosis is unclear. We therefore tested the ability of FVIII to accelerate arterial clotting following 3-minute $FeCl_3$ application to the carotid artery. Representative flow tracings are shown in Figure 4.1A, 1B. Control mice developed occlusive thrombi in 8.1 minutes (median, Figure 4.1F). Surprisingly, although mice with hypercoagulability due to elevated fibrinogen demonstrate significantly shortened TTO under these conditions[22], mice with elevated FVIII did not (7.1 minutes median, $p=0.6$, Figure 4.1F).

3 minute



2 minute



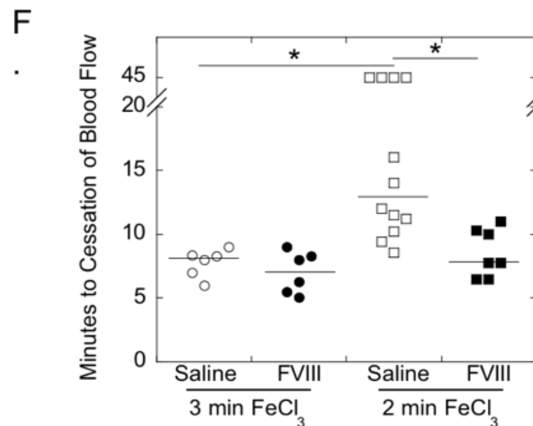


Figure 4.1 Elevated FVIII shortens the TTO after 2-minute, but not 3-minute $FeCl_3$ injury to the carotid artery. C57Bl/6 mice were infused with saline or FVIII to 285% of normal. Thrombosis was induced by 10% $FeCl_3$ application to the carotid artery for the indicated time (3 or 2 minutes) and the TTO was determined by flow probe. In vessels that did not occlude, the TTO was recorded as 45 minutes. (A-E) Representative flow tracings following 3- (A-B) or 2- (C-E) minute $FeCl_3$ application to control (saline-infused, A, C and D) and FVIII-infused (B, E) mice. Gray shaded area represents time of vessel drying, $FeCl_3$ administration and vessel washing, during which flow could not be monitored. Brackets indicate the time from $FeCl_3$ placement to the time of vessel occlusion. (F) Quantification of TTO data. Each point represents a separate mouse. Lines show median values to accommodate censored data at 45 minutes. *$p<0.05$

Given studies demonstrating differential sensitivity to intrinsic pathway components with extent of $FeCl_3$ injury[35, 36], we then tested a second set of conditions in which $FeCl_3$ was applied to the carotid artery for only 2 minutes. Representative flow tracings are shown in Figure 4.1C-E. Compared to 3-minute $FeCl_3$ injury, control mice took significantly ($p<0.005$) longer to form stable thrombi following 2-minute $FeCl_3$ injury (13 minutes, median, Figure 4.1F). Interestingly, unlike the 3-minute injury model, mice with elevated FVIII exhibited a significantly ($p<0.005$) shorter TTO than control mice in this 2-minute injury model (7.8 minutes, median, Figure 4.1F). Infusing BSA did not shorten the TTO versus control mice (data not shown). These data indicate elevated FVIII promotes arterial thrombosis following short, but not long, application of $FeCl_3$.

Elevated FVIII increases thrombin generation *in vivo* following short, but not longer, $FeCl_3$ treatment.

Our TTO data indicated duration of $FeCl_3$ application influenced the impact of FVIII on thrombogenesis. To characterize the effect of $FeCl_3$ injury and FVIII on *in vivo* procoagulant activity (PCA, thrombin generation), we measured circulating TATs. Uninjured mice had a TAT level of 17.4 ± 2.26 ng/mL. Control mice subject to either 3- or 2-minute $FeCl_3$ treatment had TAT levels of 195 ± 108 or 66.2 ± 63.1 ng/mL, respectively (Figure 4.2). TAT levels in control mice were significantly ($p<0.05$) higher following 3-minute injury than 2-minute injury, indicating 3-minute $FeCl_3$ application causes higher PCA than 2-minute $FeCl_3$ application. Compared to controls, elevated FVIII did not increase TAT levels in the 3-minute injury model (222 ± 85.2 ng/mL, $p=0.5$), but caused significantly higher TAT levels than controls after 2-minute (175 ± 115 ng/mL, $p<0.05$) $FeCl_3$ application (Figure 4.2).

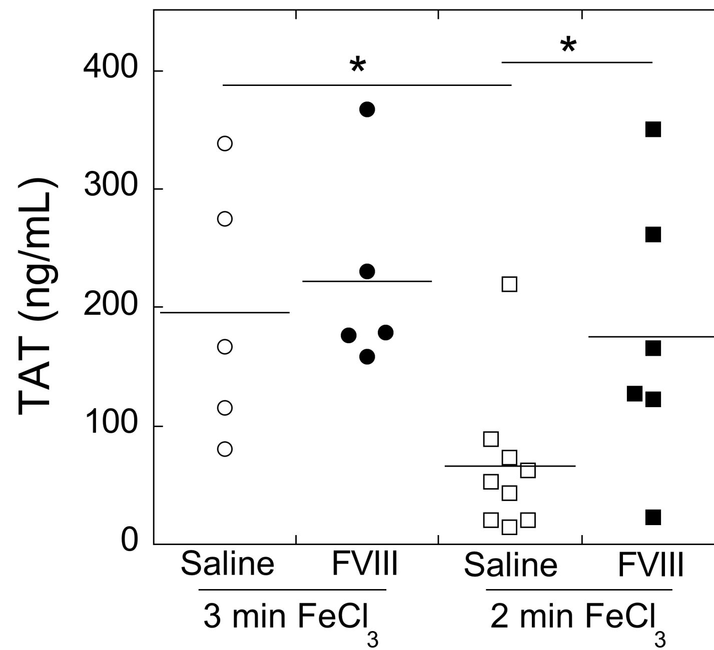


Figure 4.2 Both time of $FeCl_3$ injury and elevated FVIII influence TAT complex formation. Citrated blood samples were collected via IVC puncture from mice subject to the $FeCl_3$ carotid artery thrombosis model. TAT levels were measured by ELISA. Samples showing hemolysis were excluded. Each point represents a separate mouse. Lines show mean values. *$p<0.05$

Across all mice, TAT levels correlated with TTO values ($R=0.388$, $p=0.055$), suggesting differences in TTO resulted from differential PCA at the site of injury.

Elevated FVIII increases thrombin generation in an *in vitro* model of mild, but not more severe, vascular injury.

Given findings associating TTO with circulating TAT levels, we hypothesized that the duration of $FeCl_3$ application determined the extent of vascular injury, and consequently, the relative impact of extrinsic (TF-dependent) and intrinsic (FVIII-dependent) contributions to thrombin generation. To specifically interrogate the relationship between vascular PCA and plasma FVIII levels on thrombin generation kinetics, we modeled vascular injury *in vitro* by culturing primary SMCs at 100, 60, and 20% confluency to model high, medium, and low levels of vascular damage, respectively. Wang et al. previously showed SMC TF has a central role in $FeCl_3$-induced vascular injury.[37] SMC cultures at 100, 60, and 20% confluency expressed 5.5±0.74, 3.7±0.66, and 0.82±0.32 pM TF, respectively. We first incubated re-calcified NPP [1 U/mL FVIII (100%)] with SMCs and measured thrombin generation (Figure 4.3). Lipids (100 µM, final) were included to compensate for different lipid concentrations in cultures with different SMC plating densities. Similar to thrombin generation triggered by 5 and 1 pM soluble TF[38], decreasing cell density (decreasing TF activity) significantly ($p<0.05$) prolonged the lag time and decreased the thrombin generation rate and peak height (Figure 4.3D-F).

We then determined the effect of FVIII on thrombin generation at each confluency level by incubating SMCs with recalcified, lipidated NPP spiked with FVIII [to 2 U/mL (200%), 3 U/mL (300%) and 4 U/mL (400%), final], Figure 4.3). Elevated FVIII non-significantly

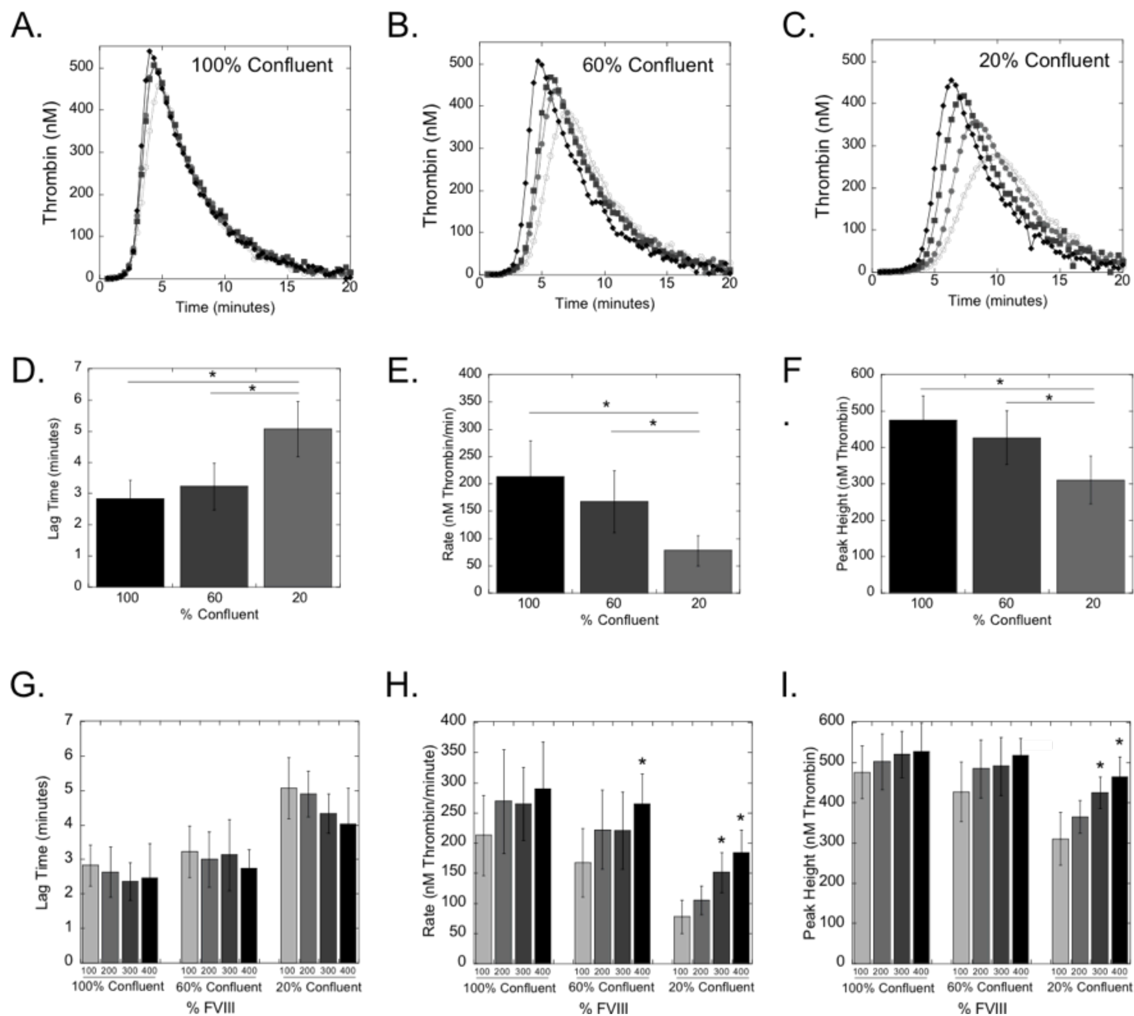


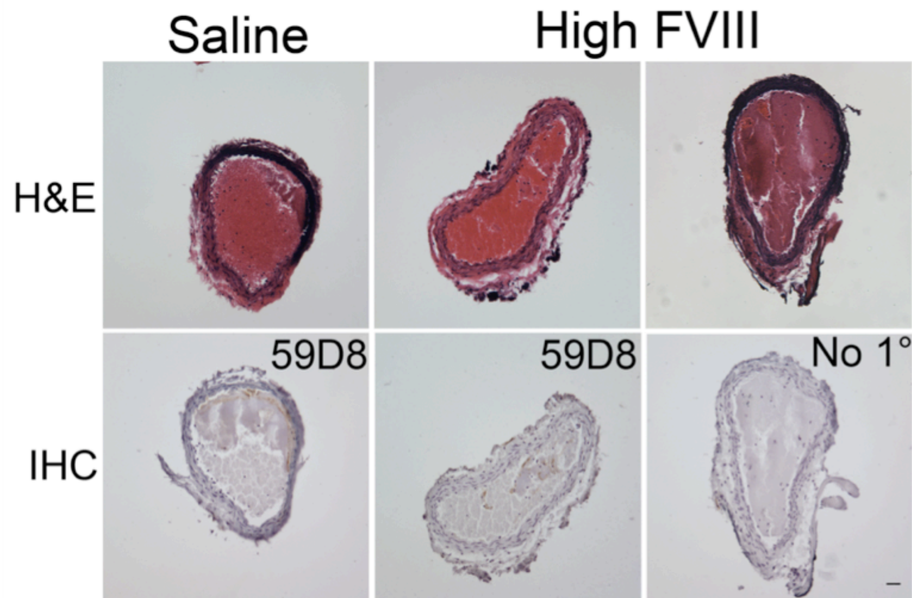
Figure 4.3 FVIII increases thrombin generation in an *in vitro* model of mild, but not severe, TF (SMC) exposure. Recalcified (16 mM, final) human NPP spiked with FVIII or HBS was added to SMC monolayers of varying densities. Thrombin generation was measured by calibrated automated thrombography, as described.[22, 33] (A-C) Thrombin generation curves representative of five independent experiments with SMC at 100% (A), 60% (B), and 20% (C) confluency. Symbols are: 100% (open circles), 200% (closed circles), 300% (closed squares), and 400% (closed diamonds) FVIII, final. (D-F) Mean values (± standard deviation) for (D) lag, (E) rate, and (F) peak height of thrombin generation for all five experiments with human NPP (1 U/mL FVIII) at different SMC confluency levels. *$p<0.05$ *versus* indicated group. (G-I) Mean values (± standard deviation) for the (G) lag, (H) rate, and (I) peak height of thrombin generation for all five experiments, at varying FVIII levels. *$p<0.05$ *versus* 1 U/mL within each confluency level.

shortened the lag time in reactions with SMCs at all confluencies (Figure 4.3G); this effect was more pronounced at lower SMC confluencies. At 100% confluency (high TF), FVIII had no effect on the rate or peak height (Figure 4.3H-I). At 60% confluency (medium TF), 400% FVIII increased the rate ($p<0.05$), but not peak height. At 20% confluency (low TF), both 300% and 400% FVIII significantly ($p<0.005$) increased the rate and peak height (Figure 4.3H-I). These results are consistent with our previous findings using lipidated TF; 400% FVIII increases the peak height to a greater extent in reactions triggered by low (1 pM) than high (5 pM) lipidated TF (164% and 115% of normal, respectively).[38] The significantly faster rate of thrombin generation in both low TF[38] and 20% SMC (Figure 4.3H-I) was consistent with the shorter TTO in the 2-minute $FeCl_3$ injury model *in vivo*.

Together, these *in vivo* and *in vitro* data show $FeCl_3$-induced vascular damage induces PCA (thrombin generation). Elevated FVIII augments thrombin generation and consequently, thrombus formation, following mild, but not more severe vascular injury.

Elevated FVIII does not alter thrombus morphology or increase thrombus fibrin(ogen) content.

We previously showed hyperfibrinogenemic mice exhibit increased thrombus fibrin deposition.[22] Since hyperfibrinogenemia promotes thrombosis by increasing thrombus fibrin(ogen) content[22] we tested whether elevated FVIII and its subsequent increase in thrombin generation in the 2-minute injury model similarly increased thrombus fibrin(ogen) content. H&E staining showed similarly extensive, occlusive thrombi with similar vessel diameters, containing distinct regions of anucleate, proteinaceous material and erythrocytes in thrombi from control and FVIII-infused mice (Figure 4.4). IHC of thrombi from both



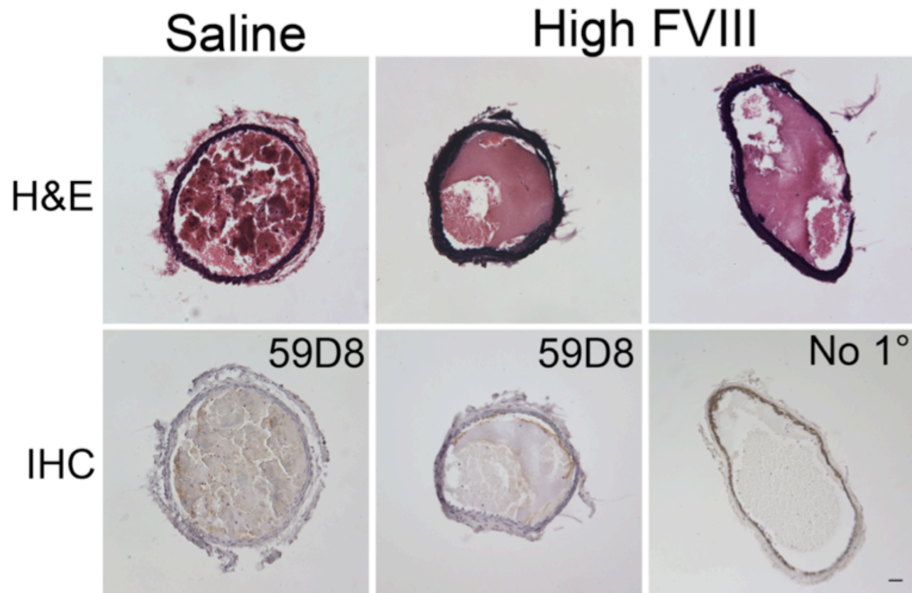


Figure 4.4 Elevated FVIII does not increase fibrin(ogen) incorporation into thrombi following $FeCl_3$ injury. Representative sections through thrombi formed in the carotid artery after (A) 2-minute or (B) 3-minute $FeCl_3$ application. The dark region on the exterior of the vein is the site of $FeCl_3$ application. H&E staining shows regions of protein and packed erythrocytes. Immunohistochemistry for fibrin (59D8) shows weak fibrin staining concentrated primarily at the luminal edge of proteinaceous regions. "No 1°" indicates antibody 59D8 was omitted from the IHC as a negative control. All images were taken at the same magnification (20X); the micron bar is 31 μm.

control and FVIII-infused mice demonstrated weak fibrin staining concentrated primarily at the luminal edge of proteinaceous regions. There were no obvious differences in fibrin staining intensity between control and FVIII-infused mice (Figure 4.4), suggesting thrombus fibrin content was unchanged by elevated FVIII. Together, these findings indicate FVIII did not shorten the TTO by increasing thrombus fibrin content.

Elevated FVIII accelerates platelet aggregation when triggered with low, but not high, TF

To determine whether FVIII-dependent, elevated thrombin generation accelerated platelet incorporation into the thrombus, we raised FVIII levels in human PRP with additional human FVIII, and triggered platelet aggregation with TRAP, collagen, ADP, or TF. Elevated FVIII had no effect on platelet aggregation initiated by TRAP, collagen, or ADP, showing that FVIII does not impact platelet function in the absence of thrombin generation (data not shown). In reactions triggered by TF, FVIII had no effect on either the rate or maximum amplitude of platelet aggregation (Figure 4.5A). Interestingly, whereas elevated FVIII had no effect on the lag time in reactions triggered with high TF, elevated FVIII dose-dependently shortened the lag time of platelet aggregation in reactions triggered by low TF ($p<0.05$, Figure 4.5A-B). These data are consistent with the FVIII-dependent shortening in TTO after 2-, but not 3-minute injury, and suggest elevated FVIII shortened the TTO by accelerating platelet activation/aggregation.

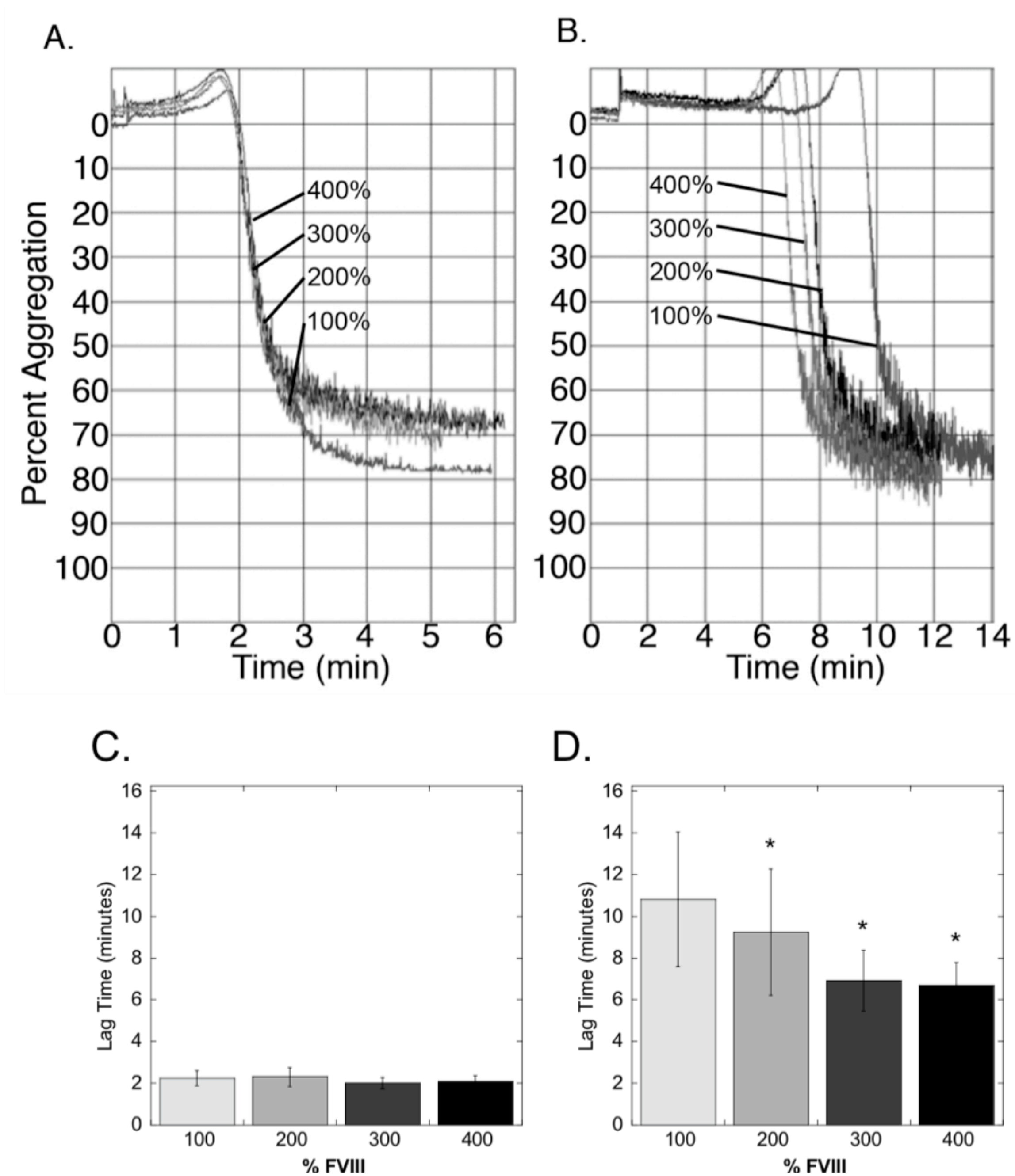


Figure 4.5 Elevated FVIII accelerates platelet aggregation when initiated with low, but not high TF. Platelet aggregation in human PRP spiked with human FVIII (final concentrations indicated) was triggered by re-calcification and addition of TF and monitored by turbidity as described in Methods and [22]. Graphs shown are from one experiment, representative of 4-6 experiments at each TF concentration. (A) High TF (1:30,000 Innovin, final). (B) Low TF (1:200,000 Innovin, final). Note difference in x-axis (time) scaling in (A) and (B). (C-D) Quantification of lag time data initiated with high (C) and low (D) TF, respectively. Graphs show mean ± standard error of the mean. *$p<0.05$ *versus* 100% FVIII

Elevated FVIII increases thrombus stability following mild, but not severe, vascular injury.

Finally, since both shorter TTO and higher PCA have previously been associated with increased thrombus stability[22, 33, 39], we examined thrombus stability in the carotid artery of mice as functions of both duration of $FeCl_3$ treatment and FVIII level. Example tracings from experiments with control (saline-infused) mice after 3- and 2-minute injury are shown in Figure 4.6A and 4.6B, respectively. Mice treated with $FeCl_3$ for 3 minutes demonstrated stable occlusion (<0.1 mL/min blood flow for 5 consecutive minutes), whereas mice treated with $FeCl_3$ for 2 minutes did not. We quantified clot stability by counting the number of embolic events (rapid increase in blood flow of at least 0.2 mL/min) in the 5 minutes following the TTO. Mice produced significantly ($p<0.05$) more emboli after 2-minute $FeCl_3$ injury than 3-minute injury (Figure 4.6C). In the 2-minute injury model, FVIII-infused mice produced significantly ($p<0.05$) fewer emboli than control mice (Figure 4.6C). However, there was no difference in number of emboli between control and FVIII-infused mice after 3-minute injury (Figure 4.6C). Across all mice, the number of emboli was significantly, positively-associated with TTO values ($R=0.495$, $p=0.019$) and significantly, negatively-associated with TAT values ($R=0.614$, $p=0.005$).

Since control and FVIII-infused mice thrombosed with $FeCl_3$ for 3 minutes demonstrated stable occlusion, we further examined thrombus stability by infusing these mice with the tissue plasminogen activator derivative, tenecteplase as described.[22] Consistent with their similar TTO, TATs, and number of emboli, there was no difference in tenecteplase-induced thrombolysis; all mice showed complete return of blood flow (data not shown). In total, these data suggest longer $FeCl_3$ treatment (increased vascular injury) produces highly stable

thrombi irrespective of intrinsic pathway components, whereas shorter treatment (less injury) produces unstable thrombi. Elevated FVIII stabilizes thrombi after mild, but not severe vascular injury.

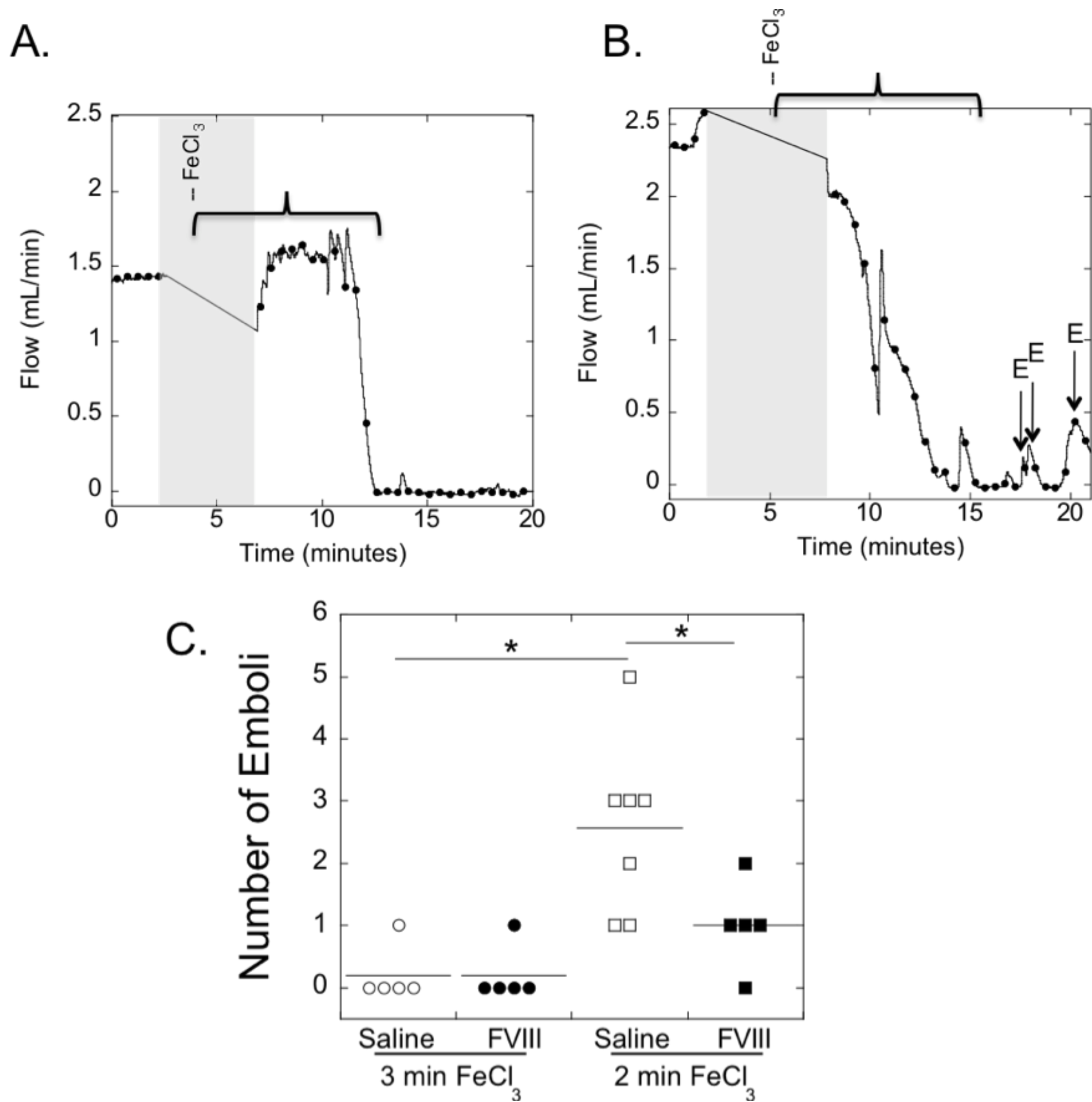


Figure 4.6 Both lengthier $FeCl_3$ treatment and elevated FVIII increase thrombus stability. C57Bl/6 mice were infused with saline or FVIII to 285% of normal. Thrombosis was induced by 10% $FeCl_3$ application to the carotid artery for the indicated time (3 or 2 minutes). Following stable occlusion, flow was monitored for 5 minutes to count embolic events (defined as a rapid increase in blood flow of at least 0.2 mL/min). (A-B) Representative flow tracings following 3- (A) and 2-minute (B) $FeCl_3$ application to control (saline-infused) mice, respectively. Gray shaded area represents time of vessel drying, $FeCl_3$ administration and vessel washing, during which flow could not be monitored. Brackets indicate the time from $FeCl_3$ placement to the time of vessel occlusion. Arrows show emboli (marked with E) in the 5 minutes after stable occlusion. (C) Quantification of embolization data. Each point represents a separate mouse. Mice that did not achieve stable occlusion were excluded. Lines show mean values. $*p<0.05$

4.5 Discussion

Epidemiologic studies have repeatedly correlated elevated plasma FVIII levels with VTE. However, the role of FVIII in arterial thrombosis is controversial. In addition, for both venous and arterial thrombosis, it is unclear whether elevated FVIII is merely a proinflammatory biomarker[14-16], a causative mechanism in the disease etiology, or both. We acutely elevated FVIII in mice and examined their susceptibility to thrombosis, and used *ex vivo* and *in vitro* methods to elucidate biochemical mechanisms for the observed effects. Our findings confirm an etiologic, prothrombotic role for elevated FVIII in veins. Moreover, our findings show elevated FVIII: 1) shortened the TTO after 2-, but not 3-minute $FeCl_3$ treatment to the carotid artery, 2) increased thrombin generation in mice after 2- but not 3-minute $FeCl_3$ treatment, 3) augmented the thrombin generation rate and peak *in vitro* with low, but not high, TF (SMC) exposure, 4) accelerated the onset of platelet aggregation initiated by low TF, and 5) promoted the formation of more stable thrombi (fewer emboli) following 2-, but not 3-minute injury in mice. Together, these findings demonstrate an etiologic role for FVIII in thrombosis, but suggest dependence of plasma FVIII activity on the extent of vascular injury.

We and others have previously shown dose-dependent effects of TF activity on thrombin generation *in vitro*.[33, 38, 40] Our data quantifying circulating TAT complexes in mice subject to 2- and 3-minute $FeCl_3$ injuries extend the *in vitro* findings with soluble and cellular TF to the *in vivo* models and provide a rationale for the differences in TTO and thrombus stability. Compared to shorter $FeCl_3$ treatment, longer $FeCl_3$ application produced higher circulating TAT levels, suggesting prolonged $FeCl_3$ treatment produced a more severe injury with higher exposure of cellular procoagulant (TF) activity. Our findings do not rule out the

possibility that longer $FeCl_3$ treatment also increased platelet deposition by exposing more subendothelial collagen than the shorter injury. However, we were able to recapitulate effects of longer and shorter $FeCl_3$ treatment on thrombin generation and platelet aggregation by varying the soluble TF concentration ([38] and Figure 4.5), or SMC confluency *in vitro* (Figure 4.3), suggesting differential exposure of procoagulant (TF) activity differentiates these treatments. Any effect on platelets of collagen exposure between the 2- and 3-minutes injury models would occur distinctly from, but potentially in addition to, effects of PCA. Importantly, our observation that TAT measurements correlate with thrombus formation (TTO) and stability measurements provides a means of quantifying differences in extent of vascular injury in different *in vivo* models. This information may enable reconciliation of differences in outcome measures between studies using different thrombosis models.

Patients with elevated FVIII demonstrate elevated circulating prothrombin fragment 1.2 and TAT complexes[5], and we and others have shown elevated FVIII increases thrombin generation *in vitro*[38, 41-43] and *in vivo*[43]. Interestingly, the *in vitro* studies show a stronger effect of elevated FVIII in reactions triggered with low TF than high TF. It is therefore of considerable interest that elevated FVIII shortened the TTO and improved thrombus stability in the carotid artery following shorter, but not longer, $FeCl_3$ application. Previous studies of intrinsic pathway components have demonstrated dose-dependent effects of $FeCl_3$ treatment on thrombosis; mice lacking intrinsic pathway factors XI or IX develop occlusive thrombi in the carotid artery following high (10%), but not lower (3.5 or 5%) $FeCl_3$ treatment.[35, 36] While previous studies hypothesized increased $FeCl_3$ injury reduces sensitivity to intrinsic factors by increasing TF/VIIa activity[35, 36], our study is the first to show a mechanism for differences in mild and severe $FeCl_3$ injury: differential PCA,

measured by TAT complex formation. Indeed, the sensitivity of intrinsic pathway components is perhaps best demonstrated by differences in the prothrombin time (PT) and partial thromboplastin time (PTT) tests. Whereas the PT detects clotting deficiencies due to extrinsic and common pathway components, its high TF concentrations mask effects of factors VIII or IX deficiency. Lower TF concentrations in a PTT reveal FVIII or factor IX deficiencies. To our knowledge, our study is the first to demonstrate an interaction between extent of vascular injury and effect of plasma *hyper*coagulability *in vivo*. This interaction suggests more severe injury (longer application of $FeCl_3$) exposes a sufficiently high amount of TF activity to negate effects of elevated FVIII. In contrast, less severe injury (shorter application of $FeCl_3$) exposes less TF activity, enabling a role for FVIII in PCA.

Our data demonstrating a etiologic role for FVIII in thrombosis are consistent with previous findings that elevated FVIII promotes Rose-Bengal-induced thrombosis[17], stabilizes thrombi (decreases microvascular thrombus embolization)[20], and normalizes the blood clotting time in vWF-deficient mice[20], and that FVIII inhibition blocks thrombus formation in murine and baboon thrombosis models[18, 19]. By comparing severe and mild vascular injuries, our study illuminates specific contributions of vascular procoagulant activity not apparent in the prior reports. Our findings differ somewhat from observations that elevated FVIII (to 250%) increases thrombus size.[17] In our study, thrombi in control and FVIII-infused mice appeared morphologically similar, with no obvious differences in thrombus size (vessel diameter), or final platelet or fibrin content. Differences between these findings may reflect the injury model ($FeCl_3$ *versus* Rose Bengal) and/or the method of quantification (IHC of fully-formed occlusive thrombi *versus* transillumination during thrombus formation which reflects the rate of thrombus growth).[17] Although we were

unable to measure the rate of thrombus growth *in vivo*, our *in vitro* data indicate faster platelet activation/aggregation (Figure 4.5) and slightly faster fibrin polymerization (data not shown), consistent with their findings. Brill *et al*. previously showed that elevated FVIII (300% of normal, 20% murine FVIII and 280% human FVIII) does not promote venous occlusion in vWF-deficient mice[20, 21], suggesting vWF is necessary for at least part of FVIII's procoagulant effects. Our study extends these findings by demonstrating a critical role for the extent of vascular injury in FVIII-dependent thrombin generation and thrombogenesis.

Besides elevated FVIII, epidemiologic studies have also correlated elevated fibrinogen levels (hyperfibrinogenemia) with thrombotic risk.[2, 8, 44] We previously showed that like elevated FVIII, elevated fibrinogen shortens the TTO in both saphenous vein and carotid artery and increases thrombus stability.[22] However, in contrast to elevated factor VIII, hyperfibrinogenemia shortens the TTO in the 3-minute $FeCl_3$ carotid injury model. Moreover, elevated fibrinogen increases thrombus fibrin content[22], whereas elevated FVIII did not. Rather, elevated FVIII accelerated platelet aggregation in a low TF/thrombin generation-dependent mechanism. Since we and others have shown that high thrombin concentrations produce dense fibrin networks that are resistant to lysis[33, 39, 45], elevated FVIII may also alter the structure of fibrin present in the thrombi of mice treated with $FeCl_3$ for 2 minutes. Unfortunately, while IHC provides quantitative information about fibrin content, which was not altered by elevated FVIII (Figure 4.4), it does not reveal information about fibrin network structure (individual fiber thickness or density). Regardless, the differences between these findings demonstrate fundamental differences in the hypercoagulable mechanisms of elevated fibrinogen and elevated FVIII, and suggest

antithrombotic strategies could be tailored for a specific hypercoagulability (blocking thrombin generation/activity *versus* blocking fibrin formation).[46]

Our findings on the relationship between vascular injury and effect of FVIII on thrombosis inform epidemiologic studies showing either moderate or no relationship between FVIII and arterial thrombosis.[9, 10, 12, 13] For example, using a broad definition of CHD that encompassed both mild and severe disease (definite, probable, or silent MI or definite CHD death), Folsom *et al*.[13] found little correlation between CHD and FVIII activity (OR=1.0). Interestingly, plaque rupture is associated with exposure of high levels of TF (reviewed in [47]). In contrast, using the presence of carotid plaque as a marker of mild or moderate disease, Pan et al.[9] showed a significant association between heart disease and FVIII (OR=2.65). In light of our data, differences in the conclusions of these studies may reflect the severity of the study inclusion event; FVIII activity likely only contributes to thrombosis when there is mild or moderate TF exposure.

Given a causative role for FVIII in thrombosis, it is tempting to speculate about the potential efficacy of antithrombotic strategies that target FVIII activity in venous and arterial thrombosis. Elevated FVIII is strongly (2- to 10.8-fold) associated with VTE, and partial inhibition of FVIII activity with a monoclonal antibody blocks thrombus formation in a murine IVC stenosis model and in baboons implanted with arteriovenous shunts.[18, 19] In addition, patients with hemophilia A are protected from venous thrombosis.[48] These data and our own findings in the saphenous vein thrombosis model suggest FVIII inhibition may protect against VTE. However, hemophilia patients are only partially protected against ischemic heart disease[49] and epidemiologic studies inconsistently associate elevated FVIII with arterial thrombosis. Together with our data, these observations suggest FVIII inhibition

would have only moderate efficacy in preventing arterial events triggered by high TF exposure.

This study has potential limitations. First, we used human FVIII to increase circulating levels in the mouse. However, our data and published studies demonstrate human FVIII is stable in murine circulation[28, 29], incorporated into murine clots[17] and supports clot formation in mice[17, 24-27]. Second, the $FeCl_3$/saphenous vein injury model is not commonly used as a model of venous thrombosis; however, our findings are consistent with published data from other venous models and effectively demonstrate the pathological activity of elevated human FVIII to thrombi formed in murine veins.[17-19] Finally, the $FeCl_3$ thrombosis model also does not fully recapitulate atherosclerotic plaque rupture in humans; however, it exposes SMC TF and produces platelet-rich thrombi similar to those seen in arterial clots.[37, 47, 50]

In conclusion, our results show that both vascular damage and plasma hypercoagulability modulate thrombogenesis. Following limited arterial injury, FVIII directly promotes thrombosis via increased thrombin generation resulting in accelerated platelet aggregation and increased thrombus stability. Elevated FVIII does not promote thrombosis following severe vascular injury. These findings provide a rationale for the controversy surrounding the role of FVIII in arterial thrombosis; FVIII's prothrombotic effects in mice depend on the extent of injury that exposes procoagulant cells and triggers thrombin generation. Although few diagnostic algorithms simultaneously consider markers of tissue damage and plasma hypercoagulability when assessing thrombosis risk, integrating these pieces of information may refine approaches to understand pathophysiologic mechanisms promoting thrombosis in humans.

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Chapter 5

Summary and Future Directions

5.1 Summary and future directions

Likely due to the ease of obtaining blood for *ex vivo* experiments, blood composition, including plasma coagulation factors, is generally well-studied. As such, deficiencies in coagulation proteins such as factor VIII or IX (hemophilia A or B, respectively) are very well-characterized and easily diagnosed. Interestingly, the role of elevated plasma coagulation factor levels (hypercoagulability) in the pathophysiology of thrombotic disorders is not well understood. Therefore, this dissertation is focused on examining the role of hypercoagulability in coagulation both *in vitro* and *in vivo*. The goal of this research was to determine whether elevated levels of specific coagulation factors contribute to risk of thrombosis.

In Chapter 2, we evaluated the effect of elevated factors XI, XI, VIII, V, X, prothrombin and fibrinogen on thrombin generation. We used a thrombin generation assay (calibrated automated thrombography, CAT) which translated changes in individual factor levels to net changes in the resulting pattern of thrombin generation. We were therefore able to demonstrate that increases in individual coagulation factors have an effect on the resultant thrombin generation profile. Because thrombin generation determines the structure of the resulting fibrin clot, changes in thrombin generation may have a profound impact on the quality of the clot that is formed. Specifically, high thrombin concentrations form dense fibrin clots with thin fibrin fibers that are resistant to lysis and therefore may be thrombogenic *in vivo*.[1-3] These studies offer insight into how different coagulation factors modulate the rate of thrombin generation and may subsequently effect clot formation and stability.

In Chapters 3 and 4, we looked more closely at elevated plasma fibrinogen (hyperfibrinogenemia) and FVIII. The goal of these studies was to determine if elevated plasma fibrinogen and FVIII is causative in the etiology of arterial and/or venous thrombosis, and the mechanism by which these hypercoagulabilities exert their effects. In both of these studies, we used an *in vivo* murine model in which the protein of interest (fibrinogen or FVIII) was infused into mice. This model allowed us to specifically, acutely elevate only one coagulation factor to a precise level (corresponding to the levels seen in thrombotic disease in humans), and therefore determine its role in thrombosis after $FeCl_3$ injury. Interestingly, hyperfibrinogenemia was prothrombotic (decreased the TTO) in both the (saphenous) vein and (carotid) artery.[4] Hyperfibrinogenemia exerted this effect by increasing thrombus fibrin content, promoting faster fibrin formation, and increasing fibrin network density, strength, and stability.[4] Importantly, hyperfibrinogenemia also increased resistance to pharmacologically-induced thrombolysis *in vivo*, demonstrating a direct, etiologic link between hyperfibrinogenemia, thrombosis, and thrombolysis in acute settings.[4]

Similarly, we acutely elevated FVIII in mice and examined their susceptibility to thrombosis, and used *ex vivo* and *in vitro* methods to elucidate biochemical mechanisms for the observed effects. Our findings confirmed an etiologic, prothrombotic role for elevated FVIII in veins (shortened TTO in saphenous vein). Interestingly, our findings in the carotid artery were in striking contrast to that of hyperfibrinogenemia; elevated FVIII shortened the TTO after 2-, but not 3-minute $FeCl_3$ treatment to the carotid artery. In addition elevated FVIII increased thrombin generation in mice after 2- but not 3-minute $FeCl_3$ treatment, augmented the thrombin generation rate and peak *in vitro* with low, but not high, TF (SMC)

exposure, accelerated the onset of platelet aggregation initiated by low TF, and promoted the formation of more stable thrombi (fewer emboli) following 2-, but not 3-minute injury in mice. Together, these findings demonstrate an etiologic role for FVIII in thrombosis, but suggest dependence of plasma FVIII activity on the extent of vascular injury.

Interestingly, both hyperfibrinogenemia and elevated FVIII are prothrombotic, but exerted their effects through different mechanisms; elevated fibrinogen increased thrombus fibrin content, density, strength and stability[4], whereas elevated FVIII accelerated platelet aggregation in a low TF/thrombin generation-dependent mechanism. The differences between these findings demonstrate fundamental differences in the hypercoagulable mechanisms of elevated fibrinogen and elevated FVIII, and suggest antithrombotic strategies could be tailored for a specific hypercoagulability (blocking thrombin generation/activity *versus* blocking fibrin formation).[5]

We have created robust and effective *in vivo* and *in vitro* methodology for examining the role of specific hypercoagulabilities in thrombosis. In the future, it will be interesting to use these methods to examine the role of elevated factors such as prothrombin. It is known that elevated prothrombin levels, typically associated with the G20210A mutation, increase risk of venous thrombosis.[6] *In vitro* with reconstituted systems and patient plasmas, hyperprothrombinemia increases the rate and peak of thrombin generation.[7-11] The high thrombin generation rates promote formation of fibrin clots composed of a fine network of thin fibrin fibers[9] and increased activation of TAFI[12]. These data suggest a direct connection between elevated prothrombin, thrombin generation, and clot stability but the role of elevated prothrombin in thrombosis *in vivo* is yet to be determined. Studies examining the role of prothrombin in a murine model of thrombosis are ongoing in our lab at this time.

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