Characterization of Prothrombotic Pathways *in Vivo*, and Discovery of Potential New Therapies for Pathological Blood Coagulation

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Dedication

To Felicia Williams, for her help and understanding over these last five years. I couldn’t have done it without you, and I look forward to many more wonderful years.

To my Grandma Joyce, who taught me that sometimes stubbornness pays off. I wish you were still here.

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Table of Contents

Page

Dedication……...….ii

Acknowledgements…….....iii

List of tables…….….vii

List of figures…….....viii

Abstract……..……..x

Chapter 1: Introduction

1.1 Blood coagulation…….……1

1.1.1 Extrinsic (tissue factor) pathway……...….2

1.1.2 Intrinsic (contact) pathway…….…2

1.2 Platelets……..……4

1.3 Thrombosis……...…….5

1.3.1 Flow……...…….6

1.3.2 Surface……7

1.3.3 Disease states……..7

1.3.3.1 Atherosclerosis…….……7

1.3.3.2 Stroke……...…8

1.3.3.3 Sepsis……..…….9

1.4 Current treatments for thrombosis……..…….10

1.4.1 Anticoagulant……...…….10

1.4.2 Antiplatelet……...….11

1.4.3 Thrombolytic…….12

1.5: Thesis overview……..12

Chapter 2: FXI deficiency and sepsis

2.1 Summary……..19

2.2 Introduction……..……20

2.3 Materials and methods…….21
2.4 Results…….…….22
2.5 Discussion……...…….24

Chapter 3: Development of a FXI inhibitor
3.1 Introduction……..….30
3.2 Materials and methods……...……..31
3.3 Results and discussion……...……..31

Chapter 4: Inhibition of FXI in baboons
4.1 Summary……..….37
4.2 Introduction…….…….38
4.3 Materials and methods……...……..39
4.4 Results……..45
4.5 Discussion……...…..50

Chapter 5: Platelet count reduction in baboons
5.1 Summary……..……62
5.2 Introduction……..….63
5.3 Materials and methods……...……..64
5.4 Results and discussion……...……..65

Chapter 6: Concluding remarks……...……..74

Chapter 7: Future direction……..…….……76

Bibliography…….…….77

CV……..……90

List of Tables

Page

1.1 The numerical system of blood coagulation factors and plasma concentrations……..……..14

2.1 Reduction of coagulopathy and leukocyte infiltration associated with factor XI (FXI) deficiency 24 hours after cecal ligation and puncture (CLP)……...……..29

4.1: FXI inhibition by aXIMab prevents occlusion of 2 mm id vascular grafts in baboons. ……53

List of Figures

Page

1.1. The blood coagulation cascade…….…….15

1.2. Intrisic (contact) factor pathway……..…..16

1.3. Representation of platelet adhesion and coagulation under flow……..17

1.4. Retention of leukocytes and platelets by thrombi formed *in vivo* under flow…….18

2.1. Cecal-ligation and puncture model of acute peritoneal sepsis……..27

2.2. Survival advantage of factor XI (FXI)-deficient mice after large bowel perforation……..……28

3.1. Anti-FXI monoclonal antibody 1A6 (aXIMab) prevents the activation of and/or the enzymatic activity of FXIa towards the chromogenic substrate S2366…….……33

3.2. aXIMab prolongs the activated partial thromboplastin time (aPTT) of pooled baboon plasma……..….34

3.3. aXIMab binds to the Apple 3 (A3) domain of factor XI……..…….35

3.4. aXIMab infusion prolongs the aPTT in a baboon……..36

4.1. Scanning electron micrograph of uncoated and collagen coated expanded polytetrafluoroethylene (ePTFE) vascular graft material……...…….54

4.2. Thrombogenic vascular graft and blood collection device……...…….55

4.3. The anti-factor XI (FXI) monoclonal antibody (aXIMab) binds human and baboon FXI, and inhibits fibrin formation in FXII-inhibited or deficient human blood under flow…….….56

4.4. Sustained inhibition of circulating FXI procoagulant activity after administration of aXIMab……57
4.5. FXI inhibition reduces platelet and fibrin deposition on collagen-coated vascular grafts……..……58
4.6. FXI inhibition reduces thrombin generation and platelet activation……59
4.7. FXI inhibition limits vascular graft-associated platelet deposition under high arterial shear……...…60
4.8. The template bleeding time is normal in baboons treated with aXIMab……..……61
5.1. Effects of anti-thrombopoietin (TPO) antiserum on platelet count and TPO levels in baboons……..68
5.2. Effects of platelet count reduction on platelet aggregation……...……69
5.3. Effects of platelet count reduction on arterial thrombus formation in baboons……...……..70
5.4. Effects of platelet count reduction on primary hemostasis in baboons……..…71
5.5. Effects of platelet count reduction on arterial thrombus formation……...…….72
5.6. Moderate platelet count reduction limits stenotic vascular-graft associated platelet deposition and prevents graft occlusion……73

Abstract

Characterization of Prothrombotic Pathways *in Vivo*, and Discovery of Potential New Therapies for Pathological Blood Coagulation

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Thrombosis and hemostasis involve a complex interplay between coagulation factors, platelets, and their underlying microenvironment. During vessel injury, platelets become activated on exposed extracellular matrix proteins or on injured endothelium where they act as a surface for coagulation amplification. Ensuing fibrin formation and sustained platelet activation results in hemostatic platelet plug development which minimizes trauma-induced bleeding. This beneficial process can spiral out of control during diseased conditions, resulting in thrombotic vascular occlusion. Elements that differentiate thrombotic and hemostatic pathways include shear force and tissue factor (TF) exposure. Since vascular thrombo-occlusion occurs at increasing shear under low TF conditions rather than the more complex flow and high TF environment of the extra-vascular wound, it is now understood that these two processes utilize divergent mechanism to maintain growth and stability. In an effort to support the advancement of safer antithrombotic treatment strategies, the goal of my studies was to explore prothrombotic pathways *in vivo* to define the elements that favor thrombus development relative to those that are crucial for normal hemostasis.

For these studies we utilized both baboon and mouse models of thrombosis. Using a mouse model of sepsis, which can result in infection related intravascular thrombosis, we found that mice lacking coagulation factor XI (FXI) had a significantly improved survival compared with wild type animals through a reduction in coagulopathy. In baboons, inhibiting FXI using a novel monoclonal antibody (aXIMab) decreased experimental thrombus formation and prevented vascular graft occlusion under high shear by destabilizing developing thrombi. FXI knockout mice and baboons treated with aXIMab showed no increased bleeding.

Platelets also play a vital role in thrombus development. While normal platelet counts vary widely in the general population, low normal counts do not predispose patients to bleeding. By transiently reducing platelet count in baboons by targeting thrombopoietin we found a strong correlation between thrombus formation and platelet number. Indeed, occlusive thrombus formation was prevented at platelet counts that did not affect bleeding.

Together our data suggest that by targeting FXI, or by reducing the platelet count, pathological thrombi developing under high shear flow may be reduced while not significantly affecting normal hemostasis.

Chapter 1: Introduction

1.1 Blood coagulation

While blood has likely fascinated humans since they were able to ponder its brilliant crimson hue, the advent of evermore sophisticated scientific tools has provided the ability to continually characterize blood components on more refined scales. In 1686, using a single-lens microscope, Marcello Malpighi was able to separate and visualize clotted blood fibers.[1] Over the last 300 plus years since this observation, more modern visualization and biochemical techniques have supplied us with many of the unanswered questions regarding blood components and blood coagulation, though many questions are still yet unresolved.

The modern sequence of blood coagulation events began to take shape in 1964 when two seminal papers were published; one by Earl Davie and Oscar Ratnoff, and another by Robert Macfarlane.[2,3] Both works outlined a waterfall or cascade model of intrinsic coagulation whereby through limited proteolysis successive activation of enzyme precursors (zymogens) lead to the eventual cleavage of fibrinogen and the formation of fibrin. The enzymes involved, known as serine proteases, selectively cleave the scissile peptide bond of the zymogen substrate in order to unmask the catalytic triad active site of the subsequent protease. This sequence of events not only provides the opportunity for stepwise amplification of the cascade, but also allows for regulation at every point. These cascade models of intrinsic blood coagulation have since expanded tremendously in complexity and have been teased apart into two separate pathways, known as extrinsic (tissue factor) and intrinsic (contact) pathways, but the general cascade schemes have remained (see Figure 1.1).

1.1.1 Extrinsic (Tissue Factor) pathway

Tissue factor (TF) is a transmembrane glycoprotein which is constitutively expressed by sub-endothelial cells of the vessel wall.[4] TF is also found in most tissue of the human body with high expression levels in the lungs, heart, and brain.[4,5] Disruption of the vascular endothelium exposes TF to blood, where it acts as a cofactor for activated factor VII (FVIIa). The TF:FVIIa complex, traditionally called the extrinsic coagulation pathway, has been proposed to be the principal initiator of coagulation *in vivo*. The importance of TF is illustrated in the fact that no TF deficient human has yet been discovered, and mice lacking TF die from perinatal bleeding.[4,6,7] FVII deficiency is very rare and results in significant bleeding similar to patients with hemophilia A and B (FVIII and FIX deficiency, respectively).[8] Thus, it is clear that the extrinsic pathway is crucial for maintaining normal hemostasis.

The role of the TF:FVIIa complex pathway in thrombosis is less clear. While TF inhibition has been shown to reduce thrombosis in animal models,[9-11] it has not been conclusively shown that TF is the main initiator and propagator of thrombus formation under all circumstances. In chapter 4, we show that inhibiting the extrinsic pathway does not produce an antithrombotic effect using an *in vitro* flow chamber model. It has been postulated that circulating TF or blood born TF microparticles supply a growing thrombus with additional TF to maintain thrombin formation,[12] although the relevance of this has been debated.[13] However, our data, as well as a growing body of experimental evidence from other labs suggest that factors of the intrinsic pathway are also crucial for thrombus propagation both *in vitro* and *in vivo*.

1.1.2: Intrinsic (contact) pathway

When mammalian blood is exposed to a negatively charged surface, a series of reactions are initiated, resulting in activation of the "contact" proteases factor XII (FXII), prekallikrein (PK), and factor XI (FXI)[14](Figure 1.2). Contact protease activation triggers coagulation- and inflammation-related host-defense systems *in vitro*; however, the importance of this to normal physiology is unclear. Most individuals with congenital contact protease deficiency have no related abnormalities. Only FXI deficiency is

associated with excessive trauma-induced bleeding in about one-third of affected individuals, indicating that FXI makes modest contributions to normal blood coagulation (hemostasis).[15] In contrast, high plasma FXI levels are an independent risk factor for arterial[16] and venous thrombosis[17] in humans, FXI deficiency protects mice from experimentally induced thrombosis,[18-20] and inhibition of FXI has antithrombotic effects in primates,[21] strongly suggesting that FXI contributes substantively to disease processes associated with abnormal coagulation (thrombosis).

In 1953 Rosenthal et al[22] first described a coagulation deficiency in plasma thromboplastin antecedent (PTA) which was later known as hemophilia C. Bleeding with PTA deficiency was only seen after surgery or trauma, with none of the spontaneous bleeding associated with classical hemophilia. Both males and females were affected, and there did not appear to be any underlying pathology associated with the disorder. Today PTA is known as FXI (Table 1), and for many years it was not considered a likely contributor to thrombosis since deficiency did not appear to predispose patients to significant bleeding. That idea began to change when Gailani and Broze[23] showed that thrombin was able to activate FXI *in vitro*, and thus proposed a revised model of the coagulation cascade whereby FXI acted as an amplifier to sustain thrombin production (see Figure 1.1). Subsequent to this observation, numerous studies have confirmed that FXI appears to be important in thrombus development. Indeed, in chapter four we show *in vivo*, using a baboon thrombosis model, that inhibiting FXI dramatically attenuates local thrombin production and renders growing thrombi prone to instability and fragmentation. This data appears to confirm the idea that FXI is important in the feedback amplification of thrombin production, and is an important constituent of growing thrombi. FXI may also be involved in inflammation, since it circulates as part of the kallikrein-kinin system in a non-covalent complex with high-molecular-weight kininogen,[24-26] which is responsible for the liberation of the vasoactive peptide bradykinin (Figure 1.2). In chapter two we show that FXI appears to be important in the development of sepsis related coagulation and possibly in inflammation, as FXI deficient mice show a survival advantage over wild type mice, and have less severe markers of coagulopathy. Thus, the story of FXI continues to evolve.

Coagulation factor XII (FXII, Hageman factor) was originally named after John Hageman, who was a patient of Oscar Ratnoff at Case Western Reserve University.[27] Mr. Hageman was found to have a significantly prolonged partial thromboplastin time upon a routine examination, however he showed no overt bleeding disorder. Mr. Hageman died in 1968 after spending a week in the hospital from trauma to the pelvis after falling from a train.[28] He died of a massive pulmonary embolism (five emboli were blocking the right and left branches of the pulmonary artery). This case likely contributed the anecdotal evidence that FXII deficiency had a prothrombotic phenotype. Recent studies using FXII deficient mice suggest that FXII may be important in thrombus formation. FXII knockout mice are in fact protected from thrombus formation and thrombotic ischemic stroke,[19] though this antithrombotic effect has yet to be confirmed in primates. FXII inhibition has also been shown to partially improve the outcome of baboons infused with a lethal dose of *E coli*,[29] though the improvement was postulated to be due to an improvement in the inflammatory response. Thus, new evidence suggests that FXII inhibition may also be an attractive strategy for antithrombotic therapy.

1.2 Platelets

Platelets circulate as anucleated cell fragments at a normal concentration of 150,000-400,000/μL. Platelet adhesion to sites of blood vessel injury and subsequent aggregation and cohesion of platelets to one another are vital to both normal hemostasis and pathological thrombosis. The adhesive and cohesive properties of platelets are regulated by a complex series of receptor mediated interactions and intracellular signaling events that allows them to maintain hemostatic functionality under a wide range of injury conditions. Under flow, von Willebrand factor (vWF) is able to bind collagen and bridge the platelet GP Ib/IX/V receptor complex to the vascular surface (Figure 1.3).[30] This is followed by platelet signaling events, resulting in an influx of calcium ions and the release of alpha and dense granules, which contain coagulation factors, fibrinogen, as well as platelet agonists such as ADP.[31,32] ADP is a weak agonist that is able to activate platelets through $P2Y_1$ and $P2Y_{12}$ receptor signaling pathways, while ADP-induced synthesis of thromboxane A2 elicits a secondary aggregation response.[33] These interactions also lead to the formation of active GP IIb-IIIa complexes on the

platelet surface, which are then able to bind vWF and fibrinogen, linking adjacent activated platelets and facilitating the formation of a stable platelet plug. While there are many agonists for platelet activation and aggregation, thrombin is the most potent physiological activator of platelets.[34] Thrombin cleaves PAR1 and PAR4 which results in intacellular signaling and platelet activation. Thrombin can also activate platelets through the GP Ib/IX/V receptor.[35]

Many of these receptor mediated pathways are also important under the complex flow conditions associated with wound environments, so while pharmacological blockade of platelet activation pathways can reduce arterial thrombosis, all current strategies elicit hemorrhagic side-effects. Another important aspect to note is that the activated platelet surface acts as a nidus for coagulation protease activation and subsequent fibrin formation. Some new evidence also suggests that FXI may be important in binding platelets through GP Ib and ApoER2, which results in platelet activation.[36] This illustrates the complex interplay between coagulation and platelets that is continuing to be uncovered.

1.3 Thrombosis

Thrombi generally develop on chronic atheroschlerotic lesions and during various disease states such as cancer and systemic infection. Thrombosis associated with heart attack and stroke is the leading cause of mortality in the developed world,[37] thus it is important to appreciate the underlying mechanisms that lead to thrombus formation. Thrombi are complex manifestations of aggregating platelets, fibrin, leukocytes, and red blood cells that form under flow within the vascular lumen. Flow is one of the most important factor in determining the complex makeup of the thrombus, as illustrated by the fact that venous thrombi formed under relatively low shear forces are rich in red cells and fibrin and thus often referred to as “red” thrombi, while those that form under arterial flow are generally rich in platelets and leukocytes and are known as “white” thrombi.[38,39] An illustration of flow effects on blood clotting is shown in Figure 1.4. Though flow is important in thrombus development, the other two components of Virchow’s triad,[40] i.e.

the surface with which the blood interacts and the blood constituents are also critical to thrombus formation.

1.3.1 Flow

As discussed previously, hemostasis and thrombosis involve different flow phenomena which results in changing convective transport of coagulation factors and platelets to the site of injury or to the thrombus surface. A regularly used term in this process to describe how fast adjacent fluid layers slide past one another is the shear rate (γ). The shear rate is zero at the midpoint of the tube or vessel axis and becomes maximum at the vessel wall. The wall shear rate is generally expressed as γ_w, and can be found using the volumetric flow rate Q and the vessel radius R.

$$\gamma_w = 4Q/\pi R^3$$

The wall shear stress, τ_w, is the force a viscous fluid exerts on the vessel wall per unit area, and is linearly related to the shear rate by a viscosity constant μ for a Newtonian fluid. Common wall shear rates for arteries are ~300-500 s^{-1} for carotid or main coronary arteries and up to 1900 s^{-1} for arterioles,[41,42] while venous shear is generally lower than 200 s^{-1}. In areas of stenosis the vascular wall shear rates can attain local maxima on the order of 10^5 s^{-1}.[42] The higher convective transport associated with greater shear is important for delivery of platelets and coagulation factors to the growing thrombus, but also in the wash-out effect of activated coagulation factors. This is likely why high arterial shear thrombi tend to be platelet rich and fibrin poor.

Flow also controls the embolization potential of arterial thrombi. The adhesion of circulating platelets is shear dependent and involves a complex set of interaction of VWF with GP Ib/IX/V complex and collagen. Other adhesion molecules are also important such as P-selectin and GPVI.[33] A defect in these mechanisms can alter platelet adhesion and destabilize thrombi, though platelet defects tend to also cause bleeding.

1.3.2 Surface

Endothelial cells, which line the entirety of the vascular lumen, actively promote the fluid phase of plasma and support platelet quiescence by secreting prostacyclin (PGI2), nitric oxide (NO), heparin sulfate, and tissue plasminogen activator (tPA) among others.[33] Endothelial cells when injured can also expose thrombogenic substances such as VWF, P-selectin, and TF.[33] Denudation of the endothelium can expose collagens and other procoagulant matrix proteins as well as TF, which are important for normal hemostatic processes to support vascular integrity but also play a significant role in thrombosis. When foreign surfaces such as bacteria during infection or prosthetic valves and vascular grafts are exposed to blood, initiation of platelet activation as well as blood coagulation through the contact pathway can occur.

1.3.3 Disease states

Many diseases produce alterations in the makeup of blood constituents and also affect the blood flow patterns and vascular surface, all of which can predispose individuals to thrombosis. The treatment for these conditions can be complicated by the need for both interventional therapy as well as prophylactic treatment strategies. In the following sections I will discuss some of the major diseases associated with thrombosis, and also discuss current treatment options.

1.3.3.1 Atherosclerosis

Coronary and cerebral artery disease continue to be the leading causes of morbidity and mortality in the developed world, accounting for up to half of all deaths in the United States.[37] The underlying cause of these diseases is the chronic development of atherosclerosis, resulting from endothelial cell injury originating from prolonged hypercholesterolemia and hypertension.[43] Endothelial cell injury initiates a cascade of responses including infiltration of monocytes, foam cell formation, and smooth muscle cell proliferation that results in atheromatous plaque formation.[33] These plaques can continue to invade the blood vessel lumen, causing severe stenoses and resulting in disturbed blood flow patterns. Though it is not completely understood, some plaques are prone to rupture, leading to heart attack and stroke from acute thrombus development.

Indeed, up to two-thirds of patients with myocardial infarction have angiographic coronary plaque lesions.[44]

While it is prudent to treat the underlying disorder by controlling cholesterol and blood pressure, preventing death and permanent disability during atherosclerosis-related thrombotic events is also an important effort. Even with the bleeding side effects, platelet inhibitors are particularly effective in preventing recurrent heart attack and stroke. Our data in chapter five indicates that reducing platelet count to low normal levels may be a new strategy to safely limit thrombus progression and improve the outcome of thrombotic events in plaque stenosed vessels.

1.3.3.2 Stroke

Stroke is the third leading cause of death in the United States and a major contributor to chronic disability.[37,45] The direct costs for therapy, drugs, and other care will total an estimated $35 billion for 2005,[45] with the majority of the money going to rehabilitate and treat the roughly 400,000 yearly stroke survivors. 85% of strokes are ischemic in nature, and 80% of ischemic strokes show the presence of occlusive clots.[46-48] Most strokes are the result of embolic thrombi that develop on atheroschlorotic plaques within the carotid arteries or from thrombi formed within the heart due to flow disturbances. The only FDA approved treatment for stroke is tPA. The benefits of using tPA to treat ischemic stroke have been well documented.[49-52] However, the benefits of tPA are partially mitigated by the narrow three-hour treatment period and the fact that tPA can cause serious intracerebral hemorrhaging (ICH).[53] Even when strict dosage guidelines are kept, there are a reported 6% of patients that still suffer from ICH.[54] These limiting factors represent a clear challenge for researchers and clinicians to overcome.

Antiplatelet therapies such as aspirin and clopidogrel have shown to be useful in the prevention or treatment of stroke.[55-57] Studies have found that aspirin therapy within 48 hours reduced mortality and stroke recurrence.[57,58] The CAPRIE study also indicated that clopidogrel may be effective in reducing the risk of ischemic events in certain patients with combined risk factors.[55] These modalities, although effective in treating

stroke patients, continue to be limited by their hemorrhagic side effects and must be used carefully to prevent ICH.

Existing anticoagulant therapies are again complicated by their tendency to cause bleeding. Drugs such as heparins and warfarin have been shown to be clinically useful in preventing the progression of cerebral thrombosis,[59] but in many cases the risks of ICH outweigh the benefits.[60-62] In order to have a safe and effective anticoagulant or antiplatelet therapy, one must be found that inhibits thrombotic progression but is not essential for normal hemostasis. Recent data suggests that FXI or FXII inhibition may be candidates for the safe treatment of ischemic stroke.[19,63]

1.3.3.3 Sepsis

Bacterial sepsis causes a systemic procoagulant state ranging from the subclinical to fulminant disseminated intravascular coagulation (DIC) with multifocal thrombosis that consumes platelets, leukocytes, and fibrinogen, manifested as hemorrhage and multiple organ failure. Infection-associated coagulation is likely maintained by contact protease activation on the surface of bacteria, and by tissue factor-expressing activated leukocytes and inflamed endothelium.[64,65] Administration of activated protein C (APC) decreases sepsis mortality probably by targeting both coagulation and inflammation.[66] Humans and mice completely deficient in protein C develop lethal perinatal consumptive coagulopathy (*purpura fulminans*). FXI likely contributes, as protein C-deficient mice that are also FXI deficient can reach adulthood.[20]

The use of APC has been FDA-approved for the reduction of mortality in adult patients with severe sepsis (sepsis associated with acute organ dysfunction) who have a high risk of death. This vitamin K-dependent protease is both anticoagulant and anti-inflammatory *in vivo*, and its administration significantly reduces mortality from severe sepsis.[66-68] However, the clinical outcome benefit is limited, and systemic anticoagulant effects of APC can contribute to bleeding in patients with an already compromised hemostatic capacity, typically caused by DIC-related consumption of platelets, leukocytes, and coagulation factors.

Activation and/or consumption of FXI during infection or inflammation, with or without the activation of other contact system proteases, has been extensively documented both in models and in patients.[69-72] Earlier studies in patients showed contact system activation in infections of various origin, and suggested that septic patients could benefit from inhibition of contact activation.[73] Experiments in baboons using inhibitory antibodies to FXII confirmed that one or more components of the contact activation complex, including FXI and prekallikrein, might contribute to the development and pathogenesis of *E. coli* induced septic shock.[29,74] Survival of *E. coli* sepsis was extended in the treatment group. Since activation of FXI by thrombin on activated platelets can bypass FXII, the resultant FXIa could, in theory, activate the coagulation cascade via FIX and release bradykinin from high-molecular-weight kininogen (HMWK, Figure 1.2),[75,76] thus causing both intravascular coagulation and acute inflammation even in the absence of FXII. Indeed, our data in chapter two suggests that targeting FXI may be a safe and effective new strategy for improving the outcome of sepsis.

1.4 Current treatments for thrombosis

Antithrombotic agents that target essential components of hemostasis can treat and reduce the progression of thrombotic diseases including heart attack and stroke. However, bleeding complications can outweigh or reverse the benefits. In the following sections I will discuss current treatment strategies for thrombosis and their mechanisms of action.

1.4.1 Anticoagulant

Heparin is one of the oldest and most widely used anticoagulant compounds in the world. Heparin was discovered in 1916 by Jay McLean by chance when he was studying alcohol extracts of the brain heart and liver, and found that after several months of storage the extracts inhibited blood coagulation. Howell and Holt in 1918 after further investigation coined the term heparin.[1] Unfractionated heparin is a high molecular weight proteoglycan consisting of a peptide core and 15 polysaccharide chains (750-1000 kD). Heparin has no direct anticoagulant effect but rather interacts with antithrombin III and increases its affinity for FXa and thrombin up to 4-orders of magnitude.[77] The treatment

of choice for venous thrombosis and pulmonary embolism is now the low-molecular-weight forms of heparin (LMWH). These have greater specificity for FXa, more predictable pharmacokinetic profiles, and less antiplatelet activity.[78]

Warfarin and other coumarin derived oral anticoagulats are widely used for the prevention of thrombosis. Warfarin is a vitamin K antagonist that prevents coagulation factors II, VII, IX, and X from folding properly by preventing vitamin K dependent γ-carboxylation of glutamic acid residues,[79,80] which is crucial for calcium binding and subsequent interaction with negatively charged surfaces, and thus hinders active protease formation. While warfarin is effective in treating thrombosis, its narrow efficacy-safety range and hemorrhagic complications can limit its usefulness.[78] Warfarin is also contraindicated during pregnancy because of its potential teratogenicity.[81]

There are other anticoagulants that can be useful in treating thrombosis, such as direct thrombin inhibitors like hirudin, as well as a new FXa inhibitor rivaroxaban. But again, as with all other existing anticoagulants that target essential components of the coagulation pathway, they produce bleeding effects. In order to have a safe and effective anticoagulant, one must be found that inhibits a portion of the coagulation cascade important for thrombotic progression, but also not essential for normal hemostasis.

1.4.2 Antiplatelet

The most commonly used antiplatelet agents are aspirin and clopidogrel. Aspirin is the oldest and most widely used of the antiplatelet drugs.[78] Aspirin irreversibly inhibits platelet cyclooxygenase which prevents the formation of the platelet agonist thromboxane A_2.[82] Aspirin is effective in preventing recurrent stroke and in preventing myocardial infarction, however severe or fatal bleeding complications increase by 0.5%/year with chronic use.[78]

Clopidogrel is a thienopyridine whose active metabolite inhibits the platelet ADP receptor $P2Y_{12}$.[83] The CAPRIE trial[55] showed clopidogrel to be slightly better than aspirin for stroke and myocardial infarction prevention. More recently the CURE trial[84]

showed that combined aspirin and clopidogrel therapy improved unfavorable outcomes by 20% over aspirin alone during unstable angina. The primary complication of clopidogrel therapy is bleeding. Antiplatelet agents tend not to be effective in preventing relatively lower shear venous thrombosis.[78]

1.4.3 Thrombolytic

Various plasminogen activators are available for the treatment of thrombosis. Tissue plasminogen activator (tPA) is a naturally occurring serine protease that is secreted by endothelial cells and acts as a direct activator of plasminogen, converting it to plasmin, which actively lyses fibrin to dissolve clots.[33] Recombinant tPA is now used for the treatment of acute thrombotic stroke and has shown effectiveness if given within three hours of stroke onset. Patients who undergo thrombolytic therapy must be screened to rule out hemorrhage and must have a systolic blood pressure no greater than 185 mmHg.[52] As with antithrombotic drugs, thrombolytics can have severe bleeding side effects.

1.5 Thesis overview

In this work I have used a primate model of acute arterial thrombosis and a mouse model of peritoneal infection and sepsis in an attempt to better characterize prothrombotic events that occur *in vivo* during disease conditions. My main goal was to contribute to the understanding of thrombosis and hemostasis in an effort to support the development of safer antithrombotic therapies. In Chapter 2, I provide evidence that FXI is important for sepsis associated mortality in mice and discuss this as a potential new strategy for safely limiting sepsis associated thrombosis in humans. Chapter 3 describes the discovery, characterization, and testing of a novel monoclonal antibody to FXI for use as a tool to probe the role of FXI in our primate thrombosis model, which I discuss further in Chapter 4. Chapter 5 presents new data suggesting that transiently reducing platelet count within the normal physiologic range may be a safe strategy for limiting occlusive arterial thrombus formation. Chapter 6 contains concluding remarks on the studies within this thesis, and Chapter 7 suggests future work. Overall, my attempt was to present evidence that thrombosis and hemostasis are not inexorably linked, and that

targeting FXI or platelet count may be attractive strategies for antithrombotic therapy, both of which seem to discriminate between the hemostatic wound environment and the environment associated with vascular thrombosis.

Table 1.1. The numerical system of blood coagulation factors and plasma concentrations.[28,85,86]

Factor	Current synonym	Former name	Plasma Concentration (μg/mL)
I	Fibrinogen	-	1500-4500
II	Prothrombin	-	100
III	Thromboplastin or Tissue Factor	-	-
IV	Calcium	-	.8-1
V	-	Proaccelerin	7
VI	Now recognized as FVa	Accellerin	-
VII	-	Proconvertin	.5
VIII	-	Antihemophilic Factor	.1-.2
IX	-	Christmas Factor	5
X	-	Stuart-Prower Factor	10
XI	-	Plasma Thromboplastin Antecedent (PTA)	4-6
XII	-	Hageman Factor	40
XIII	-	Fibrin stabilizing Factor	30

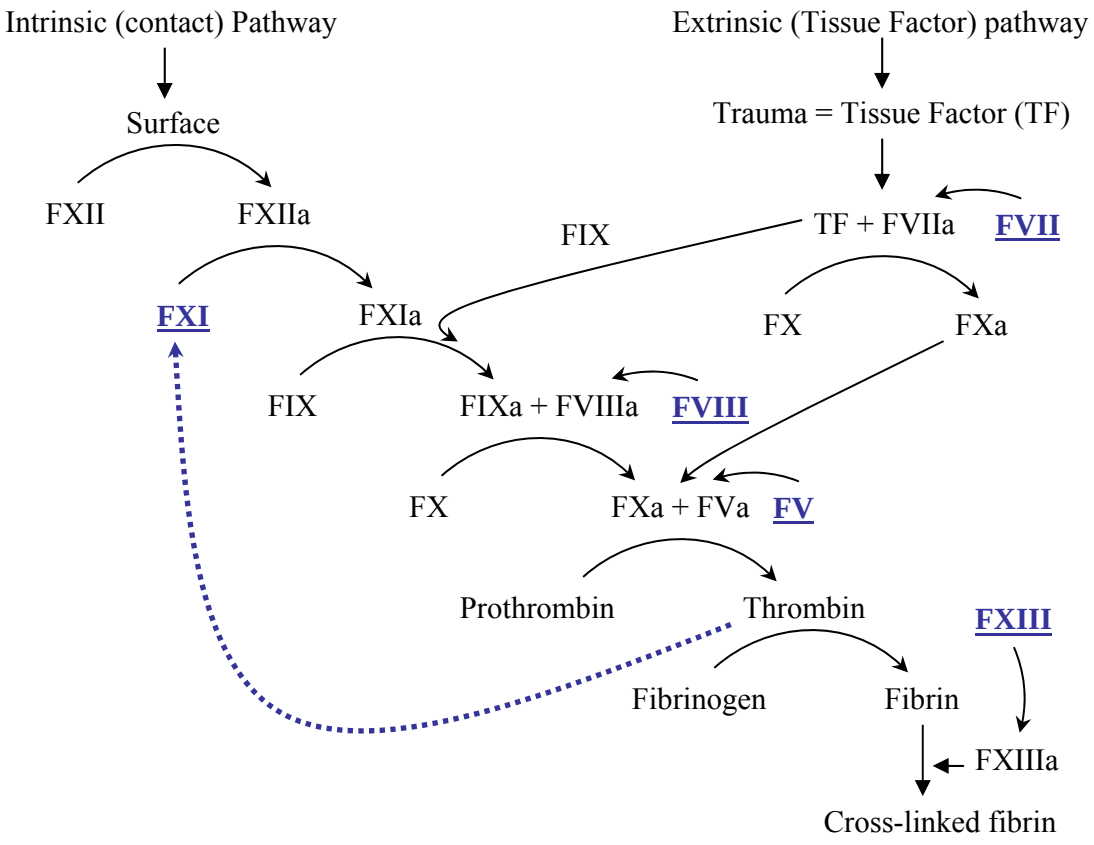

Figure 1.1. The blood coagulation cascade. Underlined factors are all activated by thrombin, which contributes to feedback amplification of the coagulation pathway.

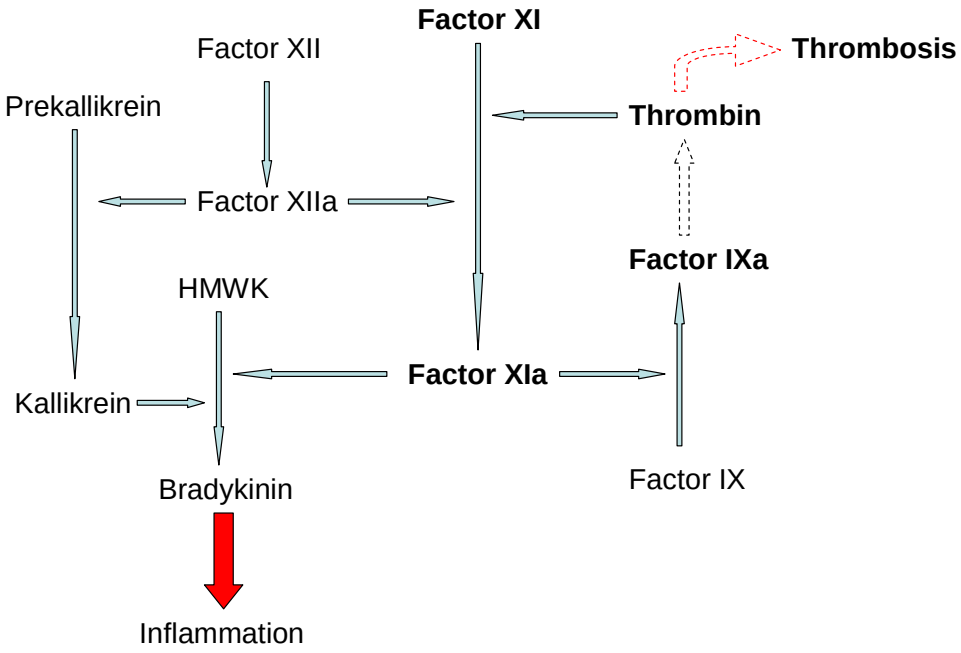

Figure 1.2. Intrisic (contact) factor pathway. This diagram highlights the role of FXI in the feedback amplification of thrombin, as well as the potential role for FXI in inflammation through the kallikrein-kinin system.

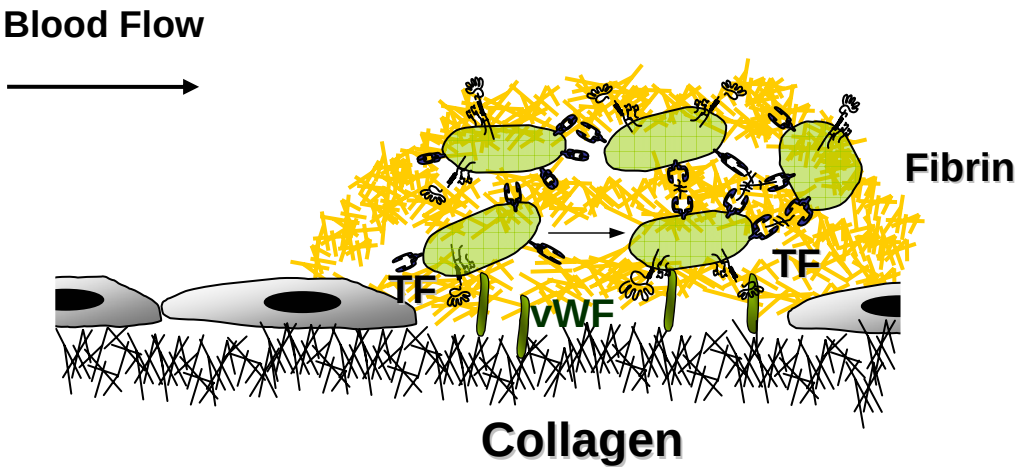

Figure 1.3. Representation of platelet adhesion and coagulation under flow. Under high shear flow, von Willebrand factor (vWF) is able to bind collagen and bridge the platelet GP Ib/IX/V receptor complex to the vascular surface. This is followed by platelet signaling events, resulting in an influx of calcium ions and the release of alpha and dense granules, which contain coagulation factors, fibrinogen, as well as platelet agonists such as ADP. These interactions also lead to the formation of active GP IIb-IIIa complexes on the platelet surface, which are then able to bind vWF and fibrinogen, linking adjacent activated platelets and facilitating the formation of a stable platelet plug. At the same instant, factors of the coagulation pathway are able to bind to the negatively charged surface of the activated platelet where they produce thrombin, through both the tissue factor (TF) and contact pathways, which cleaves fibrinogen to generate fibrin. This figure was adapted from one provided by Dr. Owen McCarty.

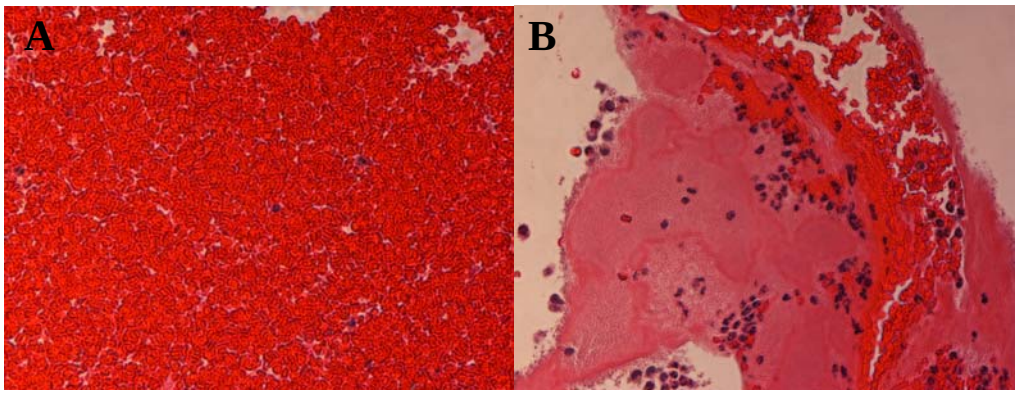

Figure 1.4. Retention of leukocytes and platelets by thrombi formed *in vivo* under flow. Standard light microscopy (phosphotungstic acid hematoxylin stain) of (A) a typical section of blood that was clotted for 60 minutes, or (B) a thrombi that was formed in the baboon arteriovenous shunt thrombosis model described in chapter 4. Thrombi are rich in fibrin, platelets, and leukocytes and had relatively fewer red cells.

-This figure was originally published in *Blood*. Gruber A, Marzec UM, Bush L, Di Cera E, Fernandez JA, Berny M A, Tucker EI, McCarty OJ, Griffin JH, Hanson SR. Relative antithrombotic and antihemostatic effects of protein C activator versus low-molecular-weight heparin in primates. 2007;109:3733-40. © 2007 by the American Society of Hematology.

Chapter 2: Factor XI Deficiency and Sepsis

2.1 Summary

Anticoagulation is a rational approach to the treatment of sepsis-associated consumptive coagulopathy, but its application is limited because of the risk of excessive bleeding. Factor XI (FXI) contributes substantially to pathological blood coagulation (thrombosis) whereas it contributing only modestly to normal hemostasis. We found that FXI-deficient mice have reduced coagulopathy and increased survival relative to FXI-expressing wild-type mice during cecal ligation and puncture-induced acute peritonitis/sepsis. This finding suggests that FXI contributes to coagulopathy and/or inflammation during sepsis, and that pharmacologic inhibition of FXI activity may alter the course and outcome of some infections.

-This research was originally published in *The Journal of Infectious Diseases*. Tucker EI, Gailani D, Hurst S, Cheng Q, Hanson SR, Gruber A. Survival advantage of coagulation factor XI-deficient mice during peritoneal sepsis. *J Infect Dis*. 2008;198:271-4.

-All data in this thesis is my work except where footnoted.

2.2 Introduction

Sepsis remains a leading contributor to mortality in critically ill people. Most patients with bacterial sepsis have abnormalities of blood coagulation, ranging from subclinical activation to fulminant disseminated intravascular coagulation (DIC) with multifocal thrombosis and hemorrhage. DIC appears to be triggered by the activation of contact proteases on the surface of bacteria,[65] and/or by tissue factor (TF)-expressing leukocytes and inflamed endothelium. During normal hemostasis, the plasma protease factor XI (FXI) may contribute to sustained thrombin generation following factor VIIa/TF-initiated coagulation, contributing to the maintenance of fibrin clot integrity and inducing platelet activation. FXI also contributes to experimental arterial and venous thrombosis,[21,87] and to DIC in mice. Protein C (PC)-deficient mice die in the peripartum period from a consumptive coagulopathy resembling *purpura fulminans* in human neonates with total PC deficiency.[88] FXI deficiency ameliorates the severity of this syndrome, with some animals surviving to adulthood.[20]

Contact proteases (FXI, factor XII [FXII], and prekallikrein) can trigger coagulation and inflammation-related host-defense systems, but their role in normal physiology is unclear. Most individuals with congenital contact protease deficiency have no abnormalities of hemostasis. FXI deficiency has been associated with moderate trauma-related bleeding in one-third of affected individuals, indicating that FXI makes modest contributions to normal hemostasis.[15] On the other hand, the plasma FXI level is an independent risk factor for thrombosis in humans, [17,63] and FXI deficiency protects mice from experimentally induced thrombosis and stroke.[19,20,89] Inhibition of FXI also has antithrombotic effects in primates,[21] suggesting that FXI contributes substantively to disease processes associated with intravascular blood coagulation. Here, we report on the effects of FXI deficiency in murine abdominal sepsis.

2.3 Materials and methods

Age-matched (4-7 month old) and sex-matched wild-type (WT or $FXI^{+/+}$) and FXI-deficient ($FXI^{-/-}$) C57BL/6 mice were used for all experiments. Generation and breeding of $FXI^{-/-}$ mice has been described elsewhere.[90] The $FXI^{-/-}$ genotype was backcrossed onto the C57BL/6 background for >9 generations. Experiments were approved by the animal care committees of the Oregon Health & Science and Vanderbilt Universities.

Primary peritoneal fecal contamination was induced in mice by standardized surgical trauma that involves ligation and puncture of the cecum (CLP, Figure 2.1) [91]. Survival was recorded for seven days post-surgery, after which all surviving mice were killed. In separate experiments, blood was drawn by cardiac puncture into a $1/10^{th}$ volume of 3.2% sodium citrate, 24 hours post-CLP or sham surgery for determining blood cell counts. Plasma was prepared from these samples and stored at -80°C pending testing.

For experimental endotoxemia, groups of 15 mice for each genotype were challenged with intraperitoneal injection of *Escherichia coli* endotoxin (25 mg/kg serotype-0111:B4, Sigma, St. Louis, MO) without general anesthesia. Spontaneous mortality 24 hours after injection was recorded, after which all survivors were killed. In a separate experiment, blood was collected by cardiac puncture into $1/10^{th}$ volume of 0.5M EDTA from groups of 10 mice for each genotype before, 6 hours, and 12 hours after injection for determining blood cell counts. Plasma was prepared from these samples and stored at -80°C pending testing.

Cell counts were measured in anticoagulated blood samples using a 9000 Hematology Series Cell Counter (Baker Instruments, Allentown, PA) for mice that underwent CLP. Clottable circulating fibrinogen was determined as a percent of the value for pooled normal mouse plasma using the Clauss method. [92] Prothrombin times (PT) were determined using an electromechanical fibrometer with plasma samples diluted in PBS. Levels of thrombin/antithrombin III (TAT) complexes and D-dimer were

determined by ELISA using commercial test kits (Dade-Behring and Diagnostica Stago, respectively). Leukocytes in the peritoneal cavity were enumerated by lavage with 3 mL of sterile saline. Lavage fluid was also plated on blood agar plates and incubated overnight at 37º C, and bacterial colonies were counted and expressed as colony-forming units (cfu) per milliliter. Identification of bacteria was conducted on blood and peritoneal swab samples collected 24 hours after CLP.

In endotoxin-injected mice, cell counts were determined using a Hemavet HV950FS analyzer (Drew Scientific, Oxford, CT). TAT complex was measured by ELISA (Enzyme Research Laboratories, South Bend, IN), using control curves constructed with murine TAT complex prepared by mixing mouse α-thrombin and antithrombin (Haematologic Technologies, Essex Junction, VT). Other laboratory parameters were measured as described above.

Data are shown as mean ± standard error of the mean (SEM), unless otherwise specified. Survival data were analyzed using Kaplan-Meier analysis, and comparisons between genotypes were done by the log-rank test. The two-tailed Student's *t*-test was used for single-pair comparisons. $P \leq .05$ was considered to indicate significance.

2.4 Results

The 15-minute surgical procedure for CLP did not cause excessive bleeding in either WT or $FXI^{-/-}$ mice. Survival after CLP is shown in Figure 2.2. All mice lived at least 24 hours, with more WT mice developing signs of illness (lethargy, piloerection, and reduced mobility), suggesting systemic infection. One week post-CLP, 6 of 47 WT mice (13%) and 24 of 52 $FXI^{-/-}$ mice (46%) were alive ($P = .0001$) (odds ratio of death for WT compared with $FXI^{-/-}$ mice, 5.9 [95% confidence interval 2.1-16.2]) and appeared to be fully recovered. WT mice died between days 2 and 4 (60 ± 2 hours, median, 62 hours), with none dying after 90 hours. In contrast, 75% of deaths in $FXI^{-/-}$ mice occurred between days 3 and 6 (79 ± 4 hours, median, 76 hours), with the latest death occurring at 142 hours. Average survival time for $FXI^{-/-}$ mice dying of sepsis was 32% longer than that for WT mice ($P < .001$). No animals (n = 6 for each genotype) died after sham

surgery, in which the cecum was exposed without puncture. FXI deficiency had no affect on mortality (70% for both genotypes) 24 hours after endotoxin injection.

Twenty-four hours after CLP, platelet counts decreased 27 ± 5% in WT mice (P = .001, compared with baseline) and by 13 ± 4% in $FXI^{-/-}$ mice (P = .05) (Table 2.1). The decrease for WT mice was significantly larger than that for $FXI^{-/-}$ mice (P = .05), suggesting more severe coagulopathy in the WT mice. Leukocyte counts decreased for WT (64 ± 5%; P < .001) and $FXI^{-/-}$ (41 ± 8%; P < .01) mice 24 hours after CLP, with the decrease being significantly greater in WT mice (P < .05). The migration of circulating leukocytes into the peritoneum was confirmed by analysis of lavage fluid after CLP, with WT mice showing more pronounced leukocyte infiltration than $FXI^{-/-}$ mice (P < .01). There were no differences in red blood cell counts in any of the groups. Sham-operated mice showed no deviations from normal baseline values (Table 2.1). The histology of livers and lungs were unremarkable 24 hours after CLP, consistent with previous reports.[93]

WT and $FXI^{-/-}$ mice (n = 10 for each) showed similar decreases in platelet counts (47 ± 4% and 41 ± 5%, respectively; P < .01 for both, compared with baseline) and blood leukocyte counts (67 ± 4% and 74 ± 2%, respectively; P < .01 for both) 12 hours after endotoxin treatment. The 6 hour time-point values were similar for all measured parameters (data not shown).

In WT mice, fibrinogen decreased from baseline 24 hours after CLP (Table 2.1), suggesting consumption or reduced production of fibrinogen. In contrast, fibrinogen levels increased considerably in $FXI^{-/-}$ mice, suggesting an acute-phase increase of fibrinogen with limited consumption. Prothrombin time, which reflects plasma coagulation-factor levels, was prolonged in WT mice 24 hours after CLP (P < .05) but was unchanged in $FXI^{-/-}$ mice. TAT levels were also higher in WT mice after CLP (P < .05), but only a modest elevation was seen in $FXI^{-/-}$ mice. D-dimer levels increased similarly for both WT mice (100 ± 10% to 302 ± 46%; P < .05) and $FXI^{-/-}$ mice (138 ±

17% to 325 ± 54%; $P < .05$) 24 hours after CLP (n = 6 for each), compared with levels in pooled normal mouse plasma.

Twelve hours after endotoxin injection, fibrinogen levels were 73 ± 11% in WT mice (n = 7) and 132 ± 20% in $FXI^{-/-}$ mice (n = 9) ($P < .05$ for WT vs $FXI^{-/-}$ mice), similar to the results for CLP. As measured by an ELISA using a murine TAT standard, baseline TAT values were 4.2 ± 0.9 ng/mL for WT mice and 6.9 ± 0.8 ng/mL for $FXI^{-/-}$ mice (n = 10 for each), levels similar to those measured by an ELISA using human standards in CLP experiments (Table 2.1). TAT levels after endotoxin injection increased >20-fold in WT mice (87.9 ± 14.8 ng/mL) but less so in $FXI^{-/-}$ mice (42.8 ± 4.9 ng/mL), whereas D-dimer increased to 249 ± 10% in WT mice and to 268 ± 15% in $FXI^{-/-}$ mice (n = 10 for each) relative to baseline.

WT and $FXI^{-/-}$ mice showed no significant differences in the number of bacterial colony-forming units (209 ± 129 vs 130 ± 35 cfu/mL, respectively; n = 8 for each) in the peritoneal cavity 24 hours after CLP. Although the mean colony-forming unit count was slightly greater in WT mice (35 cfu/mL) the median counts were higher in $FXI^{-/-}$ animals (110 cfu/mL). There was no growth in lavage fluid from sham-operated mice. Cultures of peritoneal swab samples from two mice for each genotype showed an abundance of α-hemolytic *Streptococcus* organisms (>50 colonies per sample) and a smaller number of other gram-negative and gram-positive bacteria (1-10 colonies per sample). α-hemolytic *Streptococcus* was the primary pathogen (10-50 colonies) in blood cultures, with *Staphylococcus aureus* and gram-negative bacteria also present (1-10 colonies per sample). The types and relative abundance of bacteria were similar for both genotypes.

2.5 Discussion

Activation and/or consumption of FXI during infection or inflammation, with or without activation of other contact-system proteases, has been documented in animal models and human patients. Early studies in humans demonstrated activation of the contact system during infections of various origins, suggesting that septic patients could benefit from inhibition of contact activation.[73] Experiments in baboons in which

inhibitory antibodies to FXII were used suggested that one or more components of the contact-activation complex may contribute to the pathogenesis of *E. coli*-induced septic shock.[29] The present study was designed to test the hypothesis that FXI contributes to the pathogenesis of traumatic fecal peritoneal infection and sepsis in mice. Because potent, monospecific inhibitors of mouse FXI are not available, we used FXI deficient mice and showed that FXI deficiency was indeed associated with significantly reduced mortality during untreated polymicrobial peritonitis/sepsis.

$FXI^{-/-}$ mice appeared to have less activation of coagulation and less migration of leukocytes into the peritoneum 24 hours after bowel perforation. Thus, in addition to coagulation, FXI may be involved in leukocyte trafficking, either directly or, via inflammatory pathways, indirectly. Given that FXI deficiency rescues PC-deficient mice [20] and that FXI is prothrombotic,[21,89] we postulate that FXI deficiency lessened the severity of sepsis-induced DIC through a reduction in thrombin generation, though a reduction in thrombin associated with FV deficiency in mice was linked with a decreased survival compared with WT in a sepsis model.[94] Despite the compelling data on platelets, fibrinogen, and TAT complexes, the importance of the antithrombotic component of FXI deficiency to the outcome of sepsis remains speculative. In addition to its role in coagulation, FXI may be involved in pathways that affect platelet and leukocyte function and trafficking as well as bradykinin generation and could, therefore, have an intensifying effect on the systemic inflammatory response syndrome and development of septic shock. Interestingly, FXI deficiency did not affect survival during experimental endotoxemia, despite evidence of a less-robust coagulopathy in the $FXI^{-/-}$ mice. This indicates that FXI plays a limited role in the pathophysiology of endotoxemia and reinforces the concept that there may be major differences in the mechanisms involved in gram-negative sepsis models that employ experimental endotoxemia and true abdominal sepsis in mice.

In an apparent contradiction to our present finding, a survival advantage seen with sepsis as well as after endotoxin administration has also been ascribed to the prothrombotic heterozygous, but not homozygous factor V Leiden mutation in mice, and even in septic human patients.[95] According to this study, the survival advantage might

have been related to a balanced increase in thrombin, thus modest secondary increase in APC generation in the heterozygotes, at least in the endotoxin challenge group. Since FXI deficiency would presumably result in decreased APC generation, this deficit might have lead to the observed survival advantage through different mechanisms, as discussed above. Investigating FXI and PC deficiencies in combination with the factor V Leiden mutation in mice could provide additional insight into the molecular mechanisms to understand the apparent contradiction.

Regardless of the mechanisms involved, the data suggest that FXI as well as related proteases and cofactors that affect its activation (high-molecular-weight kininogen, plasma prekallikrein, and FXII) may be attractive targets for pharmacological inhibition in treatment of sepsis-induced DIC. The relatively mild bleeding diathesis associated with severe FXI deficiency suggests that targeted FXI inhibition would be safer in this setting than conventional anticoagulation.

In conclusion, safe and effective new treatments for severe sepsis are needed, and our data demonstrating a survival benefit in a severe form of bacterial infection provide rationale for developing FXI-specific inhibitors for further investigation into the effects of FXI and FXI inhibition in infections and inflammation.

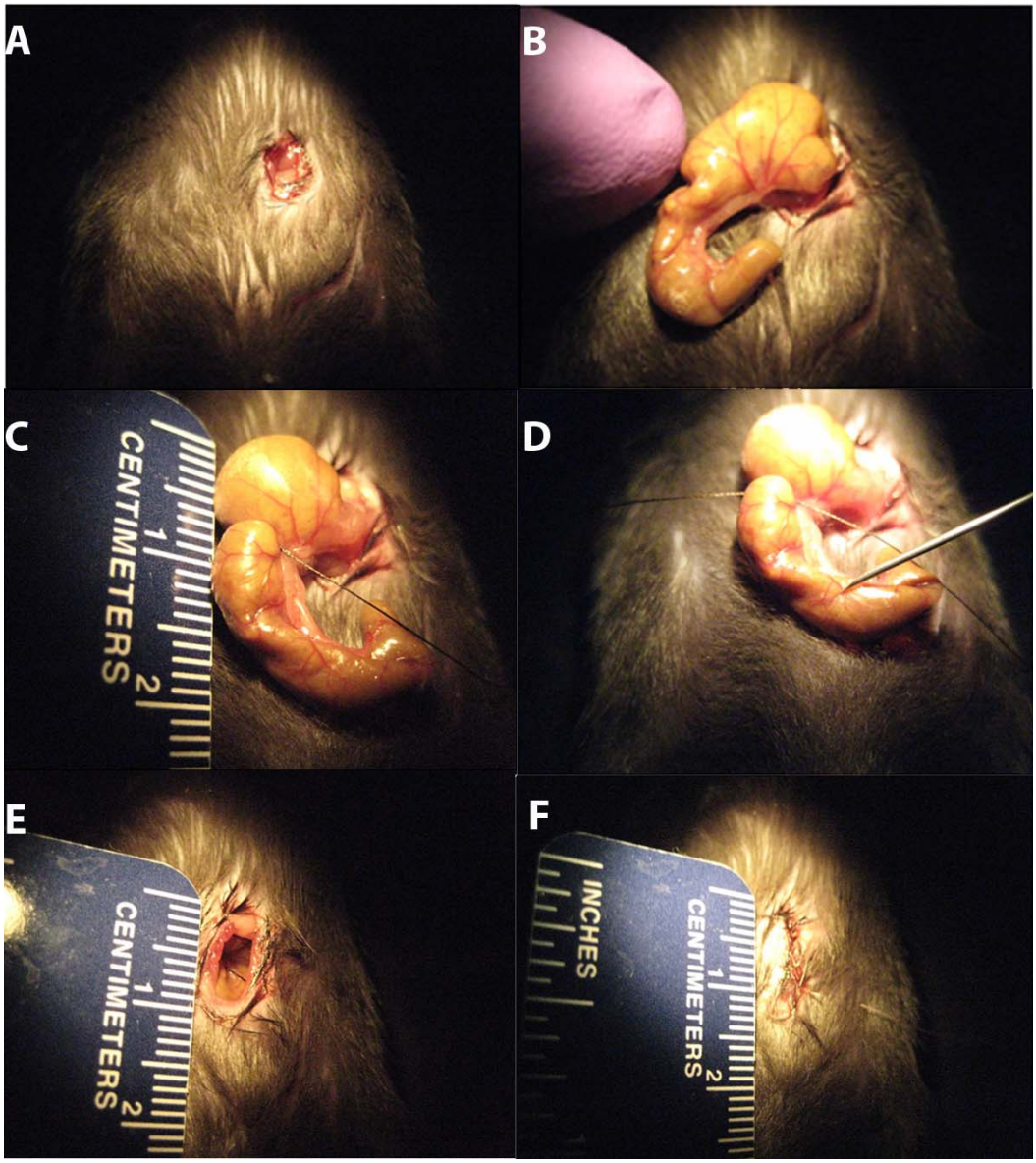

Figure 2.1. Cecal-ligation and puncture model of acute peritoneal sepsis.

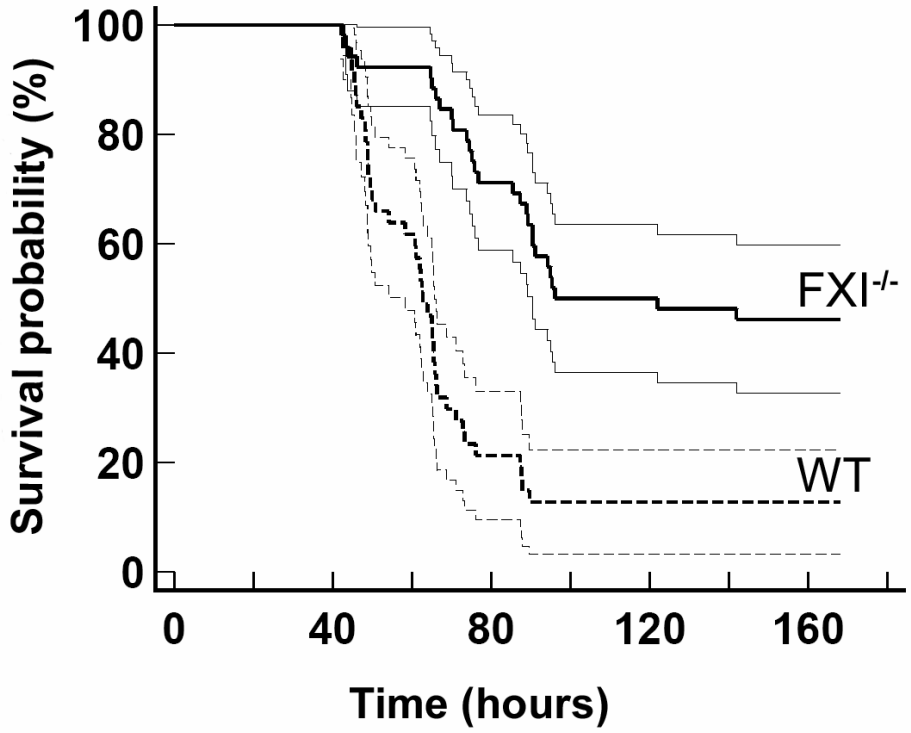

Figure 2.2. Survival advantage of factor XI (FXI)-deficient mice after large bowel perforation. Kaplan-Meier survival curves (with 95% confidence interval curves) are shown for the survival of 4-7-month-old age- and sex-matched wild-type (WT, or $FXI^{+/+}$; n = 47) and $FXI^{-/-}$ (n = 52) C57Bl/6 mice after cecal ligation and puncture. Curves were compared by log-rank test, which showed a significant survival advantage for $FXI^{-/-}$ mice (P = .0001).

Table 2.1: Reduction of coagulopathy and leukocyte infiltration associated with factor XI (FXI) deficiency 24 hours after cecal ligation and puncture (CLP).

Group	Platelet count, $10^5/\mu L$	WBC count in blood, $10^3/\mu L$	Fb level, % of normal	PT, s	TAT, ng/mL	WBC count in plf, $10^3/\mu L$
Baseline						
$FXI^{-/-}$ mice	9.8 ± 0.4 (5)	7.9 ± 0.6 (5)	75.1 ± 4.1 (28)	30.6 ± 0.6 (8)	4.1 ± 0.7 (8)	2.1 ± 0.4 (4)
WT mice	9.6 ± 0.4 (5)	8.7 ± 0.2 (5)	83.7 ± 5.5 (25)	30.3 ± 0.5 (8)	4.2 ± 1.4 (10)	1.9 ± 0.2 (6)
CLP						
$FXI^{-/-}$ mice	8.6 ± 0.4 (8)[a]	4.7 ± 0.6 (8)[a,b]	212.4 ± 24.3 (8)[b,c]	30.0 ± 3.2 (6)	4.8 ± 0.8 (10)[a]	8.8 ± 0.4 (8)[b,c]
WT mice	7.0 ± 0.5 (8)[b]	3.1 ± 0.4 (8)[b]	58.6 ± 12.3 (8)	36.3 ± 2.0 (8)[d]	22.5 ± 7.7 (12)[d]	13.3 ± 0.9 (8)[b]
Sham surgery						
$FXI^{-/-}$ mice	10.6 ± 1.1 (6)	7.5 ± 0.7 (6)	87.9 ± 4.6 (6)	31.0 ± 1.5 (6)	3.7± 0.2 (6)	1.9 ± 0.3 (6)
WT mice	11.0 ± 0.6 (6)	7.2 ± 1.3 (6)	97.1 ± 13.8 (6)	30.5 ± 2.8 (6)	5.5 ± 1.7 (6)	1.9 ± 0.4 (6)

NOTE. Data are mean ± SE values (no. of mice). Significance was determined by Student's *t* test (2-tailed). Fb, fibrinogen; plf, peritoneal lavage fluid; PT, prothrombin time; TAT, thrombin/antithrombin III complex; WBC, white blood cell; WT, wild type.

[a] $P \leq .05$, vs. WT CLP.
[b] $P \leq .001$, vs. WT baseline.
[c] $P \leq .001$, vs. WT CLP.
[d] $P < .05$, vs. WT baseline.

Chapter 3: Development of a FXI inhibitor

3.1 Introduction

Factor XI (FXI) is 160 kD disulfide linked homodimer whose monomers are made up of a heavy chain consisting of 4 polypeptide repeats called apple domains and a catalytic light chain. FXI can become activated by factor XIIa, thrombin, as well as autoactivated, which in turn activates factor IX and contributes to the coagulation cascade. Prekallikrein is a highly homologous protein of the kalikrein-kinin system that is important in the liberation of the vasoactive inflammatory peptide bradykinin. FXI may also be involved in bradykinin generation since it binds to high-molecular-weight kininogen (HMWK), the originator of the bradykinin peptide. In order to further explore the function of FXI we generated a potent neutralizing antibody to FXI.

3.2 Materials and methods

Generation of neutralizing anti-factor XI antibody

Hybridomas were derived from Balb/c mice immunized with purified human FXI using standard procedures.[96] Hybridomas were screened using solid phase ELISA against human FXI, and those that showed binding were subcloned twice by limiting dilution. The clone that produced the most potent neutralizing antibody, which inhibited the activation of FXI and/or the activity of FXIa, was selected based on prolongation of the clotting time of recalcified normal human plasma (NHP) and normal baboon plasma (NBP) by the cell culture supernatant. The cell line (1A6.1.1) producing the anti-FXI monoclonal antibody (aXIMab) was grown in a CL1000 bioreactor according to the manufacturer's protocol (Integra Biosciences), and the antibody was purified from the media using cation exchange and protein A chromatography.

3.3 Results and discussion

Cell culture supernatant from the 1A6 cell line potently inhibited the FXIa dependent cleavage of the chromogenic substrate S2366 *in vitro* (Figure 3.1). Purified aXIMab, tested within a concentration range from 0-40 nM, prolonged the aPTT (Hemosil^{TM} SynthASil, Instrumentation Laboratory) similarly in both NHP and NBP in a concentration-dependent manner (Figure 3.2) without affecting the PT (Innovin®, Dade Behring). Human and baboon FXI in plasma was recognized by aXIMab as a single band at 160 kDa on Western blots (Figure 4.3). The antibody specifically recognized the third apple (A3) domain of the FXI heavy chain, as assessed by immunoblotting of recombinant FXI/prekallikrein chimeras, as described (Figure 3.3).[97] The IC_{50} and IC_{99} of aXIMab in vitro was 2.5 nM and 10 nM, respectively, in an aPTT based clotting assay using FXI deficient human plasma (George King Bio-Medical) with serial dilutions of NBP as standards.[98] aXIMab infusion (2 mg/kg) into a single baboon prolonged the aPTT for more than 7 days, suggesting potent activity *in vivo* (Figure 3.4).

These data show that the monoclonal antibody derived for the 1A6.1.1 cell line is a potent neutralizer of FXI procoagulant activity. Indeed, since the A3 domain on FXI

includes the binding sites for FIX activation as well as platelet Gp Ib,[97,99] and aXIMab binds specifically to this region, this antibody may be a useful tool in further characterizing the role of FXI both *in vitro* and *in vivo*.

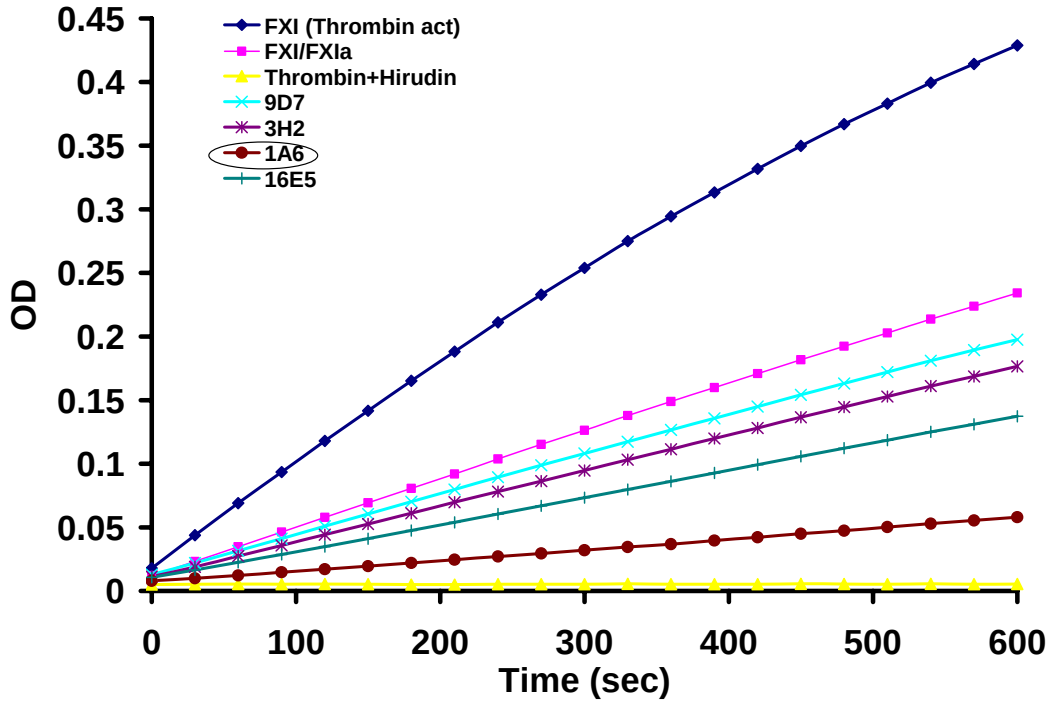

Figure 3.1. Anti-FXI monoclonal antibody 1A6 (aXIMab) prevents the activation of and/or the enzymatic activity of FXIa towards the chromogenic substrate S2366. 70 µl of assay buffer: (50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% BSA, 1µg/ml dextran sulfate) were added to a 96-well microtiter plate along with IgG serum samples from designated anti-FXI monoclonal antibody producing cell lines. 94 nM (final concentration) of purified FXI was then added to all wells except thrombin control wells. 10 nM of thrombin was then added to all wells except FXI control, followed by a 10 minute incubation at 37° C. Hirudin was added to inhibit thrombin activity (43 nM). 30µl of the reaction mixture was added to 70µl of 600µM S-2366 chromogenic substrate and the FXIa activity was tracked by reading the absorbance @405nm.

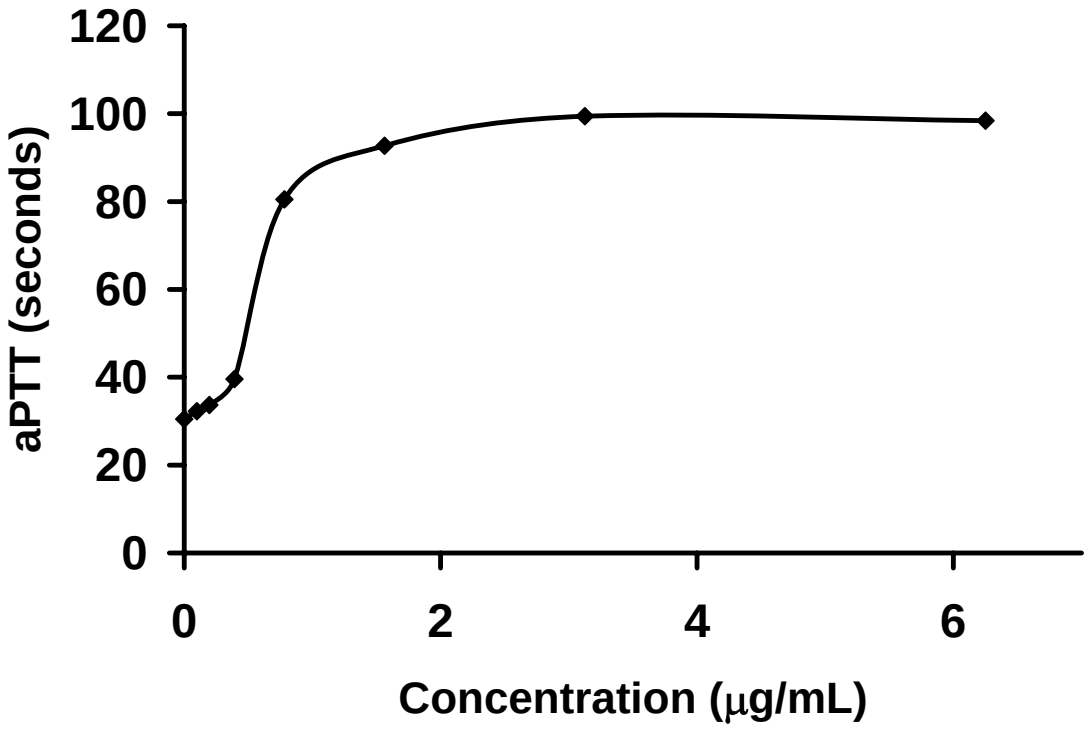

Figure 3.2. aXIMab prolongs the activated partial thromboplastin time (aPTT) of pooled baboon plasma. Concentration dependent prolongation of the aPTT with no change in prothrombin time (not shown). Citrated, pooled baboon plasma was mixed with varying concentrations of aXIMab and times were measured using an Amelung KC-4 coagulometer. Times are the average of two independent measurements at each antibody concentration.

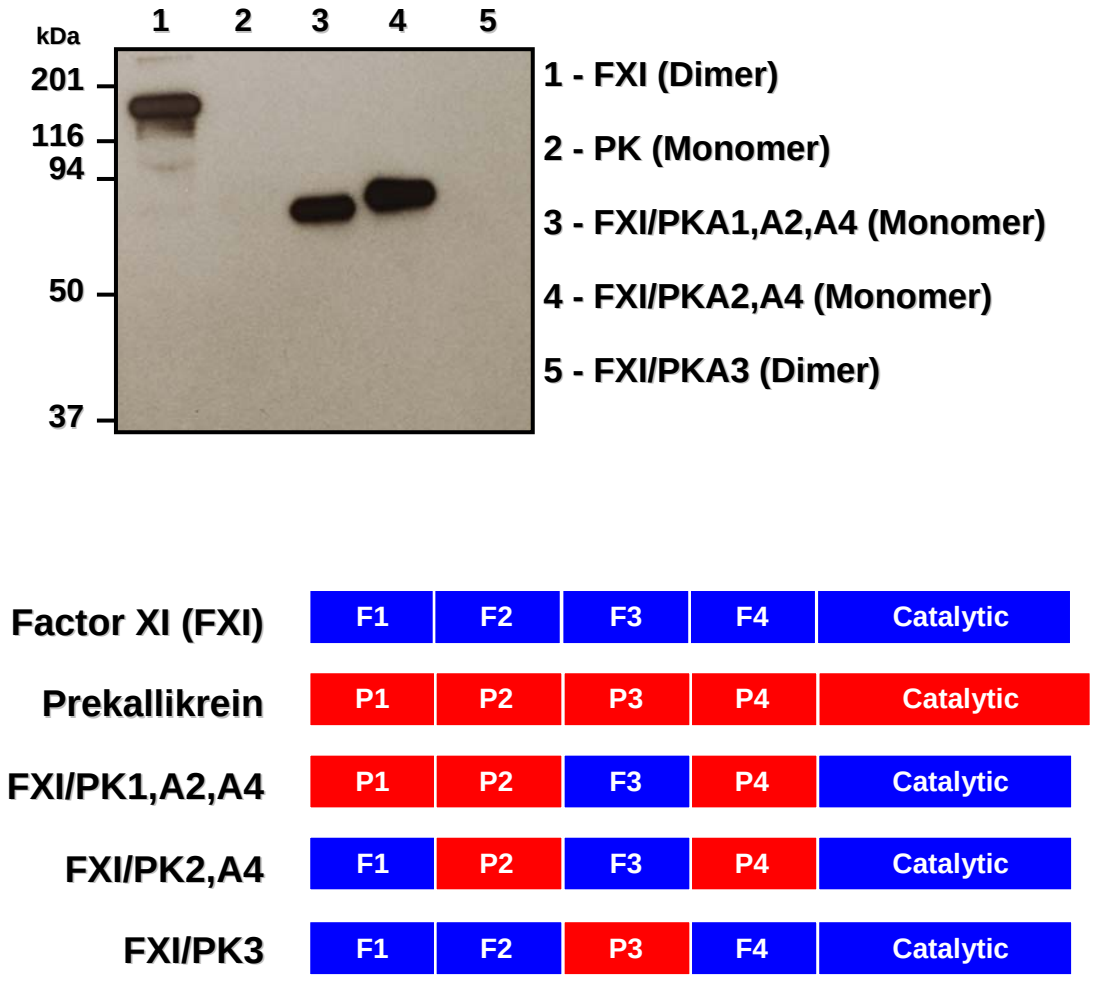

Figure 3.3. aXIMab binds to the Apple 3 (A3) domain of factor XI. The antibody specifically recognized the A3 domain of the FXI heavy chain, as assessed by immunoblotting of recombinant FXI/prekallikrein chimeras, as described.[97]

-This data was provided by Dr. David Gailani, Vanderbilt University.

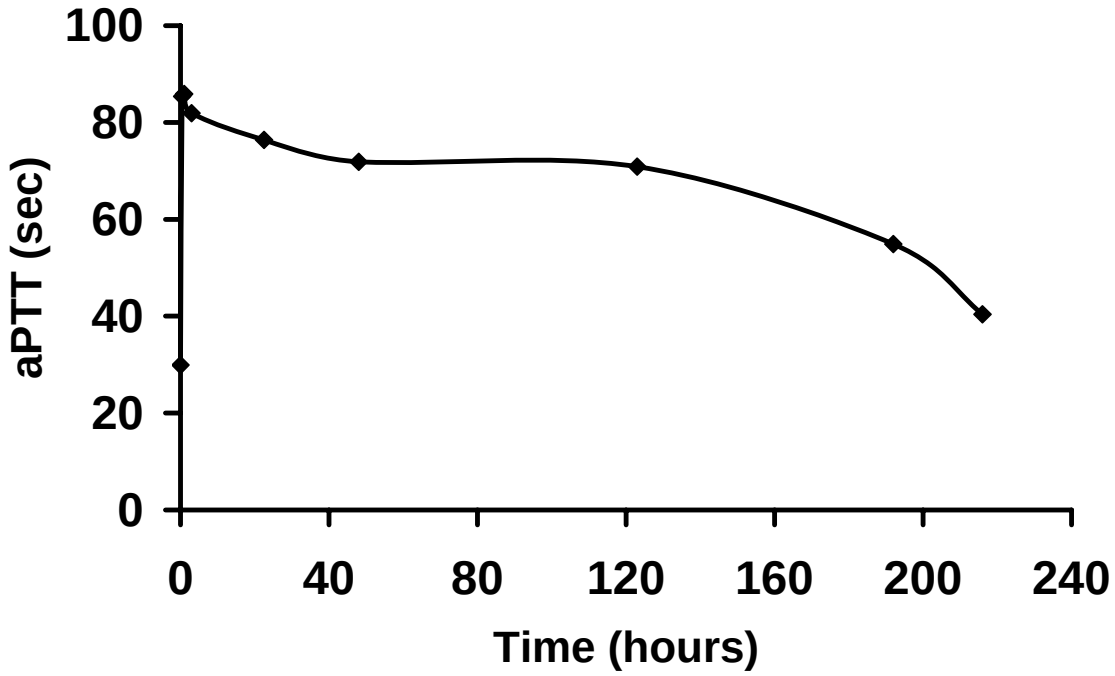

Figure 3.4. aXIMab infusion prolongs the aPTT in a baboon. An intravenous infusion of aXIMab (2 mg/kg) was given to a single baboon and blood samples were drawn over the next 9 days. Plasma was frozen and then assayed for clotting time. Each point is from a single measurement.

Chapter 4: Inhibition of FXI in baboons

4.1 Summary

The protease thrombin is required for normal hemostasis and pathologic thrombogenesis. Since the mechanism of coagulation factor XI (FXI)-dependent thrombus growth remains unclear, we investigated the contribution of FXI to thrombus formation in a primate thrombosis model. Pre-treatment of baboons with a novel anti-human FXI monoclonal antibody (aXIMab; 2 mg/kg), inhibited plasma FXI by ≥99% for 10 days, and suppressed thrombin-antithrombin (TAT) complex and β-thromboglobulin (βTG) formation measured immediately downstream from thrombi forming within collagen-coated vascular grafts. FXI inhibition with aXIMab limited platelet and fibrin deposition in 4 mm diameter grafts without an apparent increase in D-dimer release from thrombi, and prevented the occlusion of 2 mm diameter grafts without affecting template bleeding times. In comparison, pre-treatment with aspirin (32 mg/kg) prolonged bleeding times but failed to prevent graft occlusion, supporting the concept that FXI blockade may offer therapeutic advantages over other antithrombotic agents in terms of bleeding complications. In whole blood, aXIMab prevented fibrin formation in a collagen-coated flow chamber, independent of factor XII and factor VII. These data suggest that endogenous FXI contributes to arterial thrombus propagation through a striking amplification of thrombin generation at the thrombus luminal surface.

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[2] This research was originally published in *Blood*. Tucker EI, Marzec UM, White TC, Hurst S, Rugonyi S, McCarty OJ, Gailani D, Gruber A, Hanson SR. Prevention of vascular graft occlusion and thrombus-associated thrombin generation by inhibition of factor XI. *Blood*. 2009;113:936-44. © 2009 by the American Society of Hematology.

4.2 Introduction

Blood coagulation during hemostasis is initiated by the tissue factor (TF)/factor VIIa complex (the extrinsic pathway) that activates factors IX and X, and ultimately produces thrombin at sites of vascular injury.[4] In thrombosis, intravascular blood coagulation may also be initiated by the extrinsic pathway.[10,100] However, impairment of the TF/factor VIIa pathway does not provide full protection from thrombosis, since symptomatic factor VII deficient subjects can develop concurrent thrombosis and severe bleeding.[101] The functions of the contact proteins (factor XI, factor XII, prekallikrein and high-molecular-weight kininogen) in hemostasis are less clear. The physiological role of factor XI (FXI) has been difficult to determine because of the variable bleeding disorder associated with FXI deficiency,[15] and because monospecific FXI inhibitors have not been widely available for experimental investigation. FXI activation is thought to proceed through thrombin- and/or factor XII-dependent mechanisms, and activated FXI (FXIa) contributes to sustained thrombin generation after initiation of blood clotting by activating factor IX. These activities ultimately promote coagulation, platelet activation, and preservation of fibrin clot integrity.[23,102] Thrombin also increases the density of fibrin networks,[103] and indirectly inhibits fibrinolysis through activation of carboxypeptidase B (thrombin-activatable fibrinolysis inhibitor, TAFI).[104] Thus, FXI may support thrombus propagation and clot stability by increasing thrombin generation.[87,105]

Compelling circumstantial evidence suggests a contributory role for FXI in the pathogenesis of thrombosis. An elevated plasma FXI level appears to be an independent risk factor for deep vein thrombosis (DVT),[17] ischemic stroke,[106] and myocardial infarction[16] in humans. While one study did not detect a reduced incidence of myocardial infraction in patients with severe factor XI deficiency,[107] the incidence of ischemic stroke appears to be significantly lower in FXI deficiency than in the general population.[63] FXI deficiency reduces occlusive thrombus formation in mouse models,[89,108] and pharmacological inhibition of FXI is antithrombotic in rabbits[109] and primates.[21] Despite these findings, FXI appears to play a supportive role in normal hemostasis, and only a fraction of the individuals with severe factor XI deficiency exhibit a mild to moderated bleeding tendency upon injury.[15,110] In contrast, hemophilia (factor VIII or IX deficiency)

or factor deficiencies in the common pathway of coagulation (factors II, V, or X) are associated with severe bleeding or are incompatible with life.[111,112] Taken together, these observations suggest that thrombosis and hemostasis, while linked in many respects, possess mechanistic differences that may allow development of more thrombosis-specific anticoagulant strategies such as targeting of FXI.

To investigate the mechanism by which FXI contributes to acute thrombus formation, baboon and flow chamber models were employed. To block FXI activity, a potent monospecific neutralizing antibody was generated. A sensitive model was developed for locally measuring soluble markers of activated coagulation, platelets, and fibrinolysis at sites of experimental thrombus formation in baboons. Platelet and fibrin accumulation during arterial thrombogenesis, and the occlusion of thrombogenic blood conduits were determined in the presence and absence of the antibody.

4.3 Materials and methods

Experimental animals

39 non-terminal studies were performed using 17 male baboons (*Papio anubis*, 9-11 kg). All studies were approved by the Institutional Animal Care and Use Committee. Thrombosis experiments were conducted on non-anticoagulated awake animals that had chronic exteriorized femoral arteriovenous shunts, as described elsewhere.[113] Baseline shunt blood flow exceeded 250 ml/min in all study animals. Anxiety was managed with low dose ketamine (< 2 mg/kg/hr). Platelet counts, red cell counts, and hematocrits were measured daily, before and after the experiments. Calculated blood loss did not exceed 4% of total blood volume on any experimental day.

Thrombosis model

Thrombus formation was initiated within chronic arteriovenous shunts in baboons by interposing a prosthetic vascular graft segment for up to 60 min, essentially as described.[113] The hypothrombogenic graft (ePTFE, WL Gore & Co., Flagstaff, AZ)[21] was coated with collagen which consistently triggers platelet-dependent thrombus formation.

Twenty mm long graft segments (internal diameters [id] of either 2 or 4 mm) were filled with equine type I collagen (1 mg/mL; Nycomed Arzenmittel, Munich, Germany) for 15 minutes, and then dried overnight under sterile airflow (Figure 4.1). The thrombogenic collagen-coated grafts were then incorporated between segments of silicon rubber tubing, deployed into the shunts (Figure 4.2), and exposed to blood flow. The flow rate through the graft was restricted to 100 mL/min (Transonics Systems flow meter, Ithaca, NY) by clamping the proximal shunt segment, thereby producing initial mean wall shear rates of 265 s^{-1} (4 mm id) or 2120 s^{-1} (2 mm id). The 4 mm grafts remained patent without flow rate reduction for 60 minutes. In the 2 mm diameter grafts, flow rates progressively declined due to occlusive thrombus formation. The grafts were removed from the shunts either at 60 minutes (4 mm id grafts) or when the flow rate fell from 100 mL/min to 20 mL/min (2 mm id grafts), signaling imminent occlusion. The time from initiation of blood flow through the graft until the flow reached 20 mL/min was taken as the occlusion time.

Thrombus formation was assessed in real time during the experiments by quantitative gamma camera imaging of radiolabeled platelet accumulation within the graft segment, and by end-point determinations of radiolabeled fibrinogen/fibrin deposition, as described.[113] Measurements of platelet-associated radioactivity on the grafts were recorded at 5-minute (4 mm id) or 3-minute (2 mm id) intervals using a GE-400T gamma scintillation camera interfaced with a NuQuest InteCam computer system. Embolic events were recorded as abrupt decreases in the number of platelets in the graft between subsequent imaging frames.

Blood sample collection

Systemic samples were collected proximal to the graft from the midstream of the shunt into 3.8% citrate (1:9, vol/vol) before graft deployment, and then at 30 and 60 minutes. One sample was processed for platelet poor plasma and used to assess FXI procoagulant activity levels, while a second sample was used for determination of systemic coagulation markers (vide infra). For the assessment of thrombosis markers generated within the 4 mm id grafts, samples were also taken from the blood stream

adjacent to the vessel wall (intraluminal boundary layer) immediately distal to the thrombus. Blood was collected at a rate of 100 μL/min during 10 minute intervals through a 0.64 mm id port located 10 mm distal to the graft (Figure 4.2). To maintain patency of the sampling port, Phe-Pro-Arg-chloromethylketone (PPACK, 0.5 mg/mL, 1 mM), which inhibits thrombin and other coagulation proteases,[114] was infused at a rate of 20 μL/min into a second 0.64 mm id port located 3 mm proximal to and in line with the collection port. Anticoagulant infusion and local blood sampling were regulated using syringe pumps (Harvard Apparatus).

Blood sample analysis

Blood cell counts were determined using a micro-60 automated cell counter (Horiba-ABX Diagnostics). Blood samples were divided into aliquots and processed according to specific test requirements. Plasma was prepared from one aliquot and activated partial thromboplastin times (aPTT) and prothrombin times (PT) were measured on site. Another aliquot was placed on ice for 10 minutes and centrifuged at 4º C for 10 minutes at 12,900 g. For β-thromboglobulin (βTG) determinations, the samples were supplemented with 4 μg/mL prostaglandin E1 (PGE_1), 4.3 mg/mL acetylsalicylic acid (ASA), and 50 μg/mL PPACK. The plasma was then frozen and stored at -80º C until assaying. Cross-reacting ELISA assays were used to determine D-dimer levels (IMUCLONE® D-Dimer, American Diagnostica), the platelet activation marker βTG (Asserachrom®, Diagnostica Stago), and thrombin-antithrombin complexes (TAT, Enzygnost-TAT, Dade-Behring).

Derivation of neutralizing anti-factor XI antibody

Since previous studies showed that polyclonal antibodies to human FXI required very high doses to achieve near-complete inhibition of FXI in baboons,[21] we generated a new reagent, a potent neutralizing anti-human FXI monoclonal antibody (aXIMab) that cross-reacts with baboon FXI. Hybridomas were derived from Balb/c mice immunized with purified human FXI using standard procedures.[96] Hybridomas were screened using solid phase ELISA against human FXI, and those that showed binding were subcloned twice by limiting dilution. The clone that produced the most potent neutralizing antibody,

which inhibited the activation of FXI and/or the activity of FXIa, was selected based on prolongation of the clotting time of recalcified normal human plasma (NHP) and normal baboon plasma (NBP) by the cell culture supernatant. The cell line producing aXIMab (1A6.1.1) was grown in a CL1000 bioreactor according to the manufacturer's protocol (Integra Biosciences), and the antibody was purified from the media using cation exchange and protein A chromatography.

Human and baboon FXI in plasma was recognized by aXIMab as a single band at 160 kDa on Western blots (Figure 4.3). The antibody specifically recognized the third apple (A3) domain of the FXI heavy chain, as assessed by immunoblotting of recombinant FXI/prekallikrein chimeras, as described (Chapter 3).[97] The IC_{50} and IC_{99} of aXIMab in vitro was 2.5 nM and 10 nM, respectively, in an aPTT based clotting assay using FXI deficient human plasma (George King Bio-Medical) with serial dilutions of NBP as standards.[98] Purified aXIMab, tested within a concentration rage from 0-40 nM, prolonged the aPTT (Hemosil™ SynthASil, Instrumentation Laboratory) similarly in both NHP and NBP in a concentration-dependent manner without affecting the PT (Innovin®, Dade Behring).

Pharmacological inhibition of FXI and platelet activity in vivo

In a pilot experiment, aXIMab (2 mg/kg) was administered to a single baboon, and blood samples were collected into citrate anticoagulant over 4 weeks to measure circulating FXI antigen (FXI:Ag) concentrations, FXI inhibitor, and FXI procoagulant activity. This dose of aXIMab was chosen with the intent to achieve sustained and near-complete inhibition of FXI, assuming an initial dilution of the antibody into 60 ml blood volume per kg body weight after injection. The maximum achievable prolongation of the aPTT was about 2.5 fold. FXI:Ag was measured by ELISA using goat anti-human FXI polyclonal capture and detection (horseradish peroxidase conjugated [HRP]) antibodies (Affinity Biologicals, Hamilton, Ontario), which also recognized baboon FXI and its complex with aXIMab. A standard curve was constructed with serial dilutions of NBP, and FXI concentrations were determined as a percentage of NBP. Western blots for FXI were performed by size fractionation of 1 μl samples of plasma under non-reducing

conditions on 7.5% polyacrylamide-SDS gels, followed by transfer to PVDF membranes. Detection was with a goat-anti-human FXI polyclonal antibody conjugated to HRP and chemiluminescence. In the same samples, the Bethesda assay[115] was used to determine excess (non-complexed) circulating FXI inhibitor (aXIMab) activity levels, and the FXI procoagulant activity was measured using a clotting assay.[98] P-selectin (Bender MedSystem, monkey sP-selectin ELISA) and fibrinogen (Clauss assay) levels were measured as global plasma markers for inflammation.

aXIMab was administered as a bolus (2 mg/kg intravenously) at least 24 hours before the thrombosis experiments. The anticoagulant effect was monitored daily, and thrombosis experiments were performed while the systemic FXI procoagulant activity was reduced by ≥99%. We previously showed that inhibition of FXI by polyclonal antibodies is safer and as effective as high dose heparin in baboons.[21] Aspirin has less pronounced effects on hemostasis than heparin, and is often used in the treatment of arterial-type platelet dependent thrombosis. The affect of ASA treatment was compared to aXIMab on the occlusion-prone 2 mm id grafts. ASA (32 mg/kg) was administered orally 2 to 4 hours before each thrombosis experiment, as described previously.[116] Four weeks were allowed for washout of each aXIMab and ASA before performing new experiments in the same animal.

Hemostasis assessment

The effects of FXI inhibition and aspirin on hemostasis in baboons were assessed using the standard template skin bleeding time test (Surgicutt®, International Technidyne Corp). Experimentally, this tests has been shown to be sensitive to the effects of therapeutic anticoagulants and anti-platelet agents in non-human primates.[117,118] All bleeding time measurements were performed by the same expert technician.

Modeling of thrombosis marker mass transport and local sampling

A 3D computational fluid dynamics model, similar to that presented by Xu et al,[119] was used to estimate the concentrations of thrombus-derived macromolecules in blood that flows over forming thrombus and transports the markers along the adjacent

vessel wall distally. The model was based on the geometry shown in Figure 4.2, with typical values for blood density and viscosity.[120] The model of local blood sampling was implemented using the finite element software ADINA (Watertown, MA). In addition, a 2D axisymmetric computational model, similar to that of Markou et al,[121] was used to estimate thrombosis marker distribution within the flow field. Computational modeling predicted that molecules of interest (βTG, fibrin D-dimer, and TAT) released or generated at sites of thrombus formation, would be concentrated (>99%) within a very thin peripheral blood boundary layer (~0.1 mm thick concentration boundary layer) along the immediately distal vessel wall (data not shown). Thus, as employed here, the local sampling method effectively sampled the entire near-wall concentration boundary layer region, immediately distal to the forming thrombus, for platelet and coagulation markers of interest.

Flow chamber coagulation studies

Glass capillary tubes coated with 100 μg/mL Horm collagen (Type I equine tendon, Nycomed Arzenmittel, Munich, Germany) were used as thrombus chambers. Whole human blood was collected into corn trypsin inhibitor (CTI, 40 μg/mL), discarding the first 1 mL to limit activation of coagulation. The blood was perfused through the thrombus chambers at a shear rate of 265 s^{-1} for 10 minutes. Prior to each experiment, blood was incubated with aXIMab (20 μg/mL), heparin (15 U/mL), a combination of an inhibitory anti-TF antibody (20 μg/mL, Genentech) and FVIIai (1 μg/mL, NovoNordisk), and/or PBS vehicle (all concentrations final).

-Modeling of the thrombosis marker mass transport for local sampling was performed by Dr. Sandra Rugonyi at Oregon Health & Science University.

In separate experiments, washed platelets and RBCs were processed as described,[122] and then mixed with FXII deficient human plasma (<1% FXII, Haematologic Technologies) to reach a hematocrit of 40% and platelet count of 300 $x10^3/\mu L$. The reconstituted blood was incubated with aXIMab (20μg/mL) or CTI (40μg/mL), recalcified with 7.5mM $CaCl_2$ and 3.75mM $MgCl_2$, and then perfused immediately through collagen coated capillary tubes. Recalcified FXII deficient human plasma without RBCs or platelets was also perfused over collagen. Images were obtained by DIC microscopy after three minutes of perfusion with modified Tyrodes buffer to wash out unbound blood components.

Data analysis

Mean values are given ± 1 SEM (standard error of the mean). Occlusion data were compared using the log-rank test. The two-tailed Student's *t*-test was used for all other single pair comparisons. A P value ≤ 0.05 was considered significant.

4.4 Results

Inhibition of FXI by aXIMab ex vivo prevents fibrin formation independent of FXIIa

When CTI-anticoagulated fresh whole human blood was perfused over collagen at arterial shear (265 s^{-1}), platelets were deposited in large aggregates which became enveloped by forming fibrin strands (Figure 4.3). In stark contrast, when CTI blood was further anticoagulated with either heparin or aXIMab, fibrin deposition was greatly reduced. In these short experiments, there was no obvious reduction in platelet adhesion to collagen by the anticoagulants. The outcome was similar when using reconstituted FXII deficient blood, with or without CTI. Fibrin was generated independent of FXII, and FXI inhibition with aXIMab interrupted fibrin thrombus formation. Inhibition of the extrinsic pathway with a combination of FVIIai and the anti-TF antibody, which nearly doubled the PT, had no effect on fibrin formation or platelet deposition. No fibrin was formed when recalcified FXII deficient human plasma alone was perfused over collagen

(data not shown), illustrating the importance of platelets and/or other blood cells in fibrin thrombus formation in this model. These data suggest that, under physiologically relevant shear flow, FXI and corpuscular blood components promote fibrin formation on collagen independent of FXIIa or TF/FVIIa.

Inhibition of FXI with aXIMab, in vivo

A baboon given aXIMab (2mg/kg) was followed for 4 weeks post-injection to assess FXI antigen (FXI:Ag), anticoagulant activity, and antibody inhibitor levels. FXI:Ag levels (complexed and native) decreased from 110% to 40% of NBP levels by 60 minutes, but steadily increased thereafter, reaching 300% of control by day 8 post treatment (Figure 4.4). FXI:Ag remained above 200% of control through day 15, after which there was a decrease to 130% by day 27. FXI activity decreased from 125% to <1% at 1 hour after aXIMab administration, and remained inhibited by >99% for 10 days after administration. FXI activity gradually increased to 136% by day 27. Maximum circulating FXI inhibitor levels were observed at 1 hour post aXIMab infusion (72 Bethesda units [BU]), which decreased to 0.6 BU by day 13, and were undetectable thereafter (Figure 4.4). Consistent with the FXI:Ag ELISA data, the 160 kDa band representing the FXI homodimer on SDS PAGE and Western blots of plasma increased in intensity between day 0 and 8 (Figure 4.4). These results indicate that the anticoagulant effect of aXIMab was due to its sustained presence in the circulation, and its ability to block FXI activation and/or activity, and not to clearance of the zymogen from the circulation. Since aXIMab separates from FXI during SDS gel electrophoresis, it remains unclear whether inhibition of FXI by aXIMab caused a rebound increase in FXI secretion or the FXI-aXIMab immune complexes were cleared slowly from the circulation. In the same samples, P-selectin and fibrinogen levels were determined as indirect measures for inflammation, with no remarkable changes from baseline detected (data not shown).

In thrombosis studies using aXIMab, FXI procoagulant activity was inhibited by 99.0% (98.8-99.5%) at 1 hour after injection and remained inhibited by 99% in all animals for at least 7 days. No observable adverse events were associated with the treatment. aPTT values were prolonged after aXIMab administration to 65.6 ± 2.0

seconds compared with 30.5 ± 0.7 seconds in control animals, while the PT values remained comparable to baseline (9.1 ± 0.1 vs 9.0 ± 0.1 sec, respectively, n = 11 for each). Platelet aggregation in platelet rich plasma in response to adenosine diphosphate (ADP) and collagen was not affected by aXIMab-treatment (data not shown).

Protection of aXIMab-treated baboons from collagen-initiated thrombus development

Administration of aXIMab reduced platelet and fibrin deposition in the 4 mm id collagen-coated vascular grafts. Significant differences in platelet accumulation between aXIMab- and vehicle-treated groups were seen as early as 10 minutes after graft exposure to blood flow ($0.13 \pm 0.03 \times 10^9$ versus $0.23 \pm 0.03 \times 10^9$ platelets, aXIMab versus vehicle control; $P < 0.05$). The differences remained statistically significant throughout the time course of thrombus propagation (Figure 4.5). Graft platelet accumulation at 60 min was 63% lower in aXIMab-treated animals than in vehicle controls ($1.38 \pm 0.26 \times 10^9$ versus $3.68 \pm 0.52 \times 10^9$ platelets, aXIMab versus control, n = 6 and 8, respectively, $P < 0.01$; Figure 4.5). Systemic platelet counts were similar in aXIMab ($341 \pm 27 \times 10^3/\mu L$) and control groups ($337 \pm 31 \times 10^3/\mu L$), and did not change significantly following thrombosis experiments. End-point fibrin deposition was reduced by 81% compared with controls (0.23 ± 0.07 mg and 1.18 ± 0.19 mg, aXIMab versus control, $P = 0.001$; Figure 4.5). Since aXIMab is monospecific for the A3 domain of FXI, these data verify that FXI plays an important role in thrombus propagation under arterial-type flow conditions in primates.

Reduced thrombin generation and platelet activation in aXIMab-treated baboons

Since inhibition of FXI could reduce thrombus formation in vivo both by limiting thrombin-mediated platelet activation and fibrin formation and/or by increasing thrombolysis, levels of βTG, TAT, and D-dimer were measured. Systemic pre-treatment βTG, TAT, and D-dimer levels were comparable in vehicle- and aXIMab-treated baboons (19.3 ± 2.6 versus 24.8 ± 3.8 IU/mL, 6.2 ± 0.4 versus 4.6 ± 0.6 μg/L, and 2.3 ± 0.2 versus 2.4 ± 0.2 μg/ml, respectively). We observed a robust >20-fold increase in TAT release into the blood stream from the graft thrombus area, as measured in samples taken locally

from the near-wall region, immediately downstream of thrombus formation in vehicle-treated baboons. Pre-treatment of baboons with aXIMab prevented the increase in local TAT levels, indicating a profound reduction in thrombin generation in the absence of FXI activity (Figure 4.6). TAT levels in plasmas obtained by local sampling were lower, by up to 98% at 40 minutes, while systemic TAT levels were 81% lower at 60 minutes compared with the untreated controls (4.3 ± 0.5 versus 22.1 ± 2.5 μg/L, n = 6 and 7, respectively, $P < 0.001$; Figure 4.6). Since thrombin does not interact with antithrombin in the presence of PPACK, all TAT in the sample was likely generated before the blood entered the sampling port. Thus, while reflecting thrombin generation, TAT levels are likely an underestimation of the total thrombin. Platelet activation at the thrombus surface, as assessed by the release of platelet α-granule βTG, was 86% lower at 30 minutes in samples taken distal to thrombi in aXIMab-treated animals compared with vehicle-treated controls (Figure 4.6). Systemic βTG levels in aXIMab-treated animals measured at 60 minutes were 42% less (23.0 ± 2.1 IU/mL, n = 6) compared with vehicle-treated controls (39.5 ± 5.5 IU/mL, n = 7; Figure 4.6, $P < 0.05$). Local D-dimer levels were not changed at 60 minutes compared with baseline systemic values in either control (2.3 ± 0.2 and 2.4 ± 0.4 μg/mL, respectively, n = 7) or aXIMab-treated animals (2.4 ± 0.2 and 2.2 ± 0.2 μg/mL, respectively n = 6; Figure 4.6). Systemic D-dimer levels assessed at 60 minutes also were unchanged from baseline systemic values in both groups (2.4 ± 0.3 and 2.3 ± 0.2 μg/mL, control versus aXIMab-treated animals). This confirms previous reports from similar baboon studies showing systemic D-dimer levels do not increase by 60 minutes after thrombus initiation, unless a thrombolytic agent is administered.[123,124] These data indicate that: 1) FXI plays an important role in thrombin generation and platelet activation during acute, arterial-type thrombus formation, and 2) endogenous local thrombolysis appears to be limited during the time course of these studies, regardless of FXI activity.

Treatment with aXIMab prevents vascular graft occlusion

Even though macroscopic thrombi formed rapidly in 4 mm id vascular grafts, none of these grafts occluded during 60 minutes of blood perfusion. The effects of inhibiting FXI activity on thrombus formation and occlusion were therefore evaluated

using smaller diameter (2 mm id), collagen-coated vascular grafts that accumulated thrombus under higher shear conditions (2120 s^{-1}). The initial platelet accumulation rate was similar in aXIMab-treated and vehicle-treated baboons. Within 12-15 minutes after initiation of blood perfusion, platelet deposition was reduced in all aXIMab-treated animals (Figure 4.7), and the number of platelets in the graft decreased abruptly, which can be explained by reduced thrombus stability and net loss of thrombus material to embolization. Treatment with aXIMab prevented graft occlusion over 60 minutes in all experiments (5 of 5 grafts remained patent), compared with the results in vehicle-treated controls in which 8 of 9 grafts occluded by 27.0 ± 3.3 min ($P < 0.01$; Figure 4.7 and Table 4.1). Fibrin accumulation was also reduced by aXIMab treatment versus the vehicle-treated controls (0.18 ± 0.02 versus 0.30 ± 0.04 mg, respectively, $P < 0.01$; Table 4.1). As expected, treatment with high dose ASA reduced platelet deposition in the 2 mm id grafts, but did not completely interrupt occlusive thrombus formation. The time to graft occlusion was prolonged by aspirin by an average of 18 minutes in 4 grafts, while 2 additional grafts remained patent throughout the 60 minute study interval. High dose ASA was therefore less effective in preventing graft occlusion than aXIMab ($P < 0.05$, aXIMab versus ASA). These data indicate that FXI plays an important role in the thrombogenic process that leads to acute occlusion of small caliber vascular grafts.

Treatment with aXIMab does not prolong the bleeding time in baboons

The template bleeding time is typically prolonged by both anti-platelet agents and anticoagulants in baboons,[117,125] yet FXI inhibition by aXIMab had no effect on the standard template bleeding time compared with vehicle-treated controls (3.5 ± 0.3 versus 3.4 ± 0.2 min, n = 18 and 14, respectively). For comparison, single-dose ASA pre-treatment nearly doubled the bleeding time to 6.4 ± 0.7 minutes (n = 10, Figure 4.8; $P < 0.01$). No re-bleeding from the template bleeding time wounds, petechiae, hematomas or other adverse bleeding events were noted in any of the aXIMab- or ASA-treated animals during one week follow-up periods of observation. Taken together, these results show that inhibition of FXI activity by a neutralizing monoclonal antibody more effectively reduces acute occlusive arterial-type thrombus propagation, with less effect on primary hemostasis, than an antihemostatic dose of aspirin in baboons.

4.5 Discussion

The present findings document that FXI is an essential contributor to local thrombin generation on the surface of thrombi during arterial-type thrombogenesis. FXI inhibition in baboons slowed platelet accumulation onto a thrombogenic surface (collagen-coated vascular grafts) under moderate arterial shear conditions (Figure 4.5). While FXI inhibition did not demonstrably affect platelet adhesion to collagen in the ex vivo chamber perfusion studies, it clearly reduced centripetal accumulation of platelet aggregates over longer periods of time in the 2 mm id grafts. The stability of thrombi formed under higher shear flow was noticeably dependent on FXI activity (Table 4.1 and Figure 4.7). Indeed, under high shear, repeated loss of platelets from the graft was distinctly evident in aXIMab-treated animals, a mechanism which likely contributed to preventing graft occlusion. These findings are consistent with the antithrombotic and anti-occlusive phenotype observed in FXI deficient mice following ferric chloride-induced arterial injury and thrombosis.[18,89,108]

FXI promotes clot resistance to fibrinolysis through thrombin-mediated activation of the metalloproteinase TAFI, which proteolytically modifies fibrin making it resistant to plasmin.[126,127] In addition, inhibition of thrombin generation by anticoagulants enhances clot lysis due to a slower forming, less dense fibrin network.[128,129] Both processes may account, at least in part, for the enhanced lysis of blood clots formed in the presence of an anticoagulant anti-factor XI antibody in the clamped jugular vein of rabbits.[87] During the present studies in baboons, the fibrin degradation product D-dimer was measured by local blood sampling in the vicinity of fibrin-, platelet-, and leukocyte-rich arterial-type thrombi. Unlike the robust changes in TAT and βTG levels, there were no changes in local D-dimer levels in vehicle- or aXIMab-treated baboons. This finding suggests that endogenous fibrinolysis may not be a dominant process on the surface of thrombi during acute arterial thrombogenesis, which is consistent with the observation that TAFI deficient mice that were expected to have a fibrinolytic phenotype were not protected against injury-induced arterial thrombus formation.[130] Since adjacent endothelial cells capable of secreting tPA are absent from the graft, the data do not rule out the possibility of reduced TAFI activity and more efficient fibrinolysis during

inhibition of FXI. Our data suggests however that the relative reduction in deposited fibrin associated with FXI inhibition was not caused by an increase in fibrinolysis.

The inhibitory effect of aXIMab on thrombogenesis and TAT formation suggests that sustained thrombus propagation on the luminal flow surface depends on FXI-mediated thrombin generation. FXI-dependent thrombin generation could occur via continued FXI activation by FXIIa, thrombin, and/or autoactivation.[23,131] FXIIa appears to play an important role in experimental thrombosis in mice.[132] However, the flow chamber data (Figure 4.3) indicates that FXI promotes thrombosis independent of FXIIa under shear flow conditions in the presence of blood cells. Although thrombin-mediated FXI activation in static plasma assays in vitro has been questioned,[133] our flow-augmented thrombosis model clearly demonstrates a FXII independent thrombogenic pathway for FXI. Since inhibition of the contact pathway (FXII) as well as the extrinsic TF pathway did not affect thrombus formation, initiation of coagulation in this ex vivo model must depend on coagulation factor autoactivation, delivery of coagulation proteases by platelets, or an as yet unrecognized mechanism.

While FXI activity decreased to <1% for over a week following aXIMab injection, total FXI:Ag levels temporarily increased several-fold above baseline following the disappearance of FXI activity in the baboon circulation. Whether this increase was due to a longer half-life of the circulating immune complexes or upregulation of FXI synthesis and/or secretion remains to be explored.

The physiological function and regulation of FXI have been difficult to elucidate. The bleeding tendency seen in FXI deficiency suggests that FXI supports hemostasis after injury to anatomical areas with pronounced fibrinolytic activity.[15] In contrast to patients with hemophilia A or B, spontaneous bleeding in patients with FXI deficiency is rare, and trauma-related bleeding is typically milder. All conventional anticoagulant, antiplatelet, and fibrinolytic agents impair hemostasis, and most produce a significant risk of life-threatening bleeding. While bleeding time prolongation is indicative of a hemostatic defect, the test is not particularly sensitive to coagulation abnormalities in

humans. However, the bleeding time has been useful and predictive of the antihemostatic effects of antithrombotic agents, when baseline and treatment values are compared in baboons.[117] In the present study, ASA significantly prolonged the bleeding time, while the same test was not sensitive to the presumably modest hemostatic abnormality produced by FXI inhibition. Clearly, development of agents that could inhibit thrombosis without impairing hemostasis would significantly improve the safety profile of antithrombotic therapy. The results of the present study suggest that a pharmacologic inhibitor of FXI may represent an example such a strategy.

In summary, this study confirms that FXI activity is not essential for primary hemostasis in otherwise healthy primates. We introduce a pharmacologically active monoclonal antibody that, by binding to the A3 domain of FXI, can achieve sustained FXI blockade, and represents a new strategy for limiting acute arterial-type thrombus growth and blood vessel occlusion. Though we did not establish a role for FXI in endogenous thrombolysis, we demonstrate that vaso-occlusive thrombus propagation is driven by FXI-dependent thrombin generation, platelet activation, and fibrin formation in primates. Furthermore, we provide *in vitro* evidence for a FXII- and FVII-independent pathway for fibrin formation in whole blood under flow that requires FXI.

Table 4.1: FXI inhibition by aXIMab prevents occlusion of 2 mm id vascular grafts in baboons.

	Number occluded	Embolic events per experiment*	Fibrin deposition, mg
Control	8/9	ND	0.30 ± 0.04
ASA	4/6	1.5 ± 0.6	0.23 ± 0.04
aXIMab	0/5	6.6 ± 0.9	0.18 ± 0.02†

Data shown are absolute values or means plus or minus SEM when applicable.
ND indicates none detected.
*Embolic events were defined as an abrupt decrease of at least 10^6 platelets in the graft between 2 subsequent radioimaging frames.
†$P < .01$ compared with control using the 2-tailed Student *t* test.

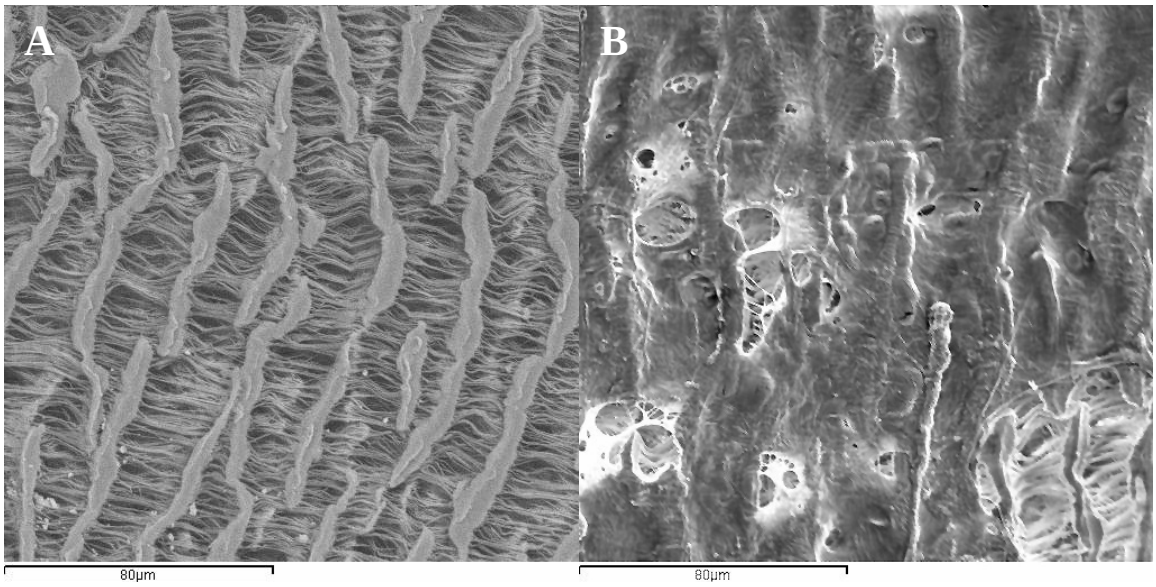

Figure 4.1. Scanning electron micrograph of uncoated and collagen coated expanded polytetrafluoroethylene (ePTFE) vascular graft material.
Hypothrombogenic vascular graft material (A) was coated with type I equine tendon collagen (B) to produce a thrombogenic surface for baboon thrombosis studies.

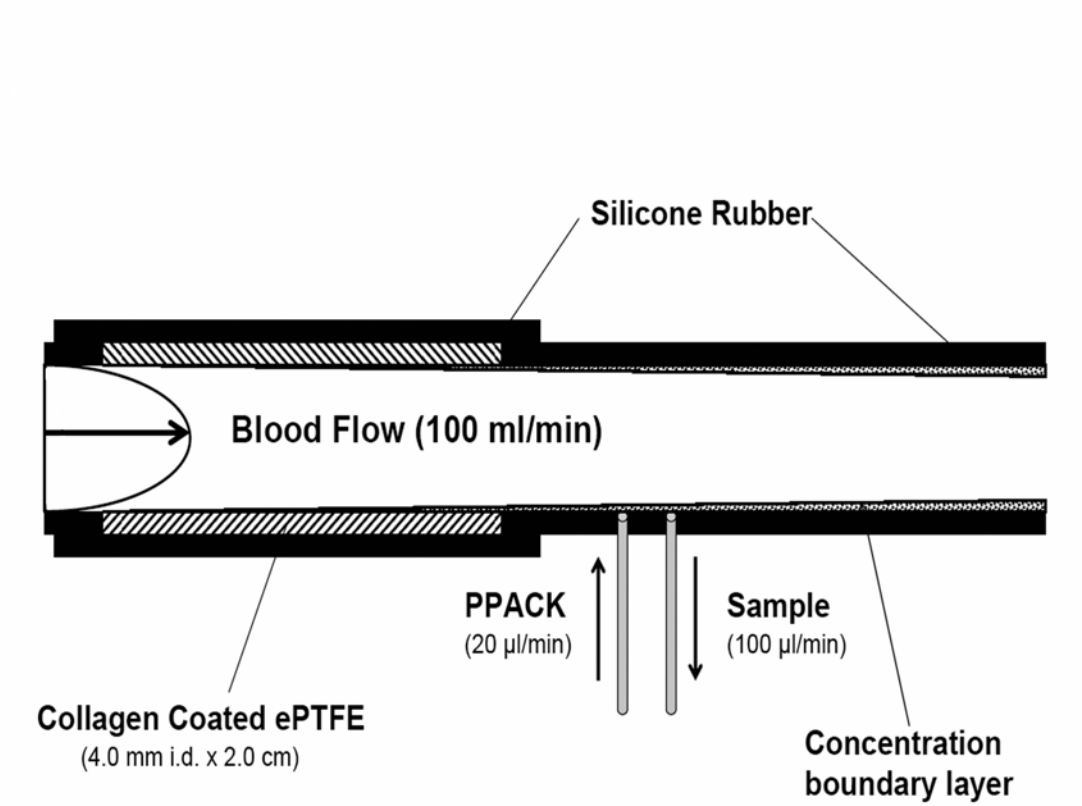

Figure 4.2. Thrombogenic vascular graft and blood collection device.
Thrombogenesis started and thrombi developed in a collagen-coated (4 mm internal diameter [id]) expanded-polytetrafluoroethylene (ePTFE) vascular graft that was deployed for 60 minutes into a chronic high flow arteriovenous shunt in healthy baboons. Blood samples were drawn from the coagulation marker concentration boundary layer by a syringe pump 1 cm downstream from acutely developing thrombi. PPACK anticoagulant was infused 3 mm proximal to the sample port to prevent the sample port from occluding during the 1 hour study period. Blood flow through the graft was maintained at a fixed rate of 100 mL/min for the entirety of each study by proximal clamping.

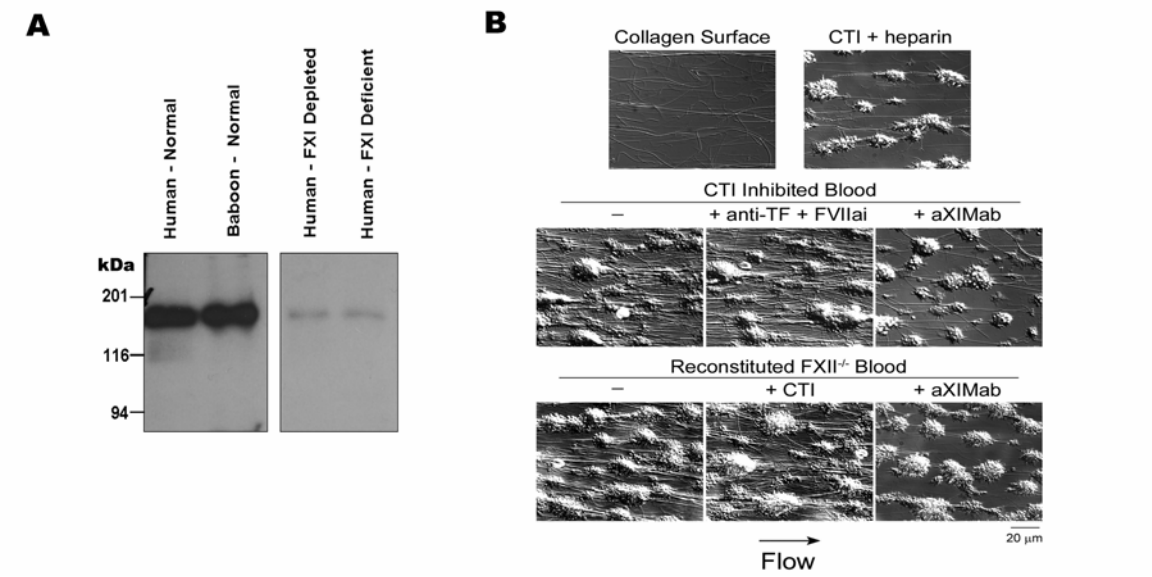

Figure 4.3. The anti-factor XI (FXI) monoclonal antibody (aXIMab) binds human and baboon FXI, and inhibits fibrin formation in FXII-inhibited or deficient human blood under flow. (A) Binding of the aXIMab to the FXI dimer (160 kDa) in platelet free NHP and NBP was demonstrated by Western blotting, developed using a secondary anti-mouse IgG antibody. aXIMab binding was minimal in FXI-depleted plasma or plasma from a FXI-deficient patient. **(B)** aXIMab prevented visible fibrin formation in FXII-inhibited or deficient blood under flow. Human whole blood, anticoagulated with corn trypsin inhibitor (CTI, 40 μg/mL) to inhibit FXIIa, or reconstituted FXII deficient human blood was perfused through collagen-coated capillary tubes (thrombus chambers) at a shear rate of 265 s^{-1} for 10 minutes. Prior to each experiment, blood was incubated with either unfractionated heparin (15 U/mL), anti-tissue factor antibody (20 μg/mL) plus FVIIai (1 μg/mL), aXIMab (20 μg/mL), CTI (40 μg/mL) for reconstituted blood where indicated, or PBS vehicle. Images were obtained via Kohler-illuminated Nomarski differential interference contrast (DIC) microscopy with a Zeiss Axiovert 200M microscope using a Zeiss 63x oil immersion 1.40 NA plan-apochromat lens. Images were captured using a Zeiss AxioCam with Slidebook 4.0 (Intelligent Imaging Innovations, Inc., Denver, CO, USA) after three minutes of perfusion with modified Tyrodes buffer to wash the thrombus of unbound cells. All experiments were performed at 37° C. Each image is representative of 2 or 3 experiments.

-Panel A was provided by Dr. David Gailani, Vanderbilt University. The experiments for panel B were performed by Tara C White-Adams in the laboratory of Dr. Owen McCarty, Oregon Health & Science University.

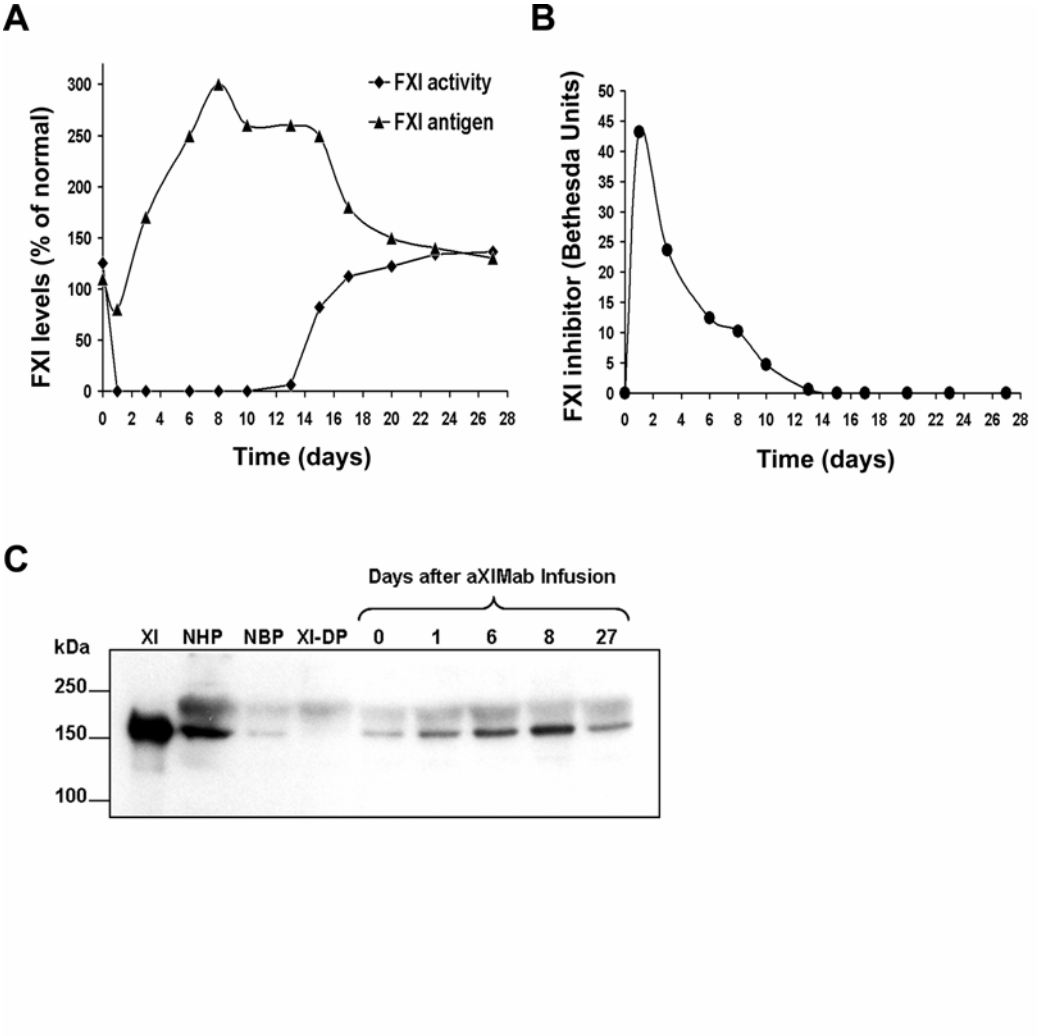

Figure 4.4. Sustained inhibition of circulating FXI procoagulant activity after administration of aXIMab. An intravenous injection of aXIMab (2 mg/kg) was given over 5 min to a single baboon. Plasma samples were collected into citrate anticoagulant and tested for (**A**) FXI procoagulant activity, FXI antigen (FXI:Ag), and (**B**) inhibitor levels over 4 weeks, with each time point being the mean of duplicate measurements. The FXI:Ag ELISA was able to detect both free and complexed FXI. Since the Bethesda assay detected only free FXI inhibitor (aXIMab), FXI:Ag and FXI activity at low inhibitor levels did not correlate until all complexes were cleared from circulation. The one hour time points for all levels were omitted for clarity. (**C**) Western blot of 1 μl NHP and NBP samples size fractionated by non-reducing 7.5% SDS-PAGE. Detection was with a polyclonal antibody against human FXI. The five lanes on the right represent samples prior to (0) or 1, 6, 8, and 27 days after infusion of aXIMab. Abbreviations: XI – 100 ng purified human FXI; NHP – normal human plasma; NBP – normal baboon plasma; XI-DP – FXI deficient human plasma.

-Panel C was provided by Dr. David Gailani, Vanderbilt University.

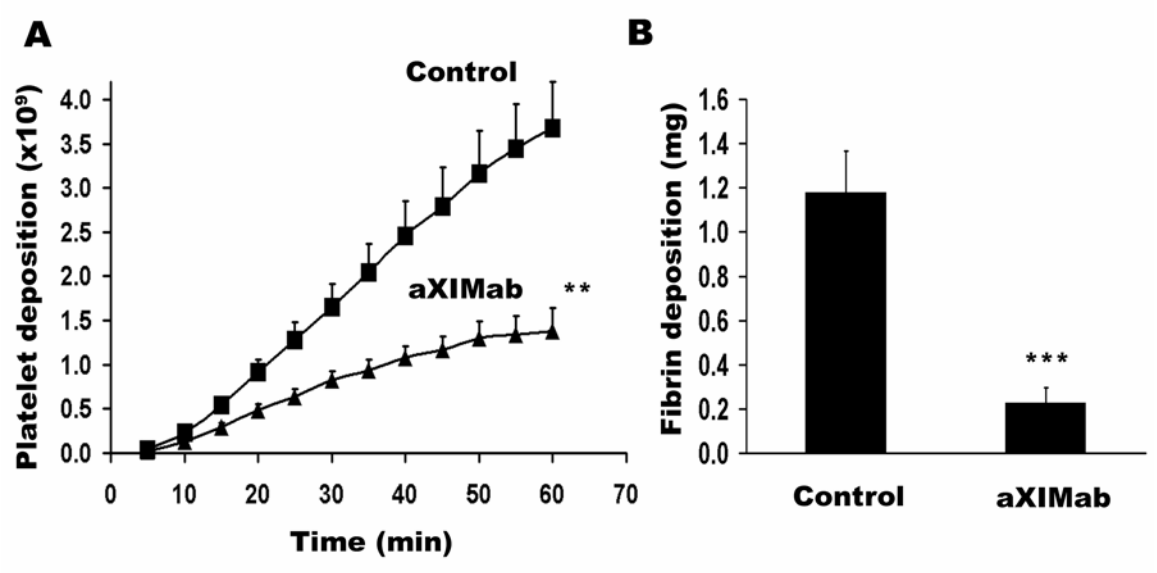

Figure 4.5. FXI inhibition reduces platelet and fibrin deposition on collagen-coated vascular grafts. Effects of FXI inhibition on (**A**) platelet and (**B**) fibrin deposition on collagen coated (4 mm id) vascular grafts. The grafts were placed in vehicle-treated (n = 8) or aXIMab treated (n = 6) animals (≥99% FXI inhibition). Blood was allowed to flow through the devices at 100 ml/min, producing an initial average wall shear rate of 265 s^{-1}. Significance levels are $^{**}P < 0.01$, $^{***}P = 0.001$, by the two-tailed Student's t-test. Values are means ± SEM.

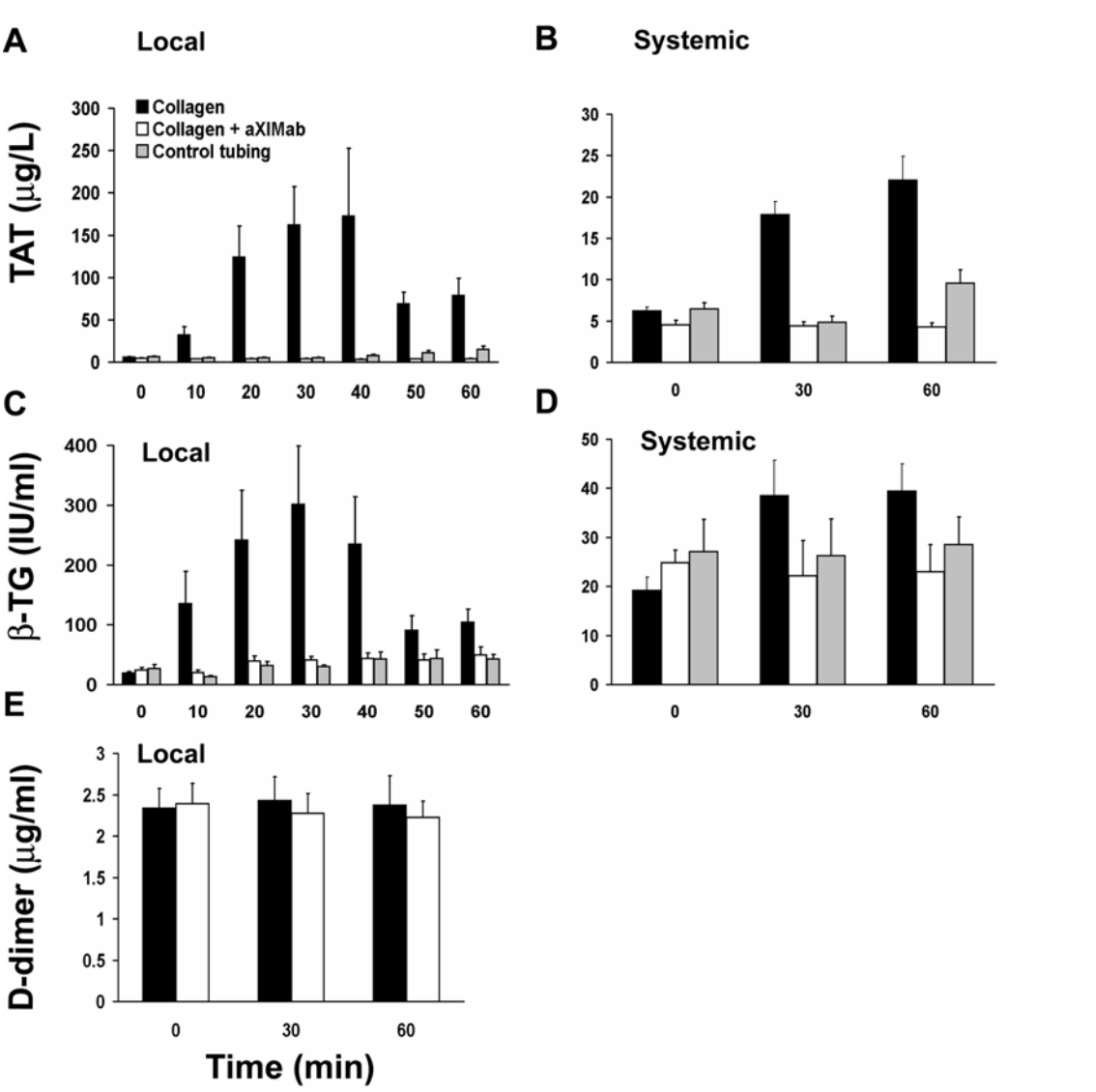

Figure 4.6. FXI inhibition reduces thrombin generation and platelet activation. Local (**A,C**) and systemic (**B,D**) TAT and βTG levels were monitored during thrombus formation, respectively. Blood samples were also tested for the fibrinolysis product D-dimer (**E**). Local values are those taken from the near wall, low flow concentration boundary layer 1 cm distal to the growing thrombus over the course of 10 min prior to its designated time, while systemic samples were taken from the arteriovenous shunt proximal to the thrombogenic device. Zero time points in all groups are from samples taken systemically immediately before each study. FXI inhibition (n = 6) reduced local thrombin formation and platelet activation, which translated into systemic reductions in both TAT and βTG compared with collagen controls (n = 7) by 60 min. No significant change in D-dimer release during acute thrombus formation (≤ 60 min) was detected in control and aXIMab treated animals. The silicone rubber tubing without the collagen-coated graft segment (n = 5) did not induce significant increases in coagulation or platelet activation during these studies. Values are means ± SEM.

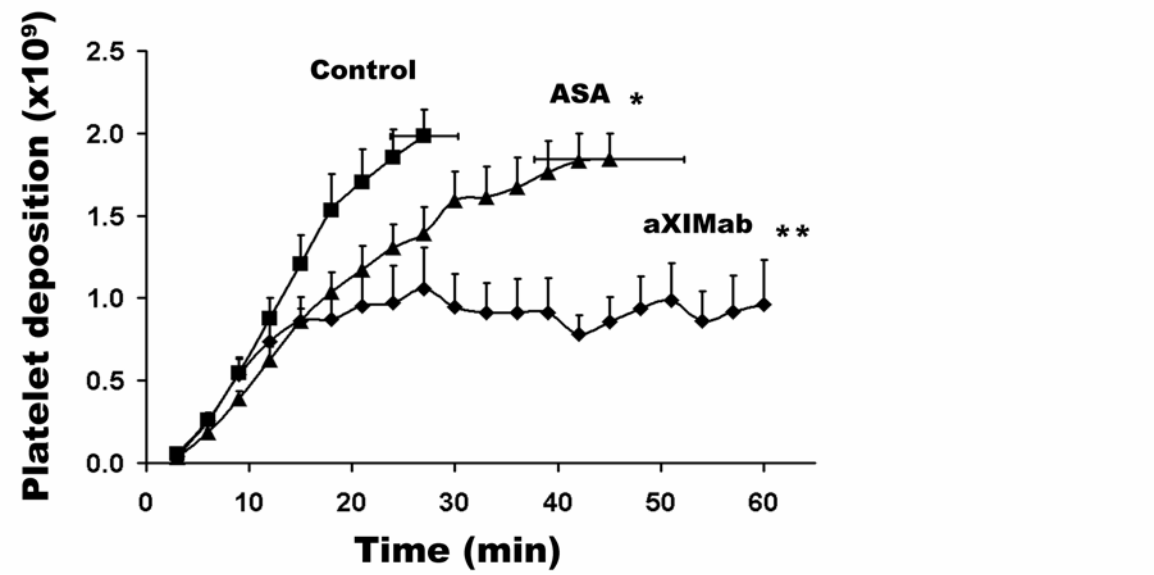

Figure 4.7. FXI inhibition limits vascular graft-associated platelet deposition under high arterial shear. Effects of FXI inhibition or ASA administration on platelet deposition on collagen-coated (2 mm id) vascular grafts are shown. Collagen-coated vascular graft segments were placed in permanent arteriovenous shunts in untreated (n = 9), ASA treated (n = 6), and aXIMab treated (n = 5) animals. Blood was allowed to flow through the grafts at a rate of 100 mL/min, producing an average initial wall shear rate of 2120 s^{-1}. The flow was maintained by the pulsatile arterial pressure until the graft occluded (defined as ≤ 20 ml/min flow rate). Thrombi that formed in the grafts in the aXIMab treated animals were unstable, embolized more frequently than in ASA-treated animals, and did not occlude the grafts for at least 60 min. Significance levels are $*P = 0.05$, $**P < 0.01$ compared with non-treatment controls, using the log-rank test with the non-occluded devices being censored. Values are means ± SEM.

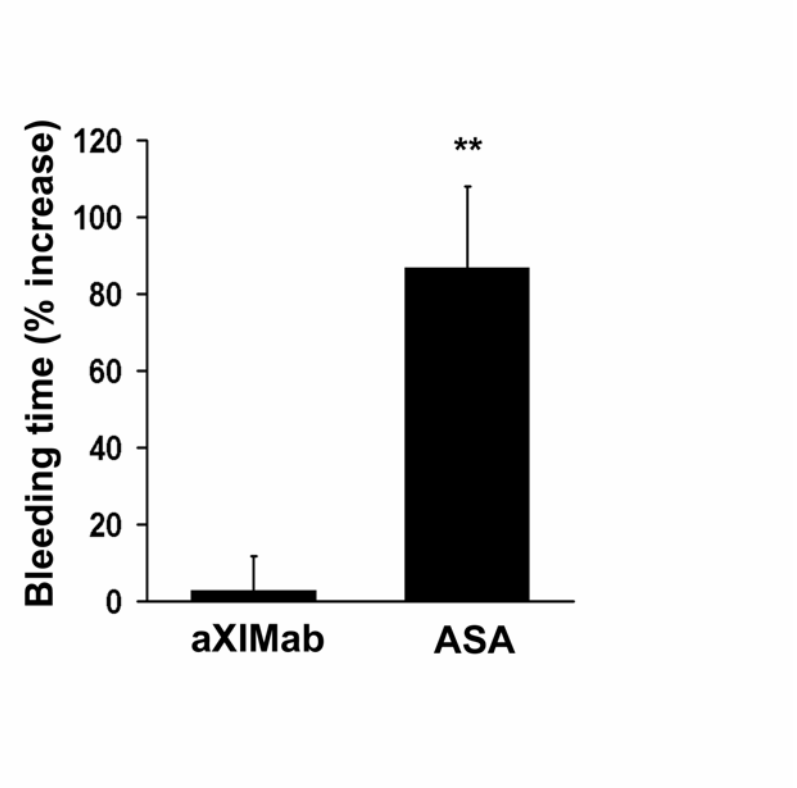

Figure 4.8. The template bleeding time is normal in baboons treated with aXIMab. Bleeding times were measured repeatedly on the volar surface of the lower arm before and during treatment with aXIMab or ASA using an FDA-approved method and device (Surgicutt®, International Technidyne Corp). ASA (n=10) prolonged the bleeding time while inhibition of FXI by aXIMab (n = 18) did not prolong bleeding times compared with controls (n = 14). The average of bleeding time changes are shown ± SEM. Significance level is $^{**}P < 0.01$, by the two-tailed Student's t-test.

Chapter 5: Platelet count reduction in baboons

5.1 Summary

Essential thrombocythemia is a clinical risk factor for thrombosis, while severe thrombocytopenia, which is antithrombotic in experimental animals, causes bleeding. We hypothesized that lowering of the platelet count within the normal physiological range could also be antithrombotic, but without affecting hemostasis. To test this hypothesis, the platelet count in baboons was transiently reduced by inhibition of thrombopoietin (TPO) with anti-TPO immune serum. The effect of lowering platelet count was evaluated in an acute vascular graft thrombosis model. The rate of platelet-rich thrombus formation in 4 mm diameter collagen coated grafts correlated strongly with platelet count ($R = .95$, $P<.0001$). Stenotic 2 mm diameter grafts occluded within an hour at normal platelet count, while graft occlusion was prevented by moderate platelet lowering without affecting bleeding time. These data suggest that elective pharmacological platelet count reduction through specific targeting of megakaryocytopoiesis may be a safe and effective approach for thromboprophylaxis.

-This research has not yet been published.

5.2 Introduction

While platelets are essential for normal hemostasis, they also play a critical role in the development and pathogenesis of atherothrombosis.[33] Systemic platelet inhibitors reduce the risk of recurrent thrombotic vascular events, but at the same instance all currently available platelet inhibitors disable hemostasis, resulting in an increased risk of severe or fatal bleeding.[55,134,135] Normal platelet count ranges from 150 x 10^3/μL to 400 x 10^3/μL, with significant hemostatic dysfunction commonly observed only during severe thrombocytopenia. In fact, given no other underlying hemostatic dysfunction, thrombocytopenic patients with a platelet count higher than 50 x 10^3/μL generally require no treatment, and are not prone to significant spontaneous bleeding.[136-139] Only when platelet counts fall below 10 x 10^3/μL are patients at significantly increased risk of spontaneous intracranial and other life-threatening forms of hemorrhage.[138,139] Data from clinical studies have consistently shown that recurrent thrombosis and mortality correlate with baseline platelet count in some cardiovascular diseases.[140-144] However, other than under severe thrombocytopenic conditions induced by antibodies directed against platelets,[145,146] it is not known whether electively lowering platelet count within the normal range, and without affecting their functional integrity, affects thrombus formation. The present study was designed to gauge the antithrombotic and antihemostatic effects of moderate platelet count reduction through inhibition of thrombopoietin (TPO), and to evaluate if this strategy could be useful for patients in need of safer thromboprophylaxis.

5.3 Materials and methods

Autoantibodies to baboon TPO (anti-TPOab) were generated by repeat injections of recombinant human TPO (rhTPO, Genentech, San Francisco, CA) to a juvenile male baboon. Eleven TPO Injections (5 μg/kg), given over 35 days, caused transient thrombocythemia followed by sustained progressive thrombocytopenia. During thrombocytopenia, blood was drawn weekly, and the immune serum was frozen at -80 C° for further experiments. Purified IgG from the serum inhibited expansion and ploidity of a TPO-dependent cell culture (IC_{50} 0.76 μg/mL),[147,148] suggesting that the presence of an anti-TPOab was the cause of thrombocytopenia in the TPO-treated baboon. An intravenous injection (30-35 mL) of the antiserum caused a transient decrease in the circulating platelet count of male baboons (*Papio anubis*, 9-13 kg) (Figure 5.1). Other blood cell counts were unaffected versus baseline values.

The effect of platelet count reduction on thrombogenesis was evaluated in 42 non-terminal experiments using 13 baboons in a vascular graft thrombosis model.[113] Accumulation of radiolabeled platelets and fibrinogen was measured in collagen-coated 4 mm internal diameter (id) or stenotic (2 mm id) expanded polytetrafluoroethylene vascular graft segments deployed into a chronic arteriovenous shunt for up to 180 minutes.[149] Initial blood flow was restricted to 100 mL/min.[113] In controls, flow remained steady in the wider grafts (data in Figures 5.3 and 5.5) while the stenotic grafts (Figure 5.6), which had a 75% central constriction in cross-sectional area, progressively occluded due to thrombus formation. The stenotic grafts were removed from the shunt at 90 minutes or when the flow rate fell to 20 mL/min, signaling imminent occlusion, and recorded as time to occlusion.

-The IC_{50} data was supplied by Stuart Bunting, Genentech, San Francisco, CA.

For platelet aggregation assays, blood was collected into 3.2% citrate (1:9, vol/vol) and processed for platelet rich plasma. The platelet count was adjusted to 150 x 10^3/μL in all samples and aggregations performed using a Chrono-Log aggregometer (Havertown, PA) with adenosine diphosphate (ADP, Sigma, St Louis, MO) and collagen (Nycomed Arzeneimittel, Munich, Germany). The aggregation results were expressed as the concentration of agonist that induced half-maximal aggregation (AC_{50}).[150] Other blood samples were collected into either 3.8% citrate (1:9, vol/vol) and processed for platelet poor plasma, or clotted for serum, and frozen at -80C. Samples were later assayed using cross-reacting ELISAs for D-dimer (IMUCLONE D-dimer; American Diagnostica, Stamford, CT) and TPO (Quantikine TPO, R&D systems, Minneapolis, MN). The effects of platelet count reduction, with or without aspirin (ASA, 32 mg/kg administered orally) or clopidogrel (1 mg/kg, daily), on hemostasis were assessed using the template skin bleeding time (BT) test (Surgicutt, International Technidyne, Piscataway, NJ). All BT data at platelet counts <70 x 10^3/μL was obtained from the baboon that developed thrombocytopenia after TPO injections. The protocol was approved by the Oregon Health & Science University Institutional Animal Care and Use Committee. Mean values are given ± 1 SEM (standard error of the mean). Occlusion data were compared using the log-rank test. The two-tailed Student's *t*-test was used for all other single pair comparisons. Pearson's correlations were calculated using MATLAB (Mathworks, Natick, MA). $P \leq .05$ was considered significant.

5.4 Results and discussion

A single intravenous injection of anti-TPO antiserum caused a transient decrease in platelet count from 289 ± 25 x 10^3/μL to 110 ± 7 x 10^3/μL, reaching the nadir by day 16 in all animals (n = 7, Figure 5.1). TPO levels decreased from a baseline of 4.6 ± 1.8 pg/mL to undetectable levels (<2.8 pg/mL, assay limit of detection) at 24 hours post infusion. TPO levels then increased to 45.1 ± 15.6 pg/mL by day 21 before decreasing steadily to undectectable levels by day 42 (Figure 5.1). Interestingly, while platelet aggregation reactivity to collagen and ADP appeared to increase as the platelet count declined (Figure 5.2), this did not seem to affect the platelet deposition rate onto the graft

surface (Figure 5.3), which had a strong linear correlation with platelet count (R^2 = .91, P < .0001). This confirms previous observations at normal platelet count that thrombus propagation is directly correlated to platelet count in the baboon thrombosis model.[67,151] In aspirin-treated animals, a similar relationship was observed (R^2 = .90, P = .01; Figure 5.3), consistent with observations that platelet count may be an independent predictor of thrombotic events in coronary artery disease patients receiving anti-platelet therapy.[142,143] Aspirin decreased platelet thrombus size by an average of 27% versus results in untreated animals at comparable platelet counts; this relationship was independent of platelet count over the ranges studied (Figure 5.3). Thus in both normal and aspirin-treated animals, a doubling of platelet count caused a 3-fold increase platelet thrombus size at 1 hour. Graft fibrin accumulation after 60 minutes averaged 1.2 ± 0.1 mgs in untreated animals, and 1.0 ± 0.1 mgs aspirin-treated animals (P > .05), consistent with aspirin's modest effect on platelet accumulation.

To determine if platelet deposition onto the graft surface would continue to increase beyond one hour, we performed several 3 hour-long studies (Figure 5.5). This data showed termination of thrombus propagation by about 60 to 80 minutes. This decrease corresponded with small successive increases in D-dimer from a baseline of 1.73 ± .31 μg/mL to 1.97 ± .26 μg/mL (n = 6 each) at 3 hours, suggesting that activation of endogenous antithrombotic mechanisms may contribute to sustained large graft patency regardless of the platelet count. BT (Figure 5.4) did not increase until the platelet count was ~100 x 10^3/μL, whereas ASA pretreatment resulted in a BT prolongation of about 3 minutes compared with untreated animals at corresponding platelet levels. Thus, while aspirin may aggravate the risk of spontaneous bleeding in thrombocytopenia,[152] our data do not suggest that moderate platelet count reduction enhances the risk of bleeding with ASA intake. Clopidogrel showed similar bleeding effects to the aspirin group (Figure 5.4)

In the stenotic grafts (Figure 5.6), platelet deposition increased rapidly at baseline platelet count (375 ± 18 x 10^3/μL, n = 4), occluding all devices in 41 ± 8 minutes. After lowering the platelet count to194 ± 38 x 10^3/μL, graft occlusion was

prevented until the end of observation at 90 minutes (n = 4, $P < .01$). This suggests that moderate platelet count reduction can have a profound impact on the dynamics of occlusive thrombus formation.

Together, these data suggest that while thrombus propagation is directly correlated with platelet count, primary hemostasis is impaired only at relatively low platelet levels ($\leq 100 \times 10^3/\mu L$), which is similar to observations of clinical bleeding.[138,139,153,154] Our data shows that the rate of thrombus growth after platelet count reduction was significantly slower than at baseline platelet count, even with high dose aspirin. The reduced thrombus propagation rate may allow time for natural pacification and/or thrombolytic mechanisms to intercede prior to thrombotic occlusion of smaller blood vessels. Thus, even moderate platelet count reduction may result in a significant decrease in vascular occlusion while not appreciably disabling hemostasis. Unlike systemic platelet inhibitors that reduce the hemostatic activity of all platelets in the circulation, development of pharmacological inhibitors of platelet production via specific targeting of megakaryocytopoiesis may allow for optimization of the prohemostatic versus prothrombotic capacity of the circulating platelet pool, and provide a safer alternative to existing strategies for reducing the incidence of thrombo-occlusive events such as heart attack and ischemic stroke.

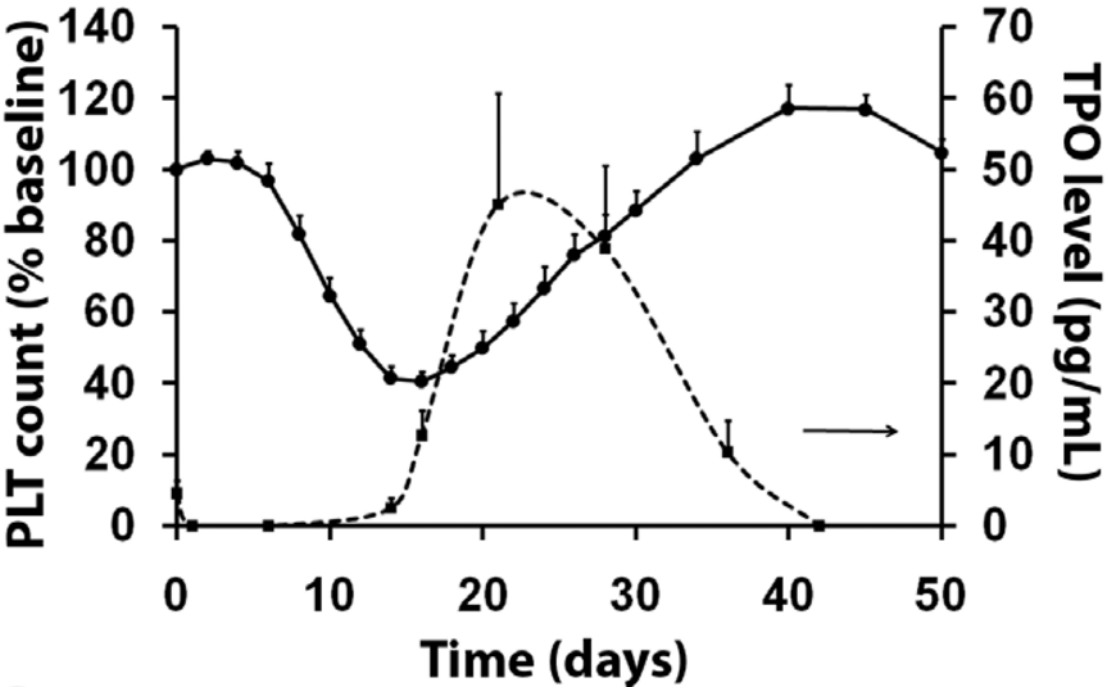

Figure 5.1. Effects of anti-thrombopoietin (TPO) antiserum on platelet count and TPO levels in baboons. Platelet (PLT) count response curve from 7 baboons infused with anti-TPO antiserum. Initial platelet counts averaged 289,000 ± 25,000/μL and decreased to 110,000 ± 7,000/μL by day 16. Circulating TPO levels were initially 4.6 ± 1.8 pg/mL, reached a maximum by day 21, and returned to normal by day 40 (n = 4).Values are mean ±1 SEM.

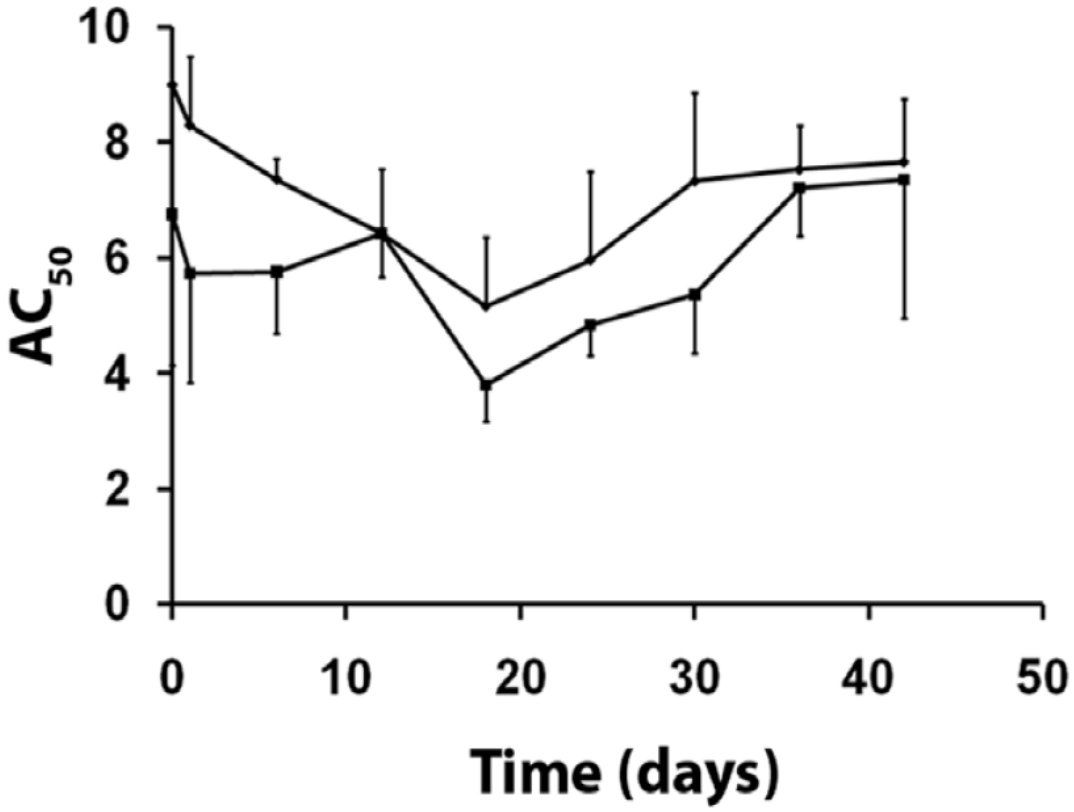

Figure 5.2. Effects of platelet count reduction on platelet aggregation. For platelet aggregation assays, blood was collected into 3.2% citrate (1:9, vol/vol) and processed for platelet rich plasma. The platelet count was adjusted to 150 x 10^3/μL in all samples and aggregations performed using a Chrono-Log aggregometer with adenosine diphosphate (ADP) and collagen. The aggregation results were expressed as the concentration of agonist that induced half-maximal aggregation (AC_{50}). Each data point is the mean plus of minus 1 SEM of 4 experiments.

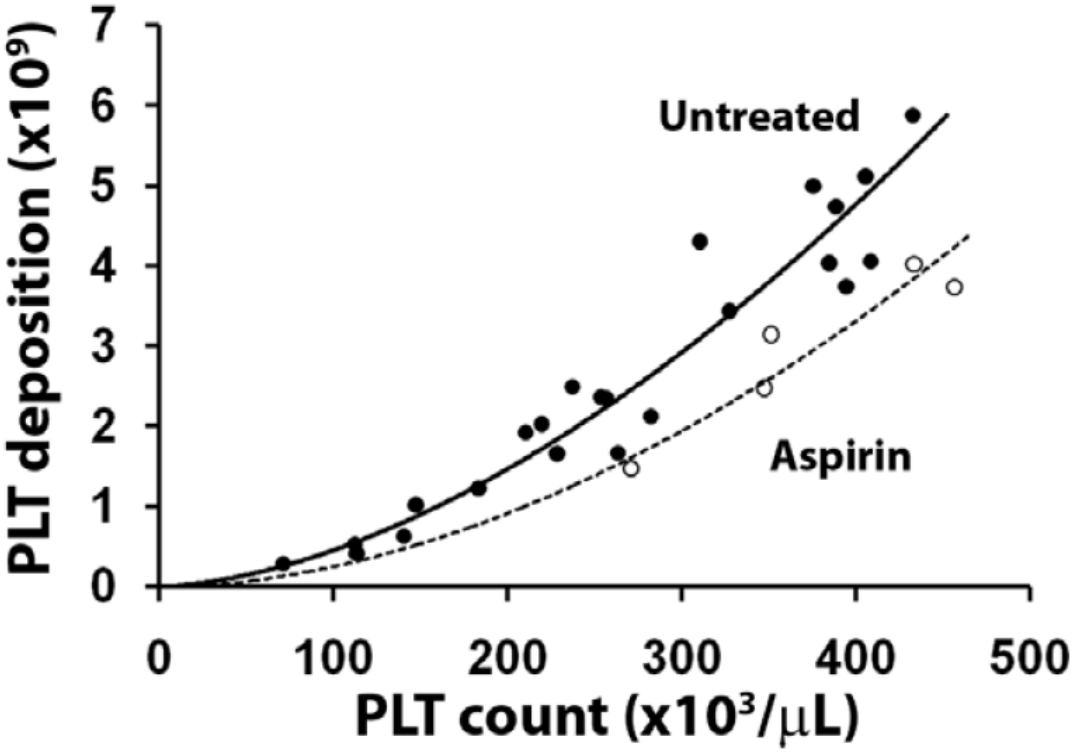

Figure 5.3. Effects of platelet count reduction on arterial thrombus formation in baboons. Platelet deposition onto collagen-coated (4 mm id x 2 cm) vascular grafts after 60 minutes (n = 23, closed circles) strongly correlated with the circulating platelet count ($R^2 = .91$, $P < .001$). The best fit curve was: $x = 5.534 \cdot y^{1.591}$. Pretreatment with oral aspirin in 5 animals (open circles) moderately reduced platelet deposition versus untreated controls. In aspirin-treated animals a strong relationship was observed between total platelet accumulation and the circulating platelet count ($R^2 = .90$, $P = .01$). The best fit curve for the aspirin data was: $3.782 \cdot y^{1.596}$.

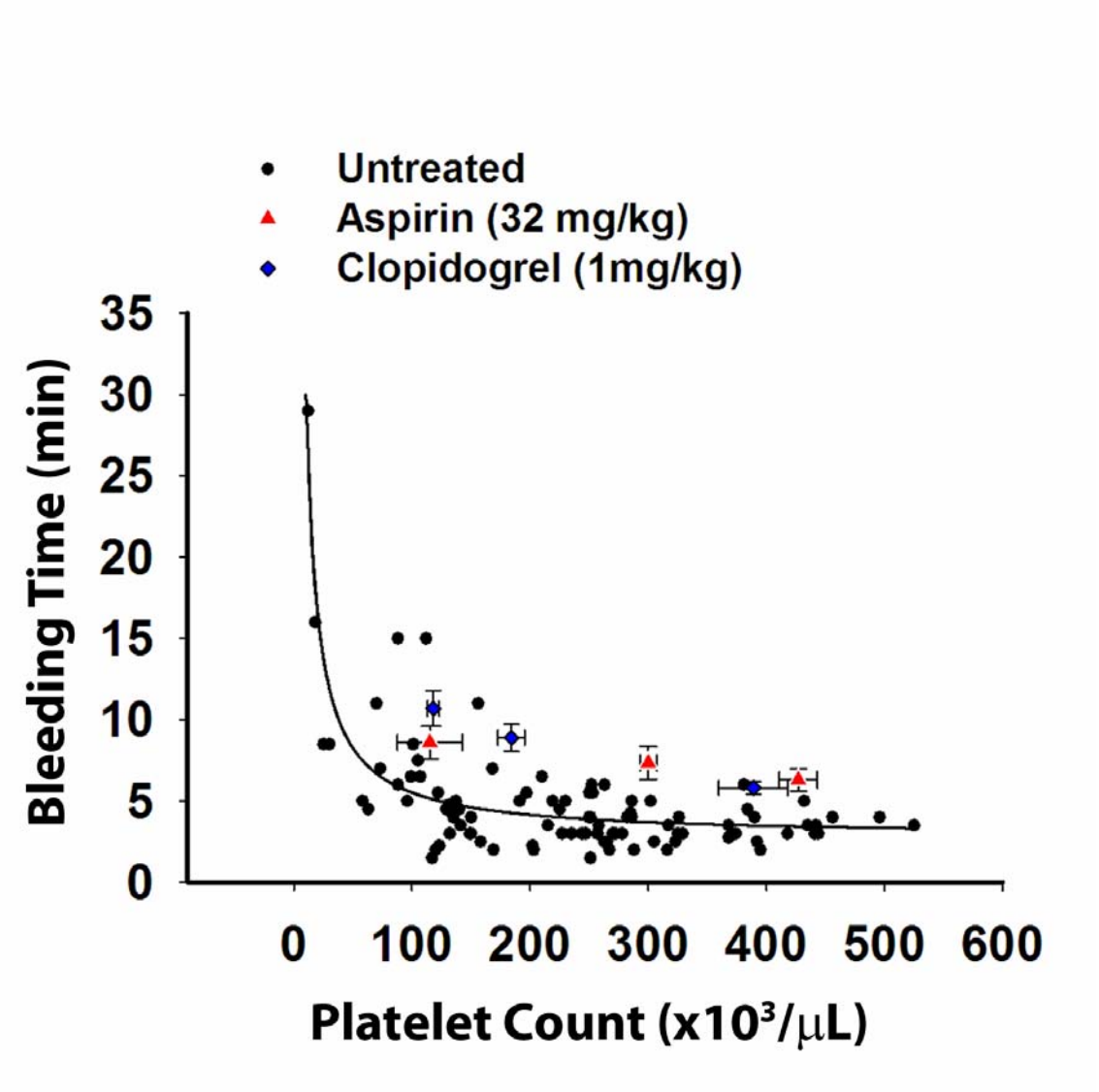

Figure 5.4. Effects of platelet count reduction on primary hemostasis in baboons. Template bleeding times and platelet counts measured in untreated and aspirin treated baboons. ASA data (▲) was grouped into tertiles of platelet count: Lower tertile (115,000 ± 27,000/μL, n = 7), middle tertile (300,000 ± 7,000/μL, n = 7), and upper tertile (427,000 ± 16,000/μL, n = 13). Clopidogrel data (♦) was also grouped into tertiles of platelet count: Lower tertile (118,000 ± 5,000/μL, n=7), middle tertile (184,000 ± 11,000/μL, n = 7), and upper tertile (389,000 ± 29,000/μL, n = 6). Values are mean ±1 SEM.

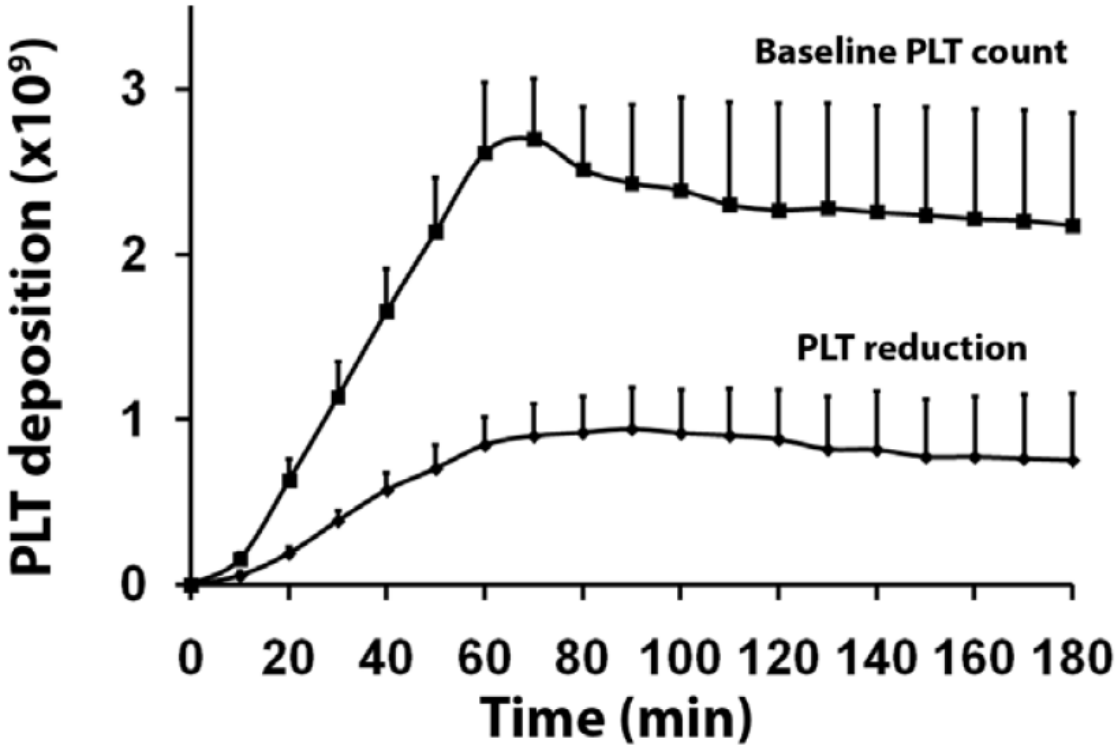

Figure 5.5. Effects of platelet count reduction on arterial thrombus formation. Platelet deposition curves (n = 3 each) from three-hour long thrombosis studies showing that platelet accumulation plateaus and begins to decrease between 60-80 minutes after flow initiation at both normal (266,000 ± 32,000/μL) and low (134,000 ± 33,000/μL) platelet counts. Values are means plus or minus SEM.

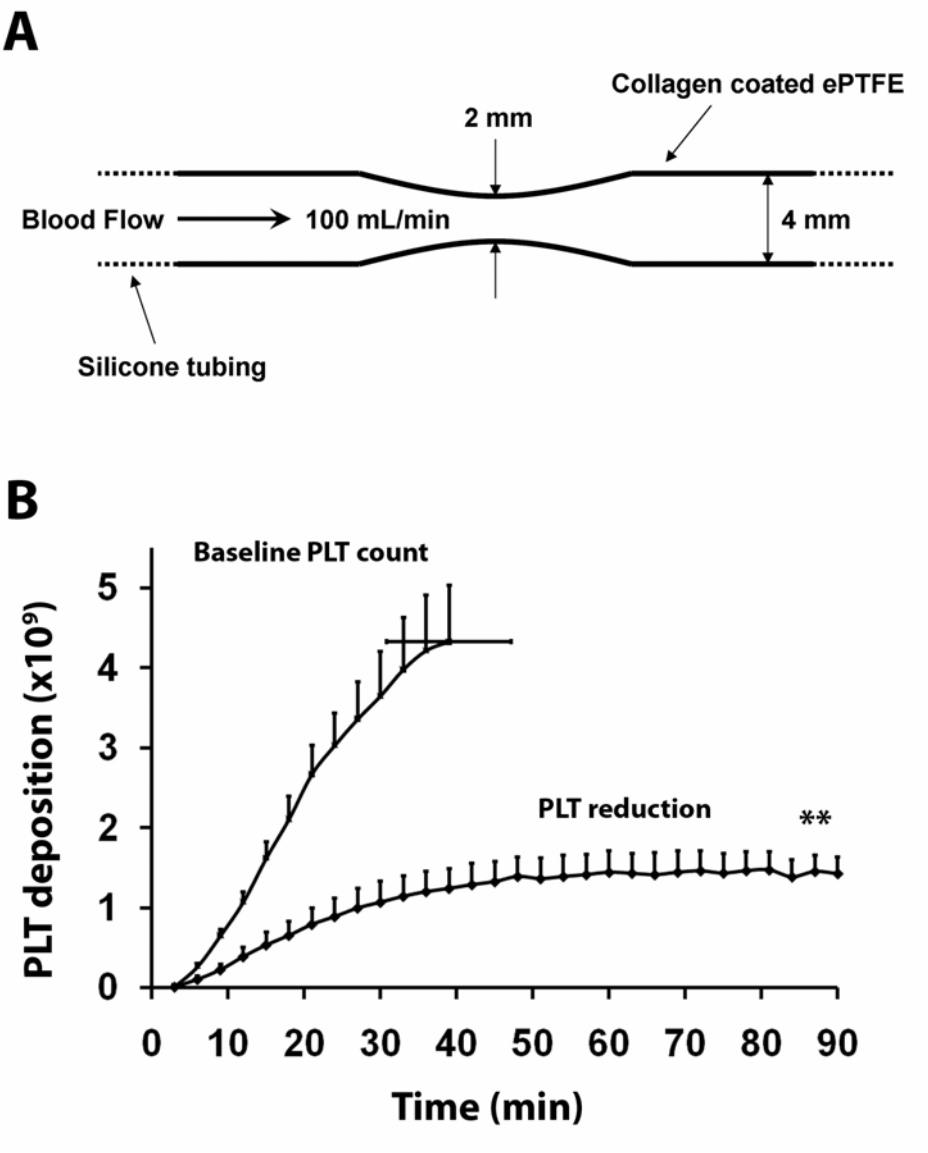

Figure 5.6. Moderate platelet count reduction limits stenotic vascular-graft associated platelet deposition and prevents graft occlusion. (A) Thrombogenesis was initiated and developed in 4 mm id, 2.5 cm long collagen coated expanded polytetrafluoroethylene (ePTFE) vascular grafts which had a centrally positioned 75% constriction in cross-sectional area. Blood flow was maintained at 100 mL/minute or until flow dropped to ≤20 mL/minute, signaling imminent occlusion and recorded as time to occlusion. For devises that did not occlude, studies were stopped at 90 minutes. **(B)** Effects of platelet (PLT) count lowering on thrombus development in collagen coated stenosed vascular grafts. At normal platelet counts (375,000 ± 18,000/μL, n = 4), all devices occluded in 41 ± 8 minutes. After moderate platelet count reduction to 194,000 ± 38,000/μL, platelet accumulation was slowed and thrombo-occlusion was completely prevented for all 90 minute studies (n = 4, ***P*<.01). Significance level was calculated using the log-rank test, with non-occluding devises being censored. Values are means plus or minus SEM.

Chapter 6: Concluding Remarks

This thesis has provided evidence suggesting that while thrombosis and hemostasis are linked in many respects, they possess subtle mechanistic differences that may allow for the development of a new class of antithrombotic agents. Our data in mice suggests that FXI is an important enzyme in infection-related coagulation and possibly contributes to an enhanced inflammatory state by supporting bradykinin generation. More *in vivo* sepsis data however must be gathered using inhibitors of FXI to confirm its role in severe infections. Inhibiting FXI in baboons reduced thrombus formation and prevented the occlusion of thrombogenic vascular grafts by destabilizing growing thrombi. FXI inhibition also dramatically attenuated local thrombin generation, as measured immediately downstream. This data supports the mechanism of FXI being involved in continued amplification of thrombin and thus in the propagation of mural thrombogenesis. Since FXII and thrombin can both activate FXI, more studies must be done to assess the relative contribution of each during thrombus formation *in vivo*.

The evolutionary role and advantage (or disadvantage) of the appearance of the contact system in mammals remains an interesting puzzle. The FXI gene or an evolutionary predecessor seems to be conserved in all mammals.[155] Since FXI deficiency is associated with trauma-related bleeding in only about a third of affected individuals, it is clear that FXI is not crucial for normal hemastasis. However, our data suggests a mechanism whereby FXI acts as a thrombo-stabilizing factor to prevent lethal hemorrhage during more severe transvascular injury, where the shear forces are high. Indeed, frequent injury surely followed our mammalian ancestors over the millennia, and the evolutionary pressures likely played a role in preserving a more robust, high-pressure hemostatic system. Today our main causes of death, heart attack and stroke, may partially

be the result of this robust system. Thus, by targeting a portion of the coagulation pathway that is designed to halt more severe forms of hemorrhage, we may be able to safely reduce the morbidity and mortality associated with thrombo-occlusive diseases.

Our data in baboons showed that transiently reducing platelet count limited arterial thrombus formation and stenotic vascular graft occlusion while not affecting hemostasis. Greater circulating platelet numbers clearly increases the convective delivery of platelets to the growing thrombus surface, but the relationship between thrombus formation and platelet count has been largely undefined to this point. It appears from these studies that high platelet counts may predispose individuals to occlusive thrombus formation. Again, a high platelet count may have been beneficial to early man in order to halt repeated injury-related vessel damage; however, modern humans are not required to compete in such a physically demanding world under normal circumstances. Thus, by reducing platelet count to low-normal levels, thrombosis may be reduced without significantly compromising normal hemostasis.

Chapter 7: Future Direction

A new platelet lowering drug is currently under early stage clinical investigation. Low dose, time released anagrelide, which is being developed by Dr. Stephen Hanson and BioVascular, Inc. Anagrelide has been FDA approved for use in the treatment of essential thrombocythemia by inhibiting the maturation of megakaryocyes, and thus inhibiting platelet production. Future development of this drug will hopefully demonstrate whether platelet count reduction is beneficial in limiting thrombotic cardiovascular disease.

Together with my adviser Dr. András Gruber we are currently seeking funding for the further development of aXIMab as a safe treatment for sepsis and other thrombotic indications. We hope to continue to explore the mechanism of FXI inhibition that leads to a survival benefit during sepsis in mice. Recently we have generated a new monoclonal antibody which is able to inhibit FXI in mice as well as all other mammalian FXI tested to date. This new reagent will allow the further delineation of the role FXI plays in disease. We are also developing, with Dr. David Gailani at Vanderbilt, ELISA assays for detection of FXI using novel antibodies that target various FXI domains and cross-react with a number of mammalian species.

This is indeed an exciting time in the development of fundamentally new antithrombotic strategies. The data presented in this dissertation as well as a growing body of other experimental evidence suggests that thrombosis and hemostasis, while linked at many levels, are mechanistically distinct. This insight will hopefully lead to a new generation of safer antithrombotic treatment strategies that may revolutionize patient care in the coming decades.

References

1. Douglas S. Coagulation history, Oxford 1951-53. Br J Haematol. 1999;107:22-32.

2. Davie EW, Ratnoff OD. Waterfall Sequence for Intrinsic Blood Clotting. Science. 1964;145:1310-1312.

3. Macfarlane RG. An Enzyme Cascade in the Blood Clotting Mechanism, and Its Function as a Biochemical Amplifier. Nature. 1964;202:498-499.

4. Mackman N, Tilley RE, Key NS. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. Arterioscler Thromb Vasc Biol. 2007;27:1687-1693.

5. Drake TA, Morrissey JH, Edgington TS. Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. Am J Pathol. 1989;134:1087-1097.

6. Toomey JR, Kratzer KE, Lasky NM, Stanton JJ, Broze GJ, Jr. Targeted disruption of the murine tissue factor gene results in embryonic lethality. Blood. 1996;88:1583-1587.

7. Bugge TH, Xiao Q, Kombrinck KW, et al. Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. Proc Natl Acad Sci U S A. 1996;93:6258-6263.

8. Tuddenham EG, Pemberton S, Cooper DN. Inherited factor VII deficiency: genetics and molecular pathology. Thromb Haemost. 1995;74:313-321.

9. Himber J, Wohlgensinger C, Roux S, et al. Inhibition of tissue factor limits the growth of venous thrombus in the rabbit. J Thromb Haemost. 2003;1:889-895.

10. Roque M, Reis ED, Fuster V, et al. Inhibition of tissue factor reduces thrombus formation and intimal hyperplasia after porcine coronary angioplasty. J Am Coll Cardiol. 2000;36:2303-2310.

11. Chi L, Gibson G, Peng YW, et al. Characterization of a tissue factor/factor VIIa-dependent model of thrombosis in hypercholesterolemic rabbits. J Thromb Haemost. 2004;2:85-92.

12. Furie B, Furie BC. Thrombus formation in vivo. J Clin Invest. 2005;115:3355-3362.

13. Butenas S, Mann KG. Active tissue factor in blood? Nat Med. 2004;10:1155-1156; author reply 1156.

14. Colman RW. Biologic activities of the contact factors in vivo--potentiation of hypotension, inflammation, and fibrinolysis, and inhibition of cell adhesion, angiogenesis and thrombosis. Thromb Haemost. 1999;82:1568-1577.

15. Asakai R, Chung DW, Davie EW, Seligsohn U. Factor XI deficiency in Ashkenazi Jews in Israel. N Engl J Med. 1991;325:153-158.

16. Doggen CJ, Rosendaal FR, Meijers JC. Levels of intrinsic coagulation factors and the risk of myocardial infarction among men: Opposite and synergistic effects of factors XI and XII. Blood. 2006;108:4045-4051.

17. Meijers JC, Tekelenburg WL, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. N Engl J Med. 2000;342:696-701.

18. Wang X, Smith PL, Hsu MY, et al. Effects of factor XI deficiency on ferric chloride-induced vena cava thrombosis in mice. J Thromb Haemost. 2006;4:1982-1988.

19. Kleinschnitz C, Stoll G, Bendszus M, et al. Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. J Exp Med. 2006;203:513-518.

20. Chan JC, Ganopolsky JG, Cornelissen I, et al. The characterization of mice with a targeted combined deficiency of protein c and factor XI. Am J Pathol. 2001;158:469-479.

21. Gruber A, Hanson SR. Factor XI-dependence of surface- and tissue factor-initiated thrombus propagation in primates. Blood. 2003;102:953-955.

22. Rosenthal RL, Dreskin OH, Rosenthal N. New hemophilia-like disease caused by deficiency of a third plasma thromboplastin factor. Proc Soc Exp Biol Med. 1953;82:171-174.

23. Gailani D, Broze GJ, Jr. Factor XI activation in a revised model of blood coagulation. Science. 1991;253:909-912.

24. Bouma BN, Vlooswijk RA, Griffin JH. Immunologic studies of human coagulation factor XI and its complex with high molecular weight kininogen. Blood. 1983;62:1123-1131.

25. Fujikawa K, Chung DW, Hendrickson LE, Davie EW. Amino acid sequence of human factor XI, a blood coagulation factor with four tandem repeats that are highly homologous with plasma prekallikrein. Biochemistry. 1986;25:2417-2424.

26. Renne T, Gailani D, Meijers JC, Muller-Esterl W. Characterization of the H-kininogen-binding site on factor XI: a comparison of factor XI and plasma prekallikrein. J Biol Chem. 2002;277:4892-4899.

27. Ratnoff OD. A quarter century with Mr. Hageman. Thromb Haemost. 1980;43:95-98.

28. Giangrande PL. Six characters in search of an author: the history of the nomenclature of coagulation factors. Br J Haematol. 2003;121:703-712.

29. Pixley RA, De La Cadena R, Page JD, et al. The contact system contributes to hypotension but not disseminated intravascular coagulation in lethal bacteremia. In vivo use of a monoclonal anti-factor XII antibody to block contact activation in baboons. J Clin Invest. 1993;91:61-68.

30. Sadler JE. von Willebrand factor. J Biol Chem. 1991;266:22777-22780.

31. Chow TW, Hellums JD, Moake JL, Kroll MH. Shear stress-induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. Blood. 1992;80:113-120.

32. Reed GL, Fitzgerald ML, Polgar J. Molecular mechanisms of platelet exocytosis: insights into the "secrete" life of thrombocytes. Blood. 2000;96:3334-3342.

33. Ruggeri ZM. Platelets in atherothrombosis. Nat Med. 2002;8:1227-1234.

34. Kahn ML, Zheng YW, Huang W, et al. A dual thrombin receptor system for platelet activation. Nature. 1998;394:690-694.

35. Mazzucato M, Marco LD, Masotti A, Pradella P, Bahou WF, Ruggeri ZM. Characterization of the initial alpha-thrombin interaction with glycoprotein Ib alpha in relation to platelet activation. J Biol Chem. 1998;273:1880-1887.

36. Tara C White, Michelle A Berny, Erik I Tucker, et al. Identification of a Role for Apolipoprotein E Receptor 2 as a Platelet Receptor for Factor XI American Society of Hematology. San Francisco: American Society of Hematology
2008.

37. Anderson RN, Smith BL. Deaths: leading causes for 2002. Natl Vital Stat Rep. 2005;53:1-89.

38. Basmadjian D, Sefton MV, Baldwin SA. Coagulation on biomaterials in flowing blood: some theoretical considerations. Biomaterials. 1997;18:1511-1522.

39. Harker LA, Slichter SJ. Platelet and fibrinogen consumption in man. N Engl J Med. 1972;287:999-1005.

40. Lip GY, Blann AD. Does hypertension confer a prothrombotic state? Virchow's triad revisited. Circulation. 2000;101:218-220.

41. Hathcock JJ. Flow effects on coagulation and thrombosis. Arterioscler Thromb Vasc Biol. 2006;26:1729-1737.

42. Wootton DM, Ku DN. Fluid mechanics of vascular systems, diseases, and thrombosis. Annu Rev Biomed Eng. 1999;1:299-329.

43. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature. 1993;362:801-809.

44. Ambrose JA, Tannenbaum MA, Alexopoulos D, et al. Angiographic progression of coronary artery disease and the development of myocardial infarction. J Am Coll Cardiol. 1988;12:56-62.

45. Heart Disease and Stroke Statistics-2005 Update. American Heart Association. 2005;Dallas, Texas.

46. Albers GW, Amarenco P, Easton JD, Sacco RL, Teal P. Antithrombotic and thrombolytic therapy for ischemic stroke: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. Chest. 2004;126:483S-512S.

47. del Zoppo GJ, Poeck K, Pessin MS, et al. Recombinant tissue plasminogen activator in acute thrombotic and embolic stroke. Ann Neurol. 1992;32:78-86.

48. Fieschi C, Argentino C, Lenzi GL, Sacchetti ML, Toni D, Bozzao L. Clinical and instrumental evaluation of patients with ischemic stroke within the first six hours. J Neurol Sci. 1989;91:311-321.

49. Hacke W, Kaste M, Fieschi C, et al. Intravenous thrombolysis with recombinant tissue plasminogen activator for acute hemispheric stroke. The European Cooperative Acute Stroke Study (ECASS). JAMA. 1995;274:1017-1025.

50. Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. N Engl J Med. 1995;333:1581-1587.

51. Hacke W, Donnan G, Fieschi C, et al. Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials. Lancet. 2004;363:768-774.

52. Albers GW, Bates VE, Clark WM, Bell R, Verro P, Hamilton SA. Intravenous tissue-type plasminogen activator for treatment of acute stroke: the Standard Treatment with Alteplase to Reverse Stroke (STARS) study. JAMA. 2000;283:1145-1150.

53. Lopez-Yunez AM, Bruno A, Williams LS, Yilmaz E, Zurru C, Biller J. Protocol violations in community-based rTPA stroke treatment are associated with symptomatic intracerebral hemorrhage. Stroke. 2001;32:12-16.

54. Tanne D, Kasner SE, Demchuk AM, et al. Markers of increased risk of intracerebral hemorrhage after intravenous recombinant tissue plasminogen activator therapy for acute ischemic stroke in clinical practice: the Multicenter rt-PA Stroke Survey. Circulation. 2002;105:1679-1685.

55. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. Lancet. 1996;348:1329-1339.

56. Steinhubl SR, Berger PB, Mann JT, 3rd, et al. Early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. JAMA. 2002;288:2411-2420.

57. CAST: randomised placebo-controlled trial of early aspirin use in 20,000 patients with acute ischaemic stroke. CAST (Chinese Acute Stroke Trial) Collaborative Group. Lancet. 1997;349:1641-1649.

58. The International Stroke Trial (IST): a randomised trial of aspirin, subcutaneous heparin, both, or neither among 19435 patients with acute ischaemic stroke. International Stroke Trial Collaborative Group. Lancet. 1997;349:1569-1581.

59. Lensing AW. Anticoagulation in acute ischaemic stroke: deep vein thrombosis prevention and long-term stroke outcomes. Blood Coagul Fibrinolysis. 1999;10 Suppl 2:S123-127.

60. Bath PM, Iddenden R, Bath FJ. Low-molecular-weight heparins and heparinoids in acute ischemic stroke : a meta-analysis of randomized controlled trials. Stroke. 2000;31:1770-1778.

61. Moonis M, Fisher M. Considering the role of heparin and low-molecular-weight heparins in acute ischemic stroke. Stroke. 2002;33:1927-1933.

62. Mohr JP, Thompson JL, Lazar RM, et al. A comparison of warfarin and aspirin for the prevention of recurrent ischemic stroke. N Engl J Med. 2001;345:1444-1451.

63. Salomon O, Steinberg DM, Koren-Morag N, Tanne D, Seligsohn U. Reduced incidence of ischemic stroke in patients with severe factor XI deficiency. Blood. 2008;111:4113-4117.

64. Giesen PL, Rauch U, Bohrmann B, et al. Blood-borne tissue factor: another view of thrombosis. Proc Natl Acad Sci U S A. 1999;96:2311-2315.

65. Herwald H, Morgelin M, Olsen A, et al. Activation of the contact-phase system on bacterial surfaces--a clue to serious complications in infectious diseases. Nat Med. 1998;4:298-302.

66. Bernard GR, Vincent JL, Laterre PF, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med. 2001;344:699-709.

67. Gruber A, Griffin JH, Harker LA, Hanson SR. Inhibition of platelet-dependent thrombus formation by human activated protein C in a primate model. Blood. 1989;73:639-642.

68. Mosnier LO, Griffin JH. Protein C anticoagulant activity in relation to anti-inflammatory and anti-apoptotic activities. Front Biosci. 2006;11:2381-2399.

69. Minnema MC, Pajkrt D, Wuillemin WA, et al. Activation of clotting factor XI without detectable contact activation in experimental human endotoxemia. Blood. 1998;92:3294-3301.

70. Wuillemin WA, Fijnvandraat K, Derkx BH, et al. Activation of the intrinsic pathway of coagulation in children with meningococcal septic shock. Thromb Haemost. 1995;74:1436-1441.

71. van Gorp EC, Minnema MC, Suharti C, et al. Activation of coagulation factor XI, without detectable contact activation in dengue haemorrhagic fever. Br J Haematol. 2001;113:94-99.

72. Espinola RG, Uknis A, Sainz IM, et al. A monoclonal antibody to high-molecular weight kininogen is therapeutic in a rodent model of reactive arthritis. Am J Pathol. 2004;165:969-976.

73. Kalter ES, Daha MR, ten Cate JW, Verhoef J, Bouma BN. Activation and inhibition of Hageman factor-dependent pathways and the complement system in uncomplicated bacteremia or bacterial shock. J Infect Dis. 1985;151:1019-1027.

74. Jansen PM, Pixley RA, Brouwer M, et al. Inhibition of factor XII in septic baboons attenuates the activation of complement and fibrinolytic systems and reduces the release of interleukin-6 and neutrophil elastase. Blood. 1996;87:2337-2344.

75. Gailani D, Ho D, Sun MF, Cheng Q, Walsh PN. Model for a factor IX activation complex on blood platelets: dimeric conformation of factor XIa is essential. Blood. 2001;97:3117-3122.

76. Scott CF, Silver LD, Purdon AD, Colman RW. Cleavage of human high molecular weight kininogen by factor XIa in vitro. Effect on structure and function. J Biol Chem. 1985;260:10856-10863.

77. Jordan RE, Oosta GM, Gardner WT, Rosenberg RD. The kinetics of hemostatic enzyme-antithrombin interactions in the presence of low molecular weight heparin. J Biol Chem. 1980;255:10081-10090.

78. DeLoughery TG. Hemostasis & Thrombosis 2nd Edition. Georgetown, Texas: Landes Biosciences; 2004.

79. Wallin R, Martin LF. Vitamin K-dependent carboxylation and vitamin K metabolism in liver. Effects of warfarin. J Clin Invest. 1985;76:1879-1884.

80. Hirsh J, Dalen J, Anderson DR, et al. Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. Chest. 2001;119:8S-21S.

81. Pauli RM, Lian JB, Mosher DF, Suttie JW. Association of congenital deficiency of multiple vitamin K-dependent coagulation factors and the phenotype of the warfarin embryopathy: clues to the mechanism of teratogenicity of coumarin derivatives. Am J Hum Genet. 1987;41:566-583.

82. Catella-Lawson F, Reilly MP, Kapoor SC, et al. Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. N Engl J Med. 2001;345:1809-1817.

83. Savi P, Zachayus JL, Delesque-Touchard N, et al. The active metabolite of Clopidogrel disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts. Proc Natl Acad Sci U S A. 2006;103:11069-11074.

84. Mehta SR, Yusuf S, Clopidogrel in Unstable angina to prevent Recurrent Events Study I. The Clopidogrel in Unstable angina to prevent Recurrent Events (CURE) trial programme; rationale, design and baseline characteristics including a meta-analysis of the effects of thienopyridines in vascular disease. Eur Heart J. 2000;21:2033-2041.

85. Lowe GD, Rumley A, Mackie IJ. Plasma fibrinogen. Ann Clin Biochem. 2004;41:430-440.

86. Kane WH, Davie EW. Blood coagulation factors V and VIII: structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders. Blood. 1988;71:539-555.

87. Minnema MC, Friederich PW, Levi M, et al. Enhancement of rabbit jugular vein thrombolysis by neutralization of factor XI. In vivo evidence for a role of factor XI as an anti-fibrinolytic factor. J Clin Invest. 1998;101:10-14.

88. Jalbert LR, Rosen ED, Moons L, et al. Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. J Clin Invest. 1998;102:1481-1488.

89. Wang X, Cheng Q, Xu L, et al. Effects of factor IX or factor XI deficiency on ferric chloride-induced carotid artery occlusion in mice. J Thromb Haemost. 2005;3:695-702.

90. Gailani D, Lasky NM, Broze GJ, Jr. A murine model of factor XI deficiency. Blood Coagul Fibrinolysis. 1997;8:134-144.

91. Hubbard WJ, Choudhry M, Schwacha MG, et al. Cecal ligation and puncture. Shock. 2005;24 Suppl 1:52-57.

92. Clauss A. [Rapid physiological coagulation method in determination of fibrinogen.]. Acta Haematol. 1957;17:237-246.

93. Ganopolsky JG, Castellino FJ. A protein C deficiency exacerbates inflammatory and hypotensive responses in mice during polymicrobial sepsis in a cecal ligation and puncture model. Am J Pathol. 2004;165:1433-1446.

94. Sun H, Wang X, Degen JL, Ginsburg D. Reduced thrombin generation increases host susceptibility to group A streptococcal infection. Blood. 2009;113:1358-1364.

95. Kerlin BA, Yan SB, Isermann BH, et al. Survival advantage associated with heterozygous factor V Leiden mutation in patients with severe sepsis and in mouse endotoxemia. Blood. 2003;102:3085-3092.

96. de StGroth SF, Scheidegger D. Production of monoclonal antibodies: strategy and tactics. J Immunol Methods. 1980;35:1-21.

97. Sun Y, Gailani D. Identification of a factor IX binding site on the third apple domain of activated factor XI. J Biol Chem. 1996;271:29023-29028.

98. Proctor RR, Rapaport SI. The partial thromboplastin time with kaolin. A simple screening test for first stage plasma clotting factor deficiencies. Am J Clin Pathol. 1961;36:212-219.

99. Baglia FA, Gailani D, Lopez JA, Walsh PN. Identification of a binding site for glycoprotein Ibalpha in the Apple 3 domain of factor XI. J Biol Chem. 2004;279:45470-45476.

100. Moons AH, Peters RJ, Bijsterveld NR, et al. Recombinant nematode anticoagulant protein c2, an inhibitor of the tissue factor/factor VIIa complex, in patients undergoing elective coronary angioplasty. J Am Coll Cardiol. 2003;41:2147-2153.

101. Gershwin ME, Gude JK. Deep vein thrombosis and pulmonary embolism in congenital factor VII deficiency. N Engl J Med. 1973;288:141-142.

102. Gailani D, Renne T. Intrinsic pathway of coagulation and arterial thrombosis. Arterioscler Thromb Vasc Biol. 2007;27:2507-2513.

103. Collet JP, Park D, Lesty C, et al. Influence of fibrin network conformation and fibrin fiber diameter on fibrinolysis speed: dynamic and structural approaches by confocal microscopy. Arterioscler Thromb Vasc Biol. 2000;20:1354-1361.

104. Mosnier LO, Bouma BN. Regulation of fibrinolysis by thrombin activatable fibrinolysis inhibitor, an unstable carboxypeptidase B that unites the pathways of coagulation and fibrinolysis. Arterioscler Thromb Vasc Biol. 2006;26:2445-2453.

105. Von dem Borne PA, Bajzar L, Meijers JC, Nesheim ME, Bouma BN. Thrombin-mediated activation of factor XI results in a thrombin-activatable fibrinolysis inhibitor-dependent inhibition of fibrinolysis. J Clin Invest. 1997;99:2323-2327.

106. Yang DT, Flanders MM, Kim H, Rodgers GM. Elevated factor XI activity levels are associated with an increased odds ratio for cerebrovascular events. Am J Clin Pathol. 2006;126:411-415.

107. Salomon O, Steinberg DM, Dardik R, et al. Inherited factor XI deficiency confers no protection against acute myocardial infarction. J Thromb Haemost. 2003;1:658-661.

108. Rosen ED, Gailani D, Castellino FJ. FXI is essential for thrombus formation following FeCl3-induced injury of the carotid artery in the mouse. Thromb Haemost. 2002;87:774-776.

109. Yamashita A, Nishihira K, Kitazawa T, et al. Factor XI contributes to thrombus propagation on injured neointima of the rabbit iliac artery. J Thromb Haemost. 2006;4:1496-1501.

110. Brenner B, Laor A, Lupo H, Zivelin A, Lanir N, Seligsohn U. Bleeding predictors in factor-XI-deficient patients. Blood Coagul Fibrinolysis. 1997;8:511-515.

111. Mannucci PM, Tuddenham EG. The hemophilias--from royal genes to gene therapy. N Engl J Med. 2001;344:1773-1779.

112. Peyvandi F, Mannucci PM. Rare coagulation disorders. Thromb Haemost. 1999;82:1207-1214.

113. Hanson SR, Griffin JH, Harker LA, Kelly AB, Esmon CT, Gruber A. Antithrombotic effects of thrombin-induced activation of endogenous protein C in primates. J Clin Invest. 1993;92:2003-2012.

114. Angliker H, Wikstrom P, Rauber P, Stone S, Shaw E. Synthesis and properties of peptidyl derivatives of arginylfluoromethanes. Biochem J. 1988;256:481-486.

115. Kasper CK, Aledort LM, Counts RB, et al. Letter: A more uniform measurement of factor VIII inhibitors. Thromb Diath Haemorrh. 1975;34:869-872.

116. Hanson SR, Harker LA, Bjornsson TD. Effects of platelet-modifying drugs on arterial thromboembolism in baboons. Aspirin potentiates the antithrombotic actions of dipyridamole and sulfinpyrazone by mechanism(s) independent of platelet cyclooxygenase inhibition. J Clin Invest. 1985;75:1591-1599.

117. Gruber A, Marzec UM, Bush L, et al. Relative antithrombotic and antihemostatic effects of protein C activator versus low-molecular-weight heparin in primates. Blood. 2007;109:3733-3740.

118. Harker LA, Marzec UM, Kelly AB, et al. Clopidogrel inhibition of stent, graft, and vascular thrombogenesis with antithrombotic enhancement by aspirin in nonhuman primates. Circulation. 1998;98:2461-2469.

119. Xu C, Wootton DM. Platelet near-wall excess in porcine whole blood in artery-sized tubes under steady and pulsatile flow conditions. Biorheology. 2004;41:113-125.

120. Fung YC. Biomechanics: Mechanical Properties of Living Tissues (ed 2nd). New York: Springer-Verlag; 1993.

121. Markou CP, Lutostansky EM, Ku DN, Hanson SR. A novel method for efficient drug delivery. Annals of Biomedical Engineering. 1998;26:502-511.

122. McCarty OJ, Calaminus SD, Berndt MC, Machesky LM, Watson SP. von Willebrand factor mediates platelet spreading through glycoprotein Ib and alpha(IIb)beta3 in the presence of botrocetin and ristocetin, respectively. J Thromb Haemost. 2006;4:1367-1378.

123. Sundell IB, Marzec UM, Kelly AB, et al. Reduction in stent and vascular graft thrombosis and enhancement of thrombolysis by recombinant Lys-plasminogen in nonhuman primates. Circulation. 1997;96:941-948.

124. Dichek DA, Anderson J, Kelly AB, Hanson SR, Harker LA. Enhanced in vivo antithrombotic effects of endothelial cells expressing recombinant plasminogen activators transduced with retroviral vectors. Circulation. 1996;93:301-309.

125. Hanson SR, Pareti FI, Ruggeri ZM, et al. Effects of monoclonal antibodies against the platelet glycoprotein IIb/IIIa complex on thrombosis and hemostasis in the baboon. J Clin Invest. 1988;81:149-158.

126. Bajzar L, Morser J, Nesheim M. TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. J Biol Chem. 1996;271:16603-16608.

127. Broze GJ, Jr., Higuchi DA. Coagulation-dependent inhibition of fibrinolysis: role of carboxypeptidase-U and the premature lysis of clots from hemophilic plasma. Blood. 1996;88:3815-3823.

128. Gruber A, Mori E, del Zoppo GJ, Waxman L, Griffin JH. Alteration of fibrin network by activated protein C. Blood. 1994;83:2541-2548.

129. Nenci GG, Parise P, Morini M, Rossini A, Agnelli G. Fibrin clots obtained from plasma containing heparin show a higher sensitivity to t-PA-induced lysis. Blood Coagul Fibrinolysis. 1992;3:279-285.

130. Nagashima M, Yin ZF, Zhao L, et al. Thrombin-activatable fibrinolysis inhibitor (TAFI) deficiency is compatible with murine life. J Clin Invest. 2002;109:101-110.

131. Broze GJ, Jr., Gailani D. The role of factor XI in coagulation. Thromb Haemost. 1993;70:72-74.

132. Renne T, Pozgajova M, Gruner S, et al. Defective thrombus formation in mice lacking coagulation factor XII. J Exp Med. 2005;202:271-281.

133. Pedicord DL, Seiffert D, Blat Y. Feedback activation of factor XI by thrombin does not occur in plasma. Proc Natl Acad Sci U S A. 2007;104:12855-12860.

134. Wiviott SD, Braunwald E, McCabe CH, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med. 2007;357:2001-2015.

135. Topol EJ, Moliterno DJ, Herrmann HC, et al. Comparison of two platelet glycoprotein IIb/IIIa inhibitors, tirofiban and abciximab, for the prevention of ischemic events with percutaneous coronary revascularization. N Engl J Med. 2001;344:1888-1894.

136. Cines DB, Blanchette VS. Immune thrombocytopenic purpura. N Engl J Med. 2002;346:995-1008.

137. McMillan R. Therapy for adults with refractory chronic immune thrombocytopenic purpura. Ann Intern Med. 1997;126:307-314.

138. George JN, el-Harake MA, Raskob GE. Chronic idiopathic thrombocytopenic purpura. N Engl J Med. 1994;331:1207-1211.

139. George JN. Platelets. Lancet. 2000;355:1531-1539.

140. Ly HQ, Kirtane AJ, Murphy SA, et al. Association of platelet counts on presentation and clinical outcomes in ST-elevation myocardial infarction (from the TIMI Trials). Am J Cardiol. 2006;98:1-5.

141. Thaulow E, Erikssen J, Sandvik L, Stormorken H, Cohn PF. Blood platelet count and function are related to total and cardiovascular death in apparently healthy men. Circulation. 1991;84:613-617.

142. Nikolsky E, Grines CL, Cox DA, et al. Impact of baseline platelet count in patients undergoing primary percutaneous coronary intervention in acute myocardial infarction (from the CADILLAC trial). Am J Cardiol. 2007;99:1055-1061.

143. Turakhia MP, Murphy SA, Pinto TL, et al. Association of platelet count with residual thrombus in the myocardial infarct-related coronary artery among patients treated with fibrinolytic therapy for ST-segment elevation acute myocardial infarction. Am J Cardiol. 2004;94:1406-1410.

144. Iijima R, Ndrepepa G, Mehilli J, et al. Relationship between platelet count and 30-day clinical outcomes after percutaneous coronary interventions. Pooled analysis of four ISAR trials. Thromb Haemost. 2007;98:852-857.

145. Moore S, Friedman RJ, Singal DP, Gauldie J, Blajchman MA, Roberts RS. Inhibition of injury induced thromboatherosclerotic lesions by anti-platelet serum in rabbits. Thromb Haemost. 1976;35:70-81.

146. Reyers I, Mussoni L, Donati MB, de Gaetano G. Severe thrombocytopenia delays but does not prevent the occlusion of an arterial prosthesis in rats. Thromb Haemost. 1981;46:558-560.

147. Matsunaga T, Tanaka I, Kobune M, et al. Ex vivo large-scale generation of human platelets from cord blood CD34+ cells. Stem Cells. 2006;24:2877-2887.

148. Proulx C, Boyer L, Hurnanen DR, Lemieux R. Preferential ex vivo expansion of megakaryocytes from human cord blood CD34+-enriched cells in the presence of thrombopoietin and limiting amounts of stem cell factor and Flt-3 ligand. J Hematother Stem Cell Res. 2003;12:179-188.

149. Tucker EI, Marzec UM, White TC, et al. Prevention of vascular graft occlusion and thrombus-associated thrombin generation by inhibition of factor XI. Blood. 2009;113:936-944.

150. Harker LA, Hunt P, Marzec UM, et al. Regulation of platelet production and function by megakaryocyte growth and development factor in nonhuman primates. Blood. 1996;87:1833-1844.

151. Hanson SR, Harker LA. Studies of suloctidil in experimental thrombosis in baboons. Thromb Haemost. 1985;53:423-427.

152. Barbui T, Buelli M, Cortelazzo S, Viero P, De Gaetano G. Aspirin and risk of bleeding in patients with thrombocythemia. Am J Med. 1987;83:265-268.

153. Andrew M, Castle V, Saigal S, Carter C, Kelton JG. Clinical impact of neonatal thrombocytopenia. J Pediatr. 1987;110:457-464.

154. Harker LA, Slichter SJ. The bleeding time as a screening test for evaluation of platelet function. N Engl J Med. 1972;287:155-159.

155. Ponczek MB, Gailani D, Doolittle RF. Evolution of the contact phase of vertebrate blood coagulation. J Thromb Haemost. 2008;6:1876-1883.

Curriculum Vitae

Erik Ian Tucker was born to Chris and Carolyn Tucker on August 13th, 1978 in Astoria, Oregon. Erik spent 5 years of his childhood in Ketchikan, Alaska, where he learned a love of the outdoors. He then returned to his place of birth where he graduated from Astoria High School in 1997. He then attended the University of Oregon in Eugene, where he studied biochemistry, earning his B.S. in 2001. After a brief stint as a wildland firefighter and as a post-baccalaureate student at Portland State University studying physics and engineering, he began his graduate career in 2004 in the Department of Biomedical Engineering at Oregon Health & Science University.

Over the course of his studies, Erik has traveled to a number of scientific meetings and was even invited to present an oral abstract at the 2007 American Society of Hematology meeting in Atlanta, GA. He was awarded a T32 NIH National Research Service Award in Molecular Hematology in 2008. Erik has two first-author and four co-author publication to date, and over 20 abstracts accepted to various scientific meetings. A list of his publications to date is included:

Publications

1., Kravtsov DV, Matafonov A **Tucker EI**, Sun M, Walsh PN, Gruber A, Gailani D. Factor XI Contributes to Thrombin Generation in the Absence of Factor XII. *Blood.* 2009. (In Press*)*

2. Tucker EI, Marzec UM, White TC, Hurst S, Rugonyi S, McCarty OJ, Gailani D, Gruber A, Hanson SR. Prevention of Vascular Graft Occlusion and Surface-Associated Thrombin Generation by Inhibition of Factor XI. *Blood*. 2009;113:936-944.

3. Tucker EI, Gailani D, Hurst S, Cheng Q, Hanson SR, Gruber A. Survival Advantage of Factor XI Deficient Mice in Peritoneal Sepsis. *J Infect Dis.* 2008;198:271-274.

4. White TC, Berny MA, **Tucker EI**, Urbanus RT, de Groot PG, Fernández JA, Griffin JH, Gruber A, McCarty OJ. Protein C Supports Platelet Binding and Activation Under Flow: Role of Glycoprotein Ib and Apolipoprotein E Receptor 2'. *J Thromb Haemost.* 2008;6:995-1002.

5. Berny MA, White TC, **Tucker EI**, Bush-Pelc LA, Di Cera E, Gruber A, McCarty OJ. Thrombin Mutant W215A/E217A Acts as a Platelet GPIb Antagonist. *Arterioscler Thromb Vasc Biol.* 2008;28:329-334.

6. Gruber A, Marzec UM, Bush L, Di Cera E, Fernández JA, Berny MA, **Tucker EI**, McCarty OJT, Griffin JH, Hanson SR. Relative Antithrombotic and Antihemostatic Effects of Protein C Activator Versus Low-Molecular-Weight Heparin in Primates. *Blood*. 2007;109:3733-3740.