

## **ABSTRACT**

**KYLE ALAN LANDSKRONER. Thrombosis and Thrombolysis: Emphasis on Hemophilia and Effect of Dissolution of Thrombi with Plasmin. (Under the direction of Dr. Neil C. Olson and Dr. Gary Jesmok.)**

The purpose of this research was to understand the mechanisms in which blood clots form and are degraded. The research studies examined the role of an endogenous protease, plasmin, which is found in all vertebrates, and the optimal doses of plasmin required to dissolve blood clots. When we investigated clot lysis with plasmin we examined blood clots from several species to which varying concentrations of plasmin were added, as well as varying methods of plasmin administration. The results of these studies not only highlighted important dose-response relationships of plasmin, but also demonstrated differences in the effect of human plasmin to dissolve blood clots compared to blood clot from the species tested. Porcine clots, in particular, were more resistant to lysis compared with human clots, while ovine clots had similar lysis compared with human clots. In addition, this research demonstrated that plasmin's effectiveness to lyse thrombi increases with an increase in clot surface area, e.g, by fragmentation, or when plasmin is administered as an intrathrombic administration. In separate studies, to investigate clot formation, we used mice that lack the FVIII protein. For these experiments we investigated the formation of blood clot formation using rotational thromboelastography (ROTEG) that measures the formation of fibrin in whole blood. This method was shown to be extremely sensitive to low levels of factor VIII protein and may have applications to classify particular phenotypes of hemophilia patients, or as a research tool to evaluate novel FVIII molecules.

**Thrombosis and Thrombolysis: Emphasis on Hemophilia and Effect of  
Dissolution of Thrombi with Plasmin**

by  
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## **Dedication**

I dedicate this dissertation to my parents, Charles and Sandra Landskroner, my brother, Kevin, my mother and father in-law, Rich and Ursula Klier, and most importantly my wife, Anne. Their support and sacrifices throughout my studies have made much of this possible.

## **Biography**

Kyle Alan Landskroner was born in Beaverton, Oregon on December, 13<sup>th</sup> 1967. He attended Beaverton High School and then the University of Oregon where he earned a Bachelor of Science Degree in Biochemistry. Following graduation, Kyle moved to Berkeley, California and began work with the Bayer Corporation. During this time Kyle studied to earn a Masters Degree in Clinical Science at San Francisco State University where he developed a rabbit model of thrombolysis for the study of plasmin as a thrombolytic. In 2001 the Bayer Corporation relocated Kyle and his wife from Berkeley to Raleigh, North Carolina to continue working on plasmin as a therapeutic for thrombotic diseases, and to enroll in a doctoral program at the College of Veterinary Medicine at North Carolina State University. Under the guidance of Dr. Gary Jesmok from Bayer and Dr. Neil Olson at NCSU, Kyle further developed the use of plasmin as a therapeutic, investigated differential lysis rates of plasmin among several species, and also developed an in vitro method to examine the role of low levels of FVIII added to mouse whole blood. This work culminated in three publications.

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made a great impression on my knowledge of imaging modalities and their use in research.

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## Abbreviations

A2AP	$\alpha_2$ -antiplasmin
A2M	$\alpha_2$ -macroglobulin
AC	Lower adenylyl cyclase
APC	Activated protein C
AT	Antithrombin
BSE	Bovine spongiform encephelopathy
COX- <u>  </u>	Cyclooxygenase enzymes ( <u>type</u> )
DSPA	<i>Desmodus rotundus</i> salivary derived PA
DVT	Deep vein thrombosis
EPCR	Endothelial protein C receptor
ET-1	Endothelin-1
ePTFE	Expanded polytetrafluoroethylene
HMWK	High molecular weight kinninogen
HSPG	Heparin sulfate proteoglycans
IP3	Inositol-trisphosphate
LMWH	Low molecular weight heparin
LT- <u>  </u>	Leukotriene ( <u>type</u> )
NO	Nitric oxide
PAF	Platelet-activating factor

PCI	Percutaneous interventions
PDE	Phosphodiesterases
PE	Pulmonary embolism
PG__	Prostaglandin ( <u>type</u> )
PS	Protein S
PTS	Post-thrombotic syndrome
scu-PA	Single-chain urokinase
SK	Streptokinase
tcu-PA	Two-chain urokinase
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
t-PA	Tissue-type plasminogen activator
TXA <sub>2</sub>	Thromboxane A <sub>2</sub>
UFH	Unfractionated heparin
u-PA	Urokinase plasminogen activator
VKOR	Vitamin K Epoxide Reductase
VTE	Venous thromboembolism
vWF	Von Willebrand Fact

## 1 Overview of Hemostasis

The ability of large multicellular organisms to form a fibrin plug to halt bleeding was a necessary adaptation for survival. Evolution from single cell organisms and invertebrates with low circulatory pressures to multicellular vertebrates with higher systemic pressures stressed the organism to adapt cellular and biochemical synergies that could clot blood under these conditions. The cellular and chemical factors, particularly platelets, coagulation proteins, and fibrinolytic proteins, enabled blood to clot regionally and then remodel the injured area through dissolution of these clots. Amino acid sequences of the coagulation proteins reveal that many of these proteins share common structures that impart slightly modified functions. While many of these proteins are present throughout invertebrates, it was the cleavage of fibrinogen via a thrombin-like protein approximately 430 million years ago that separated a relatively simple coagulation system to a more complex, highly regulated event (1-3). Furthermore, platelets, anuclear cells that participate in many functions during the initiation and propagation of clot formation and also release inflammatory mediators, are only found in mammals. Nonmammalian species have a similar nucleated “platelet-like” cell, e.g., hemocytes, amoebocytes, and thrombocytes, but are less specialized for hemostasis (4).

The complexity of coagulation results from numerous feedback loops and inhibitory mechanisms that balance coagulation and fibrinolysis. The understanding of how a limited amount of proenzymes and substrates convert large amounts of soluble fibrinogen to insoluble fibrin were landmark discoveries and cleverly described coagulation as a “cascade” in 1964 (5, 6). Ten years later the cascade was subsequently modified to include the observation of the tissue factor (TF) pathway, also known as the extrinsic pathway (7, 8).

The intrinsic and extrinsic pathway descriptions are frequently used to examine factor deficiencies and coagulopathies; however, the in vivo mechanisms for the initiation of clot formation occur predominantly via the tissue-factor mediated pathway. An overview of the pathway is outlined in Figure 1.1. Temporally, the cascade can be described by the generation of thrombin by both the overall amount and the speed in which it is generated. Two phases, the initiation phase and propagation phase, underscore the how, what, and where thrombin is generated and suppressed.

The initiation phase begins with FVIIa which is present in small quantities (approximately 1% of total FVII) in circulation. FVIIa has the interesting property of being relatively inert until it combines with membrane bound TF (expressed on fibroblasts, monocytes, and macrophages), whereby it undergoes a

conformational change and can activate circulating FX to FXa. The formation of TF:FVII:Xa has been termed the extrinsic Xase complex.

The next important event is the activation of FV to FVa. How FV becomes activated to Va is currently under investigation, but it is believed to occur via the direct activation of prothrombin (Factor II) to thrombin (Factor IIa) via FXa (9). Thrombin produced in this manner can activate membrane-bound FV to FVa on the surface of the tissue-factor bearing cell. This newly created FVa can then participate in prothrombinase complex (10). This complex combines FXa and FVa on the surface of the tissue-factor bearing cell to generate sufficient amounts of thrombin to activate platelets, convert Factor VIII to VIIIa, and will continue to convert additional amounts of Factor V to FVa to initiate the propagation phase of coagulation. Occurring simultaneously is the assembly of Factor IX on the TF:FVIIa complex (on the tissue factor bearing cell) which results in the conversion of Factor IX to IXa which can be transferred to nearby activated platelet.

On the platelet surface, assembly of Factor IXa with Factor VIIIa forms the intrinsic Xase complex which converts large amounts of Factor X to FXa. Approximately 50-100 times more Xa is formed during the intrinsic Xase complex compared to extrinsic Xase complex. This FXa can then be assembled

with FVa, to form the prothrombinase complex. The prothrombinase complex, now assembled on the platelet surface, can convert prothrombin to thrombin to produces 300,000 times more thrombin than Xa alone. In addition, the rate of thrombin generated is increased approximately 30-50 fold over a tissue-factor bearing cell (11, 12). This may be due to the combination of the unique platelet surface for factor assembly, the increased surface area of the platelet, and the number of activated platelets present compared to tissue-factor bearing cells. The thrombin formed can then cleave fibrinopeptides A and B from the fibrinogen molecule that results in the formation of fibrin protofibrils that ultimately stack and form crosslinked fibrin. Most, if not all, of these reactions assemble on, or near, phospholipids, i.e., phosphatidylserines, provided by the platelet (13, 14) and are required *a priori* for efficient conversion of the coagulation enzymes and substrates. These surface reactions and binding to cofactors are responsible for increasing activation rates by 100,000 fold, or greater (15). These reactions are not only dependent on the type of phospholipid but also on the source as anyone who has had the opportunity to work with clotting assay reagents can attest (16-18).



receptors (also known as the  $\alpha_{IIb}\beta_3$  receptor). Upon binding, the platelet undergoes shape changes within the cytoskeleton and storage granules release ADP, ATP, histamine, thromboxane A<sub>2</sub> (TXA<sub>2</sub>), epinephrine, serotonin, and other vasoactive substances. The release of the granule contents augments platelet aggregation in a positive-feedback loop as these substances not only recruit platelets to the site of injury, but platelets have receptors for these substances that cause shape change and degranulation. In particular, both thrombin and ADP are potent agents for platelet aggregation via the PAR1-4 receptors for thrombin and the P2Y<sub>1</sub> and P2T<sub>AC</sub> receptors for ADP (22).

In addition to platelets, endothelial cells also play a major role in hemostasis. Intact endothelium helps to maintain patency of blood vessels by serving as a barrier to the procoagulant surfaces that lie beneath it, thus limiting access and permeability to procoagulant proteins. In addition, the endothelial cells produce antithrombotic substances, particularly nitric oxide (NO), that regulate vasodilation and antiplatelet activities (23-25), endothelin-1 (ET-1) that is a potent vasoconstrictor (26), and PGI<sub>2</sub>, a prostaglandin with potent antiplatelet activity (27). Damaged endothelium exposes the underlying collagen, vitronectin, and fibronectin, components of the subendothelium which act as surfaces for platelets to bind. Endothelial cells also have thrombin receptors and

regulate leukocyte adhesion, release of plasminogen activator inhibitor (PAI-1), and proliferative responses such as angiogenesis (28, 29).

Admittedly, absent from these descriptions of the coagulation cascade are the roles of the proteins designated as part of the intrinsic coagulation cascade. Currently, we define the importance of a coagulation protein on what happens in its absence. For example, in Hemophilia A the absence of the FVIII protein results in a bleeding phenotype, or mutations in Factor V (Factor V Leiden mutations) are associated with an increased risk of thrombosis. In contrast, loss of kinninogen, FXII, or prekallekrein does not result in a coagulopathy. While homozygous individuals for FXI deficiency can have severe bleeding tendencies, the absolute level of FXI is not predictive of bleeding (30, 31). In addition, the intrinsic system, also called the contact-activation system is not necessary for the in vivo generation of thrombin and both factors XI and IX can be directly activated by thrombin via the TF:FVIIa complex. Interestingly, while the lack of protein may not manifest into a coagulation phenotype there is some evidence that overproduction of these proteins may result in thrombosis. In particular, increased plasma concentrations of FXIa and FXIIa may be associated with an increase in coronary artery thrombosis (32-34). In addition, FXI has been shown to play a role in protecting thrombi from fibrinolysis. Therefore, it is seductive to believe that these proteins may not necessarily contribute to the

acute formation of thrombi, but may have a more insidious role to increase the potential for thrombosis and/ or for clot remodeling.

### **1.1 Specific Role of FVIII**

Many of the cellular surfaces act as a template for coagulation factors to assemble. One such cofactor, FVIII, does not possess any enzymatic function, but helps coordinate the assembly of FIXa on the phospholipid membrane to convert large amounts of FX to FXa. Loss of functional activity of the FVIII molecule results in increased bleeding from vasculature that cannot easily be sealed with platelets and is manifested in Hemophilia A. These patients, depending on the severity of the disease, can have <1% of normal FVIII levels. Patients with Hemophilia A have normal template bleeding times, a measurement that involves a very shallow incision into the forearm of a patient, yet have reoccurring bleeding into the joints, unusually large blood loss after tooth extraction procedures, and internal bleeding from typically non-traumatic injuries. The differences in bleeding events may relate to the relative importance of platelets, or in the local milieu of coagulation proteins and cell types when sealing an injured blood vessel. Small, low-pressure wounds can be sufficiently controlled with platelet activation, vasoconstriction, and moderate fibrinogen conversion; whereas, large, higher-pressure wounds require platelets and sufficient fibrin-fibrin and fibrin-platelet cross-linking to seal the wound. Thus,

patients with hemophilia are capable of forming clots, but the clots take longer to form which is due to the decrease in thrombin generation in the propagation phase of coagulation (35, 36). Additionally, FVIIIa activity in vivo is predicated on the exposure of a phospholipid surface either from activated platelets or damaged endothelial cells since the release of FVIIIa from its carrier protein, vWF, does not occur unless the complex is first bound to a phospholipid surface (37, 38).

Factor VIII is a large protein that is synthesized with 6 domains (A1:A2:B:A3:C1:C2) and then secreted from hepatocytes. Upon secretion the molecule is processed to a heavy (90-200 KDa) and light chain (80 KDa) linked together through a calcium ion bridge and is bound to vWF (39). Upon proteolytic cleavage by thrombin, or to a lesser extent factor Xa, the FVIII molecule dissociates from vWF and enables the FVIIIa molecule to bind to activated platelets and to complex with FIX. In addition, thrombin removal of the remaining B region also occurs (40). The B-region is not required for coagulant activity and both the full-length and B-deleted forms of FVIII have been produced by recombinant technology for commercial production. The FVIII molecule is relatively unstable in plasma and spontaneously dissociates, or is inactivated by activated protein C (APC) when not bound to vWF. This can be seen in vWF deficient animals as clearance of FVIII is increased by 80% (41). FVIII bound to

vWF is unable to bind phospholipid or platelets and is protected from APC inactivation (42). However, vWF does bind platelets and collagen and the multimeric forms allow the tethering of vWF from these surfaces. This is especially important for the formation of blood clots under flow conditions where the vWF serves as an anchor for factor assembly and platelet binding. Therefore, vWF plays an integral role in FVIII activity, by directing it to sites of injury and protecting it from degradation.

## **1.2 Specific Role of Fibrinogen**

Clot formation culminates with the formation of insoluble fibrin from the limited proteolysis of fibrinogen. Fibrinogen is a large protein ( $M_r$  340,000) that consists of two copies of three subunits ( $\alpha$ ,  $\beta$ , and  $\gamma$ ). At a concentration of approximately 3 mg/ml (5  $\mu$ M) it is the most abundant coagulation protein and was one of the first molecules visualized by electron microscopy (EM). The “dumbbell” appearance by EM is due to the  $B\beta$  and  $\gamma$  chains at each end of the molecule, which form the D domains and a central N terminus globular domain that forms the E domain. Fibrin formation occurs when thrombin specifically, and sequentially, hydrolyzes the Arg-Gly bonds on the  $A\alpha$  or  $B\beta$  chains of fibrinogen. The release of fibrinopeptide A on the fibrinogen molecule allows D domains on two fibrinogen molecules to overlap. This event forms a protofibril that, upon further cleavage of fibrinopeptides A and B, begins to band together, stack, and elongate profibril chains. These chains result in the formation of fibrin

strands. Also of importance in the protofibril extensions are the crosslinking reactions that occur simultaneously with fibrinopeptide A release. FXIII, after conversion to FXIIIa by thrombin, is a transglutaminase that covalently links adjacent fibrin strands and increases the integrity of the clot and resistance to proteolytic degradation.

### **1.3 Inhibitors of Coagulation Proteins**

Concomitant with coagulation are the mechanisms of anticoagulation and fibrinolysis. The exquisite balance of this system is necessary for maintaining normal hemostasis; however, during and following injury the balance shifts between these three processes. If the injury is too severe, resulting in an acutely irreversible propensity to clot or bleed, pharmacologic and/or surgical intervention may be required. In the absence of a severe injury, five molecules in particular have important roles in controlling unrestrained production of thrombin: tissue factor pathway inhibitor (TFPI), antithrombin (AT), APC, and thrombomodulin (TM).

Recalling the reactions outlined in Figure 1.1 we can see that there are several instances whereby thrombin is formed. Initially, the generation of small amounts of thrombin is initiated by the TF:FVIIa:Xa complex. These reactions are inhibited by the protein, tissue factor pathway inhibitor (TFPI) (43). TFPI is a

protease inhibitor, primarily expressed from microvascular endothelial cells and megakaryocytes, which binds and inactivates Xa and VIIa through two separate Kunitz domains. TFPI circulates while bound to lipoproteins or endothelial cells, and the lung and heart appear to contain the greatest levels of TFPI (44, 45). Interestingly, increases in the levels of TFPI occur during inflammation or shear stress; however, its level is unaffected by TNF-alpha, endotoxin or IL-1(46, 47). The role of TFPI in regulation of TF:VIIa:Xa generation of thrombin is unclear as in vitro experiments have demonstrated that increasing TFPI by 150% had no impact on thrombin generation, while decreasing the TFPI to 50% of normal had a modest shortening of clotting times (48). Further complicating the role of TFPI is that patients with significantly altered levels of TFPI do not have deranged bleeding or thrombosis (49), while in vivo animal modeling has demonstrated that administration of TFPI significantly reduces DIC in sepsis and models (50, 51). Clearly, the role of TFPI alone and in conjunction with other thrombin inhibitors requires further investigation in our understanding of thrombin generation in disease. This may be due to the limited thrombin generated by the extrinsic tenase pathway compared to the 50-fold increase in thrombin generated by the intrinsic tenase complex. Thus, while initiation of clotting is regulated by TFPI, propagation and amplification appear to be unaffected (52).

Alternatively, AT, a serine proteinase inhibitor, not only inhibits thrombin, but also inhibits FIXa, FXa, FXIa, FXIIa, high molecular weight kinninogen (HMWK), and the TF:FVIIa complex. Because of AT's ability to inhibit many of the coagulation proteins, and at many points in the cascade, it is an important regulator of the coagulation cascade. However, AT by itself is a relatively weak inhibitor of thrombin and is dependent on cofactors and forms a ternary complex with the cofactor (heparin sulfate proteoglycans (HSPG) and the coagulation factor. This is then followed by the dissociation of the heparin molecule after factor inhibition (53, 54). The HSPG cofactor augments the activity of AT approximately 100 fold, but requires a pentasaccharide motif that must be a minimum of 18 subunits in length (55). The requirement of the HPSGs for AT's inhibition of thrombin is also the mechanism by which heparin is used pharmacologically as an anticoagulant. AT primarily regulates free thrombin and FXa since fibrin bound thrombin or bound FXa is protected from AT inhibition (56). This feature is important as AT prevents indiscriminant fibrin formation and accretion of existing thrombi.

Other important inhibitors of the coagulation system are thrombomodulin (TM), activated Protein C (APC), and protein S (PS). During the activation of prothrombin to thrombin, via the FVa:FXa (prothrombinase) complex, excess thrombin formed can combine with a transmembrane protein, TM. The complex

of thrombin and TM changes thrombin's role from acting as procoagulant to anticoagulant. This change in activity is accomplished through the complex's activation of PC to APC (activated PC). The importance of thrombomodulin in this reaction increases thrombin's activation of PC by 1000 fold, and a further increase in the formation of APC occurs in the presence of calcium  $Ca^{+}$ , and when PC is bound to the endothelial protein C receptor (EPCR) (57). Once APC dissociates from EPCR it can then bind to Protein S (PS) to inhibit to FVa and FVIIIa through limited proteolysis. Regulation of APC occurs through its inhibition by  $\alpha_1$ -proteinase inhibitor, protein C inhibitor, or  $\alpha_2$ -macroglobulin (A2M).

Clearly, the regulation of thrombin formation and APC formation are highly regulated events that are governed by their relative dissociation constants and clearance mechanisms. When thrombin is elevated the TM:APC system is operational and when the TM:APC concentration increases sufficiently it is then inhibited by proteinase inhibitors (58). The above inhibitors are highlighted in Figure 1.3.1.



exists. Central to the dissolution of the thrombi is an enzyme, plasmin. Plasmin is formed from the zymogen (plasminogen) after activation by a plasminogen activator, e.g., tissue-type plasminogen activator (t-PA), urokinase plasminogen activator (u-PA). Plasminogen circulates as glu-plasminogen at a concentration of 2.4  $\mu\text{M}$  (216  $\mu\text{g/ml}$ ) and upon hydrolysis of the Arg561-Val562 peptide bond by t-PA, or u-PA, the two-chain plasmin molecule is formed. During this activation glu-plasminogen, the primary form in circulation, is converted to lys-plasminogen, and then to lys-plasmin. The lys-plasminogen can be activated to plasmin at 20 times the rate of glu-plasminogen. Lys-plasmin carries out the truncation of glu-plasminogen to lys-plasminogen thereby increasing its own rate of formation (59).

Plasmin is a relatively non-specific protease and degrades fibrin, or other proteins such as fibrinogen, FV, and FVIII on the carboxyl side of a lysine-arginine bond. In addition, the platelet receptors, GPIIb/IIIa, and Ib, can also be diminished by plasmin, either through proteolysis or by promoting internalization (60, 61). Some specificity is conferred by five Kringle (K) regions, named for their structural similarity to the pastry, which provide affinity to lysine binding sites on target molecules (62); however, many proteins contain lysine binding regions. Because of plasmin's affinity for a variety of proteins its free concentration is highly regulated. In fact, plasmin does not normally exist freely

in circulation as it is rapidly inhibited by  $\alpha_2$ -antiplasmin (A2AP), a reaction that has been described as one of the fastest reactions recorded at greater than  $1 \times 10^7$  M/s (63, 64). The regulation of plasmin is primarily by A2AP, but another proteinase inhibitor, A2M, also provides a back-up mechanism for plasmin inhibition. These inhibitors are present in large amounts in the circulation, for example: A2M is present at 3  $\mu$ M and A2AP is present at 1  $\mu$ M. Therefore, if all of the plasminogen was converted to plasmin (approximately 2.4  $\mu$ M), there would be sufficient buffering capacity to neutralize free plasmin since inhibition by these inhibitors occurs in a 1:1 stoichiometry. Importantly, when plasmin is bound to fibrin it is protected from inhibition from its cognate inhibitor, thus permitting uncontested degradation of fibrin.

Fibrin degradation occurs processively as it binds, degrades, and exposes new sites for other plasmin molecules to bind. The degradation of fibrin by plasmin is also believed to occur laterally across fibrin and in a random fashion. The result is a transection of fibers that produces short pieces of fibrin, but with unchanging width (65). However, there have also been studies that suggest that plasmin degrades in an outside-in fashion that degrades individual fibers to produce a thin fiber from a thicker fiber (66). It is plausible that the two are not mutually exclusive and may reflect the continual reorganization of the fibrin strands with time. The degradation of the strands also is regulated by levels of thrombin

activated fibrinolysis inhibitor (TAFI) which binds newly created carboxyl-terminal lysines during plasmin digestion. TAFI, activated by both thrombin and plasmin (and also degraded by plasmin) inhibits lysis of fibrin by preventing plasmin(ogen) from binding to recently digested fibrin (67). Experimentally, TAFI has been shown to inhibit lysis by t-PA, u-PA, streptokinase (SK), and DSPA (*desmodus rotundus* salivary derived PA) a recombinant form of the enzyme in bat saliva (68). Therefore, many complex mechanisms exist to regulate plasmin's activity in terms of not only preventing non-specific degradation of proteins, but also in limiting its role in fibrinolysis.

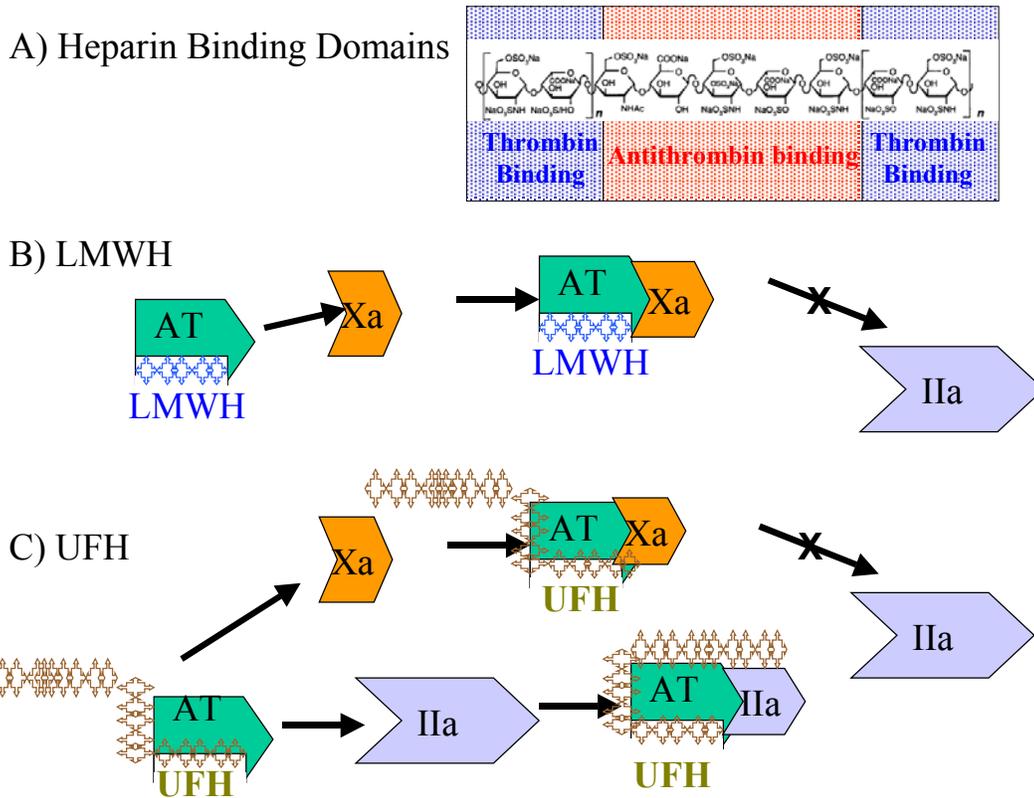
## **1.5 Common Drugs to Modulate Hemostasis: Anticoagulants**

### **1.5.1 Indirect Thrombin Inhibitors:**

Indirect thrombin inhibitors exert their pharmacologic actions by augmenting AT's affinity for thrombin or Xa. AT is a potent inhibitor to unbound thrombin and Xa, but only when combined with heparan sulfate molecules on endothelial cells, unfractionated heparins (UFH), size excluded heparins, also known as animal derived low molecular weight heparins (LMWH), e.g., enoxaprin, dalteparin, ardeparin, or synthetic heparins, e.g., fondaparinux. All of these compounds form an interaction with AT that increases AT's ability to neutralize thrombin, Xa, or both.

Heparin was found to have potent anticoagulant activities in 1916, well before it was determined that its cofactor was AT in 1968 (54, 69). Derived from bovine or porcine intestines, UFH possess differing chain lengths of heparin sulfate glycosaminoglycans. The reason for the differing sizes is due to the enzymatic cleavage of these chains during the manufacturing process that produces fragments from 7 KDa to 25 KDa, whereas, LMWH have a narrower spectrum of fragments that are between 4-6 KDa (70). Additionally, for UFH one-third of these heparin fragments are pharmacologically active as compared to 15-25% of LMWH. To exhibit potent antithrombin properties a unique pentasaccharide sequence and another thirteen saccharide units are required (Figure 1.5.1). These long chains are needed to essentially “wrap” around the AT:thrombin complex to prevent dissociation. AT and heparin also inhibits Xa, but the requirement for a longer heparin chain is not necessary. Only the shorter pentasaccharide sequence is necessary when AT and Xa are in a complex. Thus, because the LMWH contain very few of the longer 18 unit length chains compared to UFH they function primarily as Xa inhibitors; whereas, UFH function as both Xa and thrombin inhibitors.

Figure 1.5.1 Heparin Molecule and Binding Domains



The primary advantage for using UFH is cost, while the advantages of LMWH are several. It is generally accepted that LMWH, because of their increased bioavailability (100 % vs 30%) and increased half-life (2-4 fold) compared to UFH, have a more predictable therapeutic profile which eliminates the frequent monitoring associated with UFH therapy. LMWH also have less affinity for platelets which diminishes platelet activation and aggregation (71-74). In

addition, LMWH have a decreased affinity for platelet factor 4 (PF4), the relevance of which is discussed below, but is related to the incidence of heparin induced thrombocytopenia (HIT) that is seen more often with UFH than LMWH.

While the mechanisms of HIT are still poorly understood the autoantibodies produced are against PF4 epitopes. Recent studies suggest that during heparin infusions there is an increase in PF4 secretion from  $\alpha$ -granules in platelets into plasma. Heparin, and/or heparan sulfates on the endothelium, bind to PF4 to form complexes that elicit antibody formation (75). The result is a decrease in platelet counts by 50% and a paradoxical thrombotic event, e.g. deep vein thrombosis (DVT), caused by the activation of platelets. Most of the thrombotic episodes occur on the venous side and are most likely due to the combination of decreased flow and common interventional procedures that damage the lumen of the vessel. Importantly, a decrease in platelet count alone is not a diagnosis for HIT and, therefore, the diagnosis is considered a clinicopathologic syndrome (76). The risk of HIT with LMWH is approximately 10 fold less compared to UFH. Furthermore, bovine lung derived heparin carries an equal or slightly greater risk to induce HIT compared to heparin derived from porcine intestinal sources. However, with the increase in bovine spongiform encephelopathy the use of bovine heparins has been discontinued (77-80).

Still, another feature on the use of both UFH and animal derived LMWH for anticoagulation is that their effects can be reversed with the use of protamine sulfate. The highly positive protamine forms a complex with heparin and prevents heparin's binding to AT. While there are side effects with the use of protamine, ranging from hypotension to fatal cardiac arrest (approximately 10% and 1% of patients treated, respectively), the ability to reverse these drugs is an attractive feature for surgical candidates who may be excluded due to the risk of bleeding (81).

Other indirect thrombin inhibitors include the synthetic heparins (fondaparinux, idraparinux). These molecules are chemically synthesized pentasaccharides that have increased affinity for AT and are >94% bound to AT in plasma (82). The clinical efficacy for synthetic LMWH versus the animal derived LMWH was significantly greater in postoperatively treating hip fracture surgeries, elective knee replacement surgery, and major orthopaedic surgery (83). Interestingly, the use of protamine sulfate as a reversal agent for synthetic LMWH is ineffective, but FVII can be used as a "bypass" agent to initiate coagulation. The newer LMWH, e.g., idraparinux, have been classified as meta-pentasaccharides and have increased affinity for AT and longer half-lives than their pentasaccharide counterparts (17 vs 80 hrs). While the latter may be attractive

for chronic anticoagulation, its use for surgical candidates, especially those with renal disease, would be problematic as no reversal agent can be administered.

Assay detection for monitoring of LMWH is another consideration for their use. Unlike UFH which is monitored with an aPTT test to monitor anticoagulation, the LMWH do not cause a prolongation in the aPTT times (at therapeutic plasma concentrations). This would appear to be paradoxical, since without Xa activation there should be little, if any thrombin generated. However, it can be explained when one considers whether Xa or thrombin is bound. When Xa is bound to phospholipid it is protected from inhibition by LMWH *and* AT; however, since AT can also bind free thrombin the aPTT is prolonged (84). In these assay systems the amount of Xa formed is bound and protected from inhibition, thus clotting can occur. Therefore, while use of LMWH requires less monitoring than the UFH, a Xa assay must be used to monitor anticoagulation (85, 86). The use of fondaparinux has been evaluated in orthopaedic surgery patients enrolled in several phase III trials for thromboprophylaxis and demonstrated a 55% reduction in venous thromboembolism (VTE) compared to enoxaprin. In addition, fondaparinux compared to placebo reduced the incidence of VTE in patients with hip-fractures and also decreased their rate of DVT within one month of surgery (35% vs 1.4%) (87).

### **1.5.2 Direct Thrombin Inhibitors**

Direct thrombin inhibitors are new class of compounds that bind specifically to thrombin and not Xa. Thrombin, in addition to its catalytic site, has two sites that are available for binding, the first, termed exosite I, is responsible for binding fibrin, while exosite II is a heparin binding site. When thrombin is bound to fibrin, heparin (or HSPG) molecules can bind to exosite II. This interaction increases the affinity of thrombin for fibrin. Because exosite I is now occupied by fibrin and exosite II is occupied by HSPG, exogenously administered heparin (LMWH or UFH) in complex with AT cannot bind to exosite II. This effectively prevents AT from inhibiting bound thrombin. Hirudin derivatives (lepirudin, desirudin) from the medicinal leech, *Hirudo medicinalis*, are bivalent thrombin inhibitors that can bind to exosite I and the catalytic site. When hirudin binds to thrombin it can displace thrombin from fibrin. Thus, in contrast to LMWH and UFH, hirudin can inhibit thrombin bound fibrin (88, 89). Another type of direct thrombin inhibitor, argatroban, binds directly to the catalytic site of thrombin to inhibit its activity; therefore, because it acts independently of the exosites it also binds bound thrombin. The relative inhibition constants (dissociation constants) for hirudin and argatroban are 0.1-2.3 pmol/L and 0.4  $\mu$ mol/L, respectively, indicating that hirudin is a more powerful inhibitor of thrombin than argatroban (90). Currently, the FDA has approved the direct thrombin inhibitor class for use

in HIT patients. Several trials have attempted to demonstrate superiority to heparin in reducing event-free survival (no death, coronary artery bypass grafting, stenting, or non-fatal MI) in non-ST-segment elevation and ST-segment elevated myocardial infarction patients; however, none have convincingly reached this endpoint.

The above mentioned direct thrombin inhibitors are all administered via the intravenous route; however, two compounds have currently been developed for oral administration. The first compound, ximelagatran, was approved in France for prevention of VTE in orthopaedic surgery patients; however, it was not approved in the US due to increased hepatotoxicity. The second compound, currently in clinical trials is dabigatran etexilate. This small molecule demonstrated a significant reduction in the prevention of VTE in patients undergoing total hip or knee replacement surgeries compared to enoxaparin. At the efficacious dose there was not a significant increase in liver enzymes and bleeding events; therefore, the drug appears to have a better therapeutic profile, with the tested doses, and further dose refinements may result in further safety benefits (91).

### **1.5.3 Vitamin K Antagonism**

Post-translational modifications of several coagulation proteins are required for their activity. Arguably, the most important of these modifications is the carboxylation of the  $\gamma$ -carbon of all glutamyl residues to form “Gla domains” by adding a carboxyl group to the amino acid as the protein moves through the endoplasmic reticulum. This reaction is carried out by a vitamin K-dependent carboxylase, and is important for the affected protein’s binding of  $\text{Ca}^{2+}$ , which helps determine conformational changes for activity and also allows their insertion into membranes (92, 93). The reduced form of vitamin K, which acts as a cofactor for the carboxylase, is continually recycled with the help of the Vitamin K Epoxide Reductase (VKOR) (94). This reductase is sensitive to warfarin and is the pharmacologic basis for warfarin’s mechanism of action. Other pathways capable of producing a reduced form of vitamin K are present and can overwhelm the contribution of VKOR when there is an increase in dietary intake of vitamin K. Therefore, patients on warfarin therapy are advised not to eat vegetables containing vitamin K as it can promote anticoagulation reversal. Prothrombin, FVII, FIX, FX, PC and PS are all vitamin K-dependent proteins, but the range of vitamin K-dependent proteins is not limited to the coagulation proteins of mammalian species as they have been found in fish, cone snails, and snake venoms, and also play roles in bone morphogenesis (93).

As mentioned above, warfarin, or other coumarins, is used to inactivate the VKOR which prevents the recycling of the vitamin K. However, this mechanism was not known when the coumarins were first developed in 1922 when a hemorrhagic disease affected cattle that ate spoiled sweet clover.

Approximately nine years later it was identified that these animals' hemorrhagic diatheses was due to a deficiency in prothrombin. Furthermore, it wasn't until 1978 that it was determined that coumarin's role was on vitamin K function (95). Surprisingly, the first uses for coumarins were as rat poisons and it was during the development to try and find a superior poison that warfarin (3-phenylacetyl ethyl, 4 hydroxycoumarin) was developed by Karl Link, who patented the agent in 1946. Warfarin was named for the owner of the patent, the Wisconsin Alumni Research Foundation.

#### **1.5.4 Platelet Inhibition**

Platelets, as described above, are important regulators in initiating and sustaining coagulation as they become intertwined within the thrombus framework. In addition, they also possess mechanisms to control coagulation as they respond to environmental stimuli. Therefore, it is not surprising that platelets have been targets for the development of anticoagulant therapies. Receptors on the platelet surface respond to specific ligand interactions and changes in blood flow that can trigger the secretion of vasoactive substances, or

ligation of these receptors can induce cytoskeletal shape changes promoting aggregation. In addition, the platelet can also be quiescent through separate, but similar ligand interactions and signaling events. Three of the most common pathways of platelet signaling are: (1) G-protein activation followed by the release of intracellular pools of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum via phospholipase C stimulation; (2) G-protein stimulation or inhibition of adenylyl cyclase resulting in an increase or decreases in cAMP, respectively; (3) and the activation of GPIIb/IIIa and GPIb/IX/V receptors that interact with either fibrin, or collagen and vWF, to increase platelet adherence and aggregation.

Activation of the G-protein molecules can be accomplished by a variety of soluble agonists. In particular, TXA<sub>2</sub> can bind the stimulatory TP receptor coupled to the G<sub>q</sub> transmembrane protein. Activation of G<sub>q</sub> stimulates phospholipase C, which increases inositol-trisphosphate (IP<sub>3</sub>), and cytoplasmic  $\text{Ca}^{2+}$  concentrations. Epinephrine binds to the  $\alpha_2$  receptor on platelets to stimulate the G<sub>i</sub> receptor. The activation of the G<sub>i</sub> receptor decreases adenylyl cyclase (AC) concentrations and decreases intracellular cAMP which stimulates platelet aggregation. The primary function of epinephrine appears to potentiate the actions of other agonists. Additionally, endothelial cells, via the production of PGI<sub>2</sub> and nitric oxide (NO), utilize the AC pathway to raise cAMP within the platelet (96). This pathway utilizes PKA and the catalytic units that dissociate

from this complex ultimately phosphorylate proteins that inhibit platelet activation. Phosphodiesterases (PDE), which degrade cAMP, have also proven to be effective targets through the use of PDE inhibitors for inhibiting platelet aggregation (e.g., dipyridamole, methylxanthines, cilostazol). Finally, following exposure to thrombin, ADP, epinephrine, or TXA<sub>2</sub>, there is an increase in ligand binding (e.g., vWF, fibrinogen) to the GPIIb/IIIa and GPIb receptors. In addition to this “outside-in” mechanism of signaling, there can also be “inside-out” signaling since these adhesion proteins have both an extracellular domain and cytoplasmic domain that are capable of regulating signals (97). The most studied receptor system for “inside-out” signaling is the GPIIb/IIIa receptor. Upon platelet activation, the receptor increases its affinity for fibrinogen and vWF. The clustering of this receptor to other GPIIb/IIIa receptors results in Syk signaling, while the clustering and ligand binding, e.g., fibrinogen, is required for FAK signaling events. Syk and FAK both phosphorylate other signaling proteins to promote platelet spreading on an exposed collagen surface (98).

In the prevention of thrombosis the goals for platelet therapy are to prevent activated platelets from binding exposed collagen and fibrin, or to passivate the platelet, so that its response to stimuli is muted. Several popular approaches are: cyclooxygenase inactivation, e.g., acetyl-salicylic acid (aspirin); GPIIb/IIIa antagonism, e.g., abciximab, eptifibatid, or tirofiban; ADP receptor antagonists,

e.g., thienopyridines (clopidogrel, ticlopidine); agents that increase cAMP, e.g., dipyridamole, cilostazol.

### **1.5.5 Cyclooxygenase Inhibition**

The mechanisms of these drugs are important in understanding their limitation as therapeutics. For acetylsalicylic acid (aspirin), which was first discovered in the 1850's and used as an antipyretic and analgesic, its platelet inhibitory effects are through the selective and irreversible modification of the cyclooxygenase enzymes (COX-1 and COX-2). By making this modification, the enzyme is inhibited and prostaglandin biosynthesis is greatly reduced. Although arachidonic acid metabolism to PGI<sub>2</sub>, and the prostaglandins (PGE<sub>2</sub>, D<sub>2</sub>, and F<sub>2</sub>) are inhibited, the primary effects on platelets is the inhibition of TXA<sub>2</sub> that inhibits platelet aggregation. In addition, the effects of aspirin on the TXA<sub>2</sub> last the lifetime of the platelet since the anuclear platelet cannot synthesize more COX-1. Importantly, while aspirin inhibits endothelial production of PGI<sub>2</sub>, an inhibitor of platelet aggregation, the endothelial cells will subsequently regain the ability to synthesize PGI<sub>2</sub> since, unlike platelets, they contain a nucleus. Thus, the risk of thrombosis while on aspirin therapy is mitigated (99). Because platelets can aggregate after exposure to several agonists, not just TXA<sub>2</sub>, the effect of aspirin on platelet inhibition is relatively weak. Therefore, much

research has gone into the development of pharmacologic agents that can inhibit platelet aggregation pre and post exposure to agonists.

### **1.5.6 GPIIb/IIIa Inhibition**

The GPIIb/IIIa antagonists have been a primary target for platelet inhibition because this receptor plays a large role in the final aggregation of platelets. Their mechanism, either by blockade of the receptor with peptides or antibodies, relies on the prevention of fibrinogen to ligate receptors. In essence, fibrinogen, which normally acts as cement between the two platelets, is excluded from the ligand. Three molecules that have been developed for this purpose are abciximab, eptifibatide, and tirofiban. Abciximab (c7E3) is a murine-derived monoclonal antibody against the GPIIb/IIIa receptor, but unlike tirofiban and eptifibatide it also has affinity for the  $\alpha_v\beta_3$ , and endothelial integrin involved in angiogenesis (100). Two major clinical trials, the Evaluation of 7E3 for the Prevention of Ischemic Complications (EPIC, 1994) and the Evaluation of PTCA to Improve Long-Term Outcome with Abciximab GPIIb/IIIa blockade (EPILOG, 1997), were designed to test the efficacy and safety of abciximab (101, 102). The EPIC trial demonstrated that a bolus plus infusion versus a bolus dose alone resulted in a decrease in death, myocardial infarction (MI), and unplanned percutaneous interventions (PCI), in patients compared to placebo. An additional heparin group was included in these studies, but all patients received

heparin and aspirin during and again at 12 hours. There was an increase in bleeding events in this trial, but it was unknown whether this was due to the additional administration of heparin. To address this question, the EPILOG trial compared three treatment regimens, an abciximab bolus followed by infusion and low-dose heparin (75 U/kg followed with additional boluses to maintain an ACT=200), heparin alone (100 U/kg) with additional boluses to maintain an ACT=300), versus low-dose heparin and placebo. The doses of heparin used in this trial were important to discern whether abciximab was the culprit for increasing bleeding events. This trial demonstrated that abciximab with heparin significantly reduced the primary endpoints: death, non-Q wave MI, and the need for urgent revascularization within 30 days. A concern with these agents is the bleeding events, both minor, e.g., at catheter insertion sites, and major, e.g., decreases in Hg or HCT that require blood transfusions, or intracerebral hemorrhages. In the EPIC trial these agents demonstrated significantly more minor bleeding compared to placebo; however, in the larger (N=2792 patients) EPILOG trial there was no differences in minor bleeding and major bleeding events were comparable (102). Additional studies have since been designed to determine the benefit of abciximab in conjunction with stenting (EPISTENT and ADMIRAL) (103, 104). Abciximab (ReoPro) is approved to prevent cardiac ischemic complications in patients undergoing PCI.

Tirofiban and eptifibatide are non-peptide and peptide, reversible antagonists of the GPIIb/IIIa receptor, respectively. Both have very different pharmacokinetic activities compared to abciximab. While abciximab has a half-life of approximately 30 minutes, tirofiban and eptifibatide are approximately 2-2.5 hours. In addition, abciximab binds to platelets with high affinity and platelet inhibition can be detected in the absence of detectable plasma concentrations of abciximab (105). Persistence of the abciximab molecule on the surface of the platelet may account for some of this effect. All of these molecules, regardless of their half-lives, must be administered with some form of an infusion regimen. Abciximab delivered only as a bolus was not effective (see EPIC trial above). The TARGET (Do Tirofiban and Reopro Give Similar Efficacy) trial is the only head to head clinical trial performed to determine efficacy similarities. The result of this trial demonstrated that abciximab significantly reduced MI, death, and urgent percutaneous revascularization techniques at 30 days. Most of the benefit was due to the significant decrease in MI rates with abciximab (5.4% vs 6.9%,  $P=0.04$ ). Bleeding events were similar for both agents (106). Eptifibatide, in the IMPACT II clinical trial (Integrilin to Minimize Platelet Aggregation and Coronary Thrombosis-II), was determined to show efficacy compared to a placebo in reducing MI, death, and percutaneous interventions at 24 hours; however, no benefit was seen at 6 months. This was in contrast to the abciximab data (EPIC and EPISTENT) that demonstrated benefits for some

patients and for some endpoints out to 3 years. After analysis of the data it was determined that the doses were not effective due to the physiologic levels of calcium in blood. These levels were inhibitory to eptifibitide's plasma concentration with the doses administered; therefore, since the dose was insufficient to produce therapeutic plasma concentrations a new trial was designed. The new trial, called ESPRIT (Enhanced Suppression of the Platelet IIb-IIIa Receptor with Integrelin Therapy), employed greater doses than the EPIC trial. For this study, the bolus dose was increased by 33% and the infused dose was increased 4 fold. Patients were also administered clopidogrel or ticlopidine, aspirin, and heparin. These changes resulted in a >90% inhibition of platelet aggregation in 90% of the patients. While bleeding events, both minor and major, were increased significantly with these doses the efficacy gained prompted the early termination of the study. Currently all agents, eptifibitide, tirofiban, and abciximab are all licensed for use for acute coronary syndrome and/or PCI (107).

### **1.5.7 ADP Receptor Antagonism**

Clopidogrel bisulfate (Plavix) and ticlopidine (Ticlid) are two ADP receptor antagonists. Their mechanism of action is to irreversibly bind the ADP receptor on platelets to prevent aggregation for the entire life of the platelet. Only certain metabolites of both drugs are active; therefore, both clopidogrel and ticlopidine

have no intrinsic in vitro activity and several hours (clopidogrel) or days (ticlopidine) are required for these drugs to reach effective therapeutic concentrations. Cells, particularly red blood cells, when damaged release ATP which is cleaved by ectonucleotidases to ADP. The ADP produced can then bind to specific receptors that are important for the signaling events involved in platelet shape changes and aggregation, two independent and potentially unlinked events. Receptors that mediate shape changes and/or aggregation are primarily of the P2X, P2Y1, and P2Y12 receptor subtypes. The P2X receptor family primarily binds ATP and although it promotes shape changes it does not induce platelet aggregation. In contrast, the P2Y1,12 G-protein receptors respond to ADP and can induce shape change and aggregation. In addition, P2Y12 receptor antagonism also increases cAMP and also inhibits GPIIb/IIIa activation through the inhibition of PI3K, a protein kinase directly involved in aggregation. Both clopidogrel and ticlopidine bind to the P2Y12 receptor (108).

Differences between these two drugs are due to their striking side-effect profiles. Clopidogrel and ticlopidine, because of their pharmacologic actions, both can increase bleeding events. However, in 30-50% of patients ticlopidine can provoke rashes, nausea, vomiting, and diarrhea. Furthermore, neutropenia also occurred in 2.1% of the subjects tested in a clinical trial that included greater than 2000 patients (109, 110). In addition, aplastic anemia and

thrombocytopenic purpura have also been documented at rates estimated to be as high as 0.025% and 0.05%, respectively. These results necessitated a warning on the packaging label for ticlopidine to inform clinicians of the potential for toxicity. Because of the potential for adverse effects for ticlopidine, time for onset of action, and the similar efficacy profile to clopidogrel the use of ticlopidine has diminished considerably (111).

### **1.5.8 cAMP Augmentation**

cAMP is an important molecule whose function is to phosphorylate cAMP dependent intracellular enzymes. These enzymes, primarily kinases, have a wide variety of roles that can increase or decrease a particular activity. The formation of cAMP is carried out with the help of G-proteins that stimulate adenylate cyclase. The adenylate cyclase then converts ATP to cAMP, then cAMP activates protein kinase A (PKA), which is then responsible for phosphorylating specific proteins. These proteins, when phosphorylated result in a decrease in platelet aggregation. Like cAMP, a second nucleotide important for platelet signaling events is cGMP. However, unlike cAMP, adenylate cyclase does not participate in the conversion of GTP to cGMP, instead a soluble molecule, guanylate cyclase, carries out the conversion. Similar to cAMP, cGMP participates in the phosphorylation of protein kinases that can regulate cellular processes, e.g., muscle contraction. Levels of cAMP and cGMP are

controlled by phosphodiesterases (PDE) that hydrolyze cAMP to AMP and cGMP to GMP. In the platelet, there are three major PDE; PDE2, PDE3, and PDE5. PDE2 and PDE5 are important regulators of both cAMP and cGMP inactivation; however, in platelets PDE3 participates only in the hydrolysis of cAMP, and cGMP acts as an inhibitor to PDE3 (112). Since high levels of cAMP and cGMP are important for inhibiting platelet aggregation and vasodilation, respectively, the use of PDE inhibitors can pharmacologically prevent primary hemostasis and increase tissue perfusion. Drugs have been developed in the attempt to inhibit specific PDE. Dipyridamole is a potent PDE5 inhibitor with primary effects of vasodilation, but also possesses antiaggregatory effects, and cilostazol, is a PDE3 inhibitor that has potent antiaggregatory effects, but also has vasodilatory properties too. The vasodilation with both these drugs is mediated by increasing cAMP within endothelial cells that also contain PDE (113). These two examples show the complexity in the interaction between different PDE and their physiologic actions. In addition, sildenafil (Viagra), is a PDE5 inhibitor (increases cGMP) that has highly selective tissue specificity and potency and exhibits both vasodilatory and platelet antiaggregatory activity (114). Therefore, the inhibition of PDE5, while not increasing cAMP directly, appears to do so indirectly because cGMP inhibits PDE3-mediated hydrolysis of cAMP (115).

The licensed use of PDE drugs as anticoagulants is limited to cilostazol and dipyridamole. Cilostazol, is an oral medication currently approved in the United States for intermittent claudication (116). It has also been studied for the prevention of stroke and for preventing restenosis after PCI and stent placement. Dipyridamole, which also inhibits adenosine uptake, is only effective when combined with aspirin therapy, or in *in vitro* studies at high doses. Adenosine, when bound to the A<sub>2</sub> receptor on platelets activates adenylate cyclase and increases cAMP. There are two mechanisms for the removal or degradation of adenosine; the first is by adenosine deaminase and the second is the reuptake by red blood cells. Dipyridamole works by inhibiting adenosine reuptake by red blood cells resulting in the inhibition of platelet aggregation. Because damaged red blood cells are major contributors of released adenosine, and *in vitro* systems used previously did not use whole blood, dipyridamole's activity was not observed at low concentrations. After it was understood that the mechanism of dipyridamole's activity was dependent on the presence of red blood cells and higher plasma concentrations were necessary, whole blood assay systems were used and a sustained release form of dipyridamole (Aggrenox) was developed (117, 118). The latter provided higher plasma concentrations due to the requirement for low gastric pH for absorption. In addition to the antiaggregatory effects on platelets dipyridamole has several endothelial effects including, increasing PGI<sub>2</sub> secretion, antioxidant effects, and

preventing smooth muscle cell proliferation. Most of these effects have been demonstrated in *in vitro* systems or in preclinical animal models (119).

### **1.6 FVIIa/TF Inhibition**

As mentioned previously, the initiation of coagulation *in vivo* is triggered by FVIIa:TF interactions. Regulation of this pathway is controlled by TFPI that limits extrinsic FXa formation and subsequent thrombin generation. Inhibition of this pathway ultimately limits the generation of intrinsic FXa and fibrin formation. Normally, TFPI is produced in small amounts and is easily overwhelmed during normal coagulation. Certain LMWHs can transiently increase TFPI 2-fold, but it is unknown to what extent this elevation contributes to anticoagulation (120). It is generally accepted that while TFPI may play a minor role in physiologic regulation of thrombosis (compared to ATIII), the contribution of FVIIa/TF initiation of coagulation is a powerful stimulus. Therefore, regulation of this complex may provide an opportunity to pharmacologically manipulate coagulation. One such agent is NAPc2, which is a polypeptide derived from the hematophagous canine hookworm, *Ancylostoma caninum* (121). NAPc2's anticoagulant mechanism is accomplished by binding to FX or FXa and then to the FVIIa/TF complex. By binding to the TF complex the initiation of coagulation is diminished because extrinsic Xa cannot be formed. This compound has been investigated in clinical trials and is currently in Phase II clinical trials for unstable angina and non-ST elevated myocardial

infarction. In addition, this drug was also tested in patients undergoing total knee replacement surgery to evaluate the incidence of DVT and pulmonary embolism (PE) (122). Because this compound binds to FX or Xa it assumes FX's half-life of approximately 50 hrs in humans. This relatively long half-life poses opportunities and challenges for its clinical use because although anticoagulation is maintained for longer periods of time (compared to LMWH and UFH), reversal of this agent is not possible and rFVIIa "bypassing" therapy is required.

### ***1.7 Common Drugs to Modulate Hemostasis: Thrombolytics***

The thrombolytics, or fibrinolytics, represent a class of molecules that dissolve blood clots to restore blood flow to an affected tissue of a limb or organ. The urgency of flow restoration depends on many factors, e.g, time of occlusion, organ affected, whether the thrombus is on the arterial versus venous side, size of the thrombus, whether the clot is focal or multifocal, and comorbidities and risk factors for the patient. Many of these factors must be weighed before determining the use of plasminogen activators or surgical interventions. In addition, the risk of serious bleeding is frequently used as a reason not to use a thrombolytic, e.g., hemorrhagic stroke. This single risk initiated the development of systemically administered, fibrin-specific agents, that were expected to hone in on the culpable thrombus and prevent unintentional lysis of circulating

coagulation proteins. While this was a popular goal, these endeavors have not greatly diminished the incidence of hemorrhagic stroke. The sections below provide a description of these agents (indirect fibrinolytics), their common properties, and the development of newer regionally administered agents (direct fibrinolytics) that may have wider therapeutic indices.

### ***1.7.1 Indirect Fibrinolytics: Plasminogen Activators***

Indirect fibrinolytic agents have one thing in common: they do not directly digest fibrin. All indirect fibrinolytics rely on the activation of plasminogen to plasmin, which will digest fibrin and other substrates containing lysine-arginine (lys-arg) bonds. Additionally, the indirect thrombolytics maintain their efficacy when administered systemically. Conversely, direct fibrinolytics (plasmin and fibrolase) are only efficacious when applied regionally, via a needle or catheter, to where the offending thrombus resides.

#### **1.7.1.1 Streptokinase**

Streptokinase (SK) was discovered by William Tillett and Garner in 1933 after the observation by Tillett that streptococci clumped when added to serum, but not plasma. He reasoned that if the streptococci were added to anticoagulated serum, the bacteria would bind all the fibrinogen and upon recalcification there would not be a fibrin clot. This was not the case, but as he left the culture tubes

on the countertop the clot dissolved in several minutes. Tillet went on to publish that differing strains of hemolytic streptococci from different patient isolates could completely dissolve approximately 600  $\mu\text{g}$  of fibrinogen within 10-50 minutes (123). Tillet coined the name for this bacterially derived enzymatic activity streptococcal fibrinolysin. In 1941 Milstone discovered that purified fibrinogen, when clotted with thrombin, was unable to be degraded by streptococcal fibrinolysin *unless* inhibitor-free serum (euglobulin) was added to the tube (124). This observation resulted in the idea that there was a plasma factor that the streptococcal protein was converting to achieve lysis. Approximately, four years later Christensen discovered that the plasma factor was indeed a precursor that the streptococcal protein acted on to produce an enzyme that degraded fibrin. The plasma zymogen was termed plasminogen and the active enzyme was termed plasmin (125). The first clinical use of systemically administered SK by Tillett in 1946 used a crudely purified preparation made by Christensen. These studies were performed without testing in animals because they argued that SK was not very active on many species' plasminogens. However, Tillett, in 1952 performed experiments in rabbits and SK proved to work well in this system (126). Demonstrating that SK, when administered systemically, was capable of lysing thrombi prompted collaboration between Tillett and Lederle Laboratories to develop a purified SK for the treatment of myocardial infarction. In 1958 the first clinical study was

performed with SK from Lancefield group C  $\beta$ -hemolytic streptococci. This trial demonstrated that among patients treated within 14 hours only 1/15 died; whereas, 3/9 patients died if treated between 20-72 hours (127). These results were very encouraging to many in the medical community; however, there were some discouraging side-effect to the use of SK. In particular, some patients had allergic reactions and others had bleeding complications due to systemic activation of plasmin with subsequent degradation of fibrinogen.

The mechanism of SK is distinct among the plasminogen activators as it forms a complex with plasminogen, or plasmin. This complex is the true activator that converts plasminogen to plasmin. This interaction has affinity for fibrin; although, compared to other PA it is relatively non-specific. In addition, plasmin bound SK is protected from inhibition by A2AP. Due to immunogenicity associated with SK, the higher incidence of bleeding associated with its use, and as less antigenic PA have become clinically available, SK is not the thrombolytic of choice in interventional medicine.

### **1.7.1.2 Plasminogen Activators (UK, t-PA, bat-PA)**

#### **1.7.1.2.1 Urokinase**

A protein derived from human urine, urokinase (UK), was the next PA to be discovered in 1952 by Astrup and Sterndorf after it was determined a year earlier by Williams that there was fibrinolytic activity in urine. This was

encouraging because these researchers hypothesized that if the protein could be purified from human urine it may not generate antibodies, or be recognized as antigenic. Because UK has a concentration of 40-80  $\mu\text{g/L}$  in human urine it was necessary to purify large amounts to test whether UK was efficacious and not antigenic. In fact, 300 gallons of urine were necessary to purify enough UK to treat one patient (128)! By the late 1960's Abbott Laboratories and Sterling-Winthorp Pharmaceuticals produced purified UK for the treatment of patients with pulmonary embolisms. In 1970 the Urokinase-Pulmonary Embolism Trial Study Group published results that demonstrated UK to be superior to heparin for restoring angiographically evident pulmonary blood flow (129, 130). Subsequent studies comparing UK to SK were also performed that also demonstrated UK's superior efficacy and lack of antigenicity (130). Abbokinase is manufactured from tissue cultures of human neonatal kidney (HNK) cells derived from stillborn fetuses or neonates that died from natural causes. Up until 1999 Abbokinase was the preferred source of UK for clinical use; however, in 1999 the United States Food and Drug Administration (FDA) halted the sale of Abbokinase due to viral contamination and manufacturing infractions within Abbott Laboratories. These deficiencies have been rectified and in 2002 the FDA reinstated Abbokinase for use in lysis of "massive pulmonary emboli and pulmonary emboli associated with unstable hemodynamics (131). "

Unlike SK, UK can convert plasminogen to plasmin without forming the intermediate plasminogen-activator complex. Therefore, it acts as a plasminogen activator. UK has two forms, a single-chain urokinase (scu-PA) or prourokinase, and a two-chain urokinase (tcu-PA). Scu-PA (54 kDa) is present in the plasma at 3-4 ng/mL and can be converted to the tcu-PA after cleavage of a single peptide bond by plasmin or kallekrein. Tcu-Pa and scu-PA have different substrate activating abilities. While tcu-PA is efficient at activating low molecular weight chromogenic substrates, scu-PA is relatively inefficient. In contrast, tcu-PA has the ability to activate plasminogen in solution while scu-PA has only limited activity. However, when comparing fibrin specificity, scu-PA dominates compared to tcu-PA (132). The differences in fibrin-specificity can be explained by scu-PA's ability to activate plasminogen only on partially degraded fibrin. This fibrin, degraded by plasmin, exposes terminal lysines that facilitate scu-PA plasminogen activation. These structures allow scu-PA to efficiently convert the bound plasminogen to plasmin on the surface of the clot. Since plasmin can also generate tcu-PA, these results were determined with a recombinant form of scu-PA that could not be converted to the tcu-PA form (133). Thus, fibrin specificity for UK is really a secondary effect due to plasminogen/plasmin concentrations. Fibrinogenolysis and plasminogen consumption, both markers of systemic plasmin activation, have also been used as a marker for fibrin specificity.

#### **1.7.1.2.2 Tissue Plasminogen Activator (t-PA)**

T-PA is secreted primarily by endothelial cells in very low quantities (5-10 ng/ml) and is the primary fibrinolytic for maintaining vascular patency. Its activity is regulated by plasminogen activator inhibitor-1 (PAI-1), which is present in concentrations of approximately 5-20 ng/ml. Both t-PA and PAI-1 concentrations can change depending on diurnal cycles, in the presence of thrombin, or in the presence of exogenously administered t-PA. Indeed, it has been shown that PAI-1 is increased during myocardial infarction and may account for the prothrombotic state (134). Like UK, t-PA is synthesized as a single-chain form and then activated to the more active two-chain form by plasmin. In contrast to UK, in the presence of fibrin the two-chain form binds in a ternary complex with plasminogen and fibrin which increases t-PA's activity approximately 400 fold (59).

Described by Astrup in 1966 (135), plasminogen activation had been shown to be accomplished by urokinase and SK and it was known that a molecule in tissue was capable of converting plasminogen to plasmin. In 1981, Rijken and Collen successfully purified the tissue plasminogen activator (136) and in 1990 Genentech licensed t-PA as a recombinant therapeutic for the treatment of acute massive pulmonary embolism. In 1993, t-PA and SK were compared in a head-to-head trial termed Global Utilisation of Streptokinase and t-PA for Occluded

Coronary Arteries (GUSTO). The 41,000 patient GUSTO trial was a landmark study in that it demonstrated that time to reperfusion of coronary vessels was important and t-PA with heparin reduced mortality by 1% (10 patients saved for every 1000 treated) and with a lower incidence of major bleeding compared to SK and heparin. Important in the interpretation of these studies was the impact of time to treatment. Patients treated with t-PA, but not SK, had a significant decrease in mortality if they were treated within three hours of symptoms (even greater if treated within the first 2 hours). After four hours the benefits were similar, but suggested an increase in the incidence of hemorrhagic stroke with t-PA after 6 hours of treatment. Nevertheless, a decrease in 30 day mortality in the t-PA treated patients was obtained, and Activase (recombinant t-PA) became the drug of choice for treatment of myocardial infarctions (137).

The success of t-PA in this clinical trial, and the reduction of bleeding, pointed towards t-PA's fibrin specificity as the pharmacodynamic property imparting safety. It was hypothesized that since t-PA only recognizes fibrin, not fibrinogen, a systemic lytic state was never achieved. This prompted many researchers, especially within Genentech, to develop and search for more fibrin-specific agents and agents with decreased clearance to allow a bolus administration. Modifying the fibrin binding and/or PAI-1 motifs via recombinant technology resulted in several t-PA variants, e.g., alteplase (Genentech), reteplase

(Centecor), tenecteplase (Genentech), and lanoteplase were developed. Several clinical trials compared these agents, but all had similar mortality rates compared to t-PA as well as similar rates of intracranial hemorrhage (approximately 0.9-1.1%) (138). For example, tenecteplase has 14-fold higher fibrin specificity and an 80 fold reduction in PAI-1 inhibition; however, the result of the Assessment of the Safety of a New Thrombolytic trial (ASSENTT-II) resulted in similar 30 day mortality and similar intracranial hemorrhage rate; thus, the hypothesis of fibrin specificity appears to be incorrect.

### ***1.7.2 Direct Fibrinolytics: Fibrinase and Plasmin***

With the discouraging results of the more fibrin-specific agents a new hypothesis on the relative importance of fibrin specificity has emerged. It is generally agreed that we are continually forming small blood clots all the time as we walk (applying pressure on our joints) and occasionally bumping into objects, etc., and the cerebral vasculature is not excluded. These small thrombi, or hemostatic plugs, provide a mechanism to prevent blood from escaping from the vessel and into the surrounding tissue, and normally the clots and vessels are remodeled. In the cerebral vasculature these hemostatic plugs are obviously important as blood leaking into the cerebral tissue can result in neuronal damage. Since the administration of these agents has always been with the goal to be fibrin specific, or to avoid inhibition, one can understand why the

incidence of intracerebral hemorrhages was not decreased. Therefore, a change in dogma from more fibrin specific to regional administration of lytic agents has emerged. This approach seeks to treat thrombi by 1) diminishing the dependence of endogenous plasminogen concentrations, and 2) by efficiently lysing thrombi at doses that do not exceed the endogenous inhibitor capacity of the patient. This approach, however, also has limitations in that the site of the thrombus needs to be accessible by catheter, and the thrombi cannot be multifocal and separated by large anatomical distances, e.g, emboli in the distal branches of the pulmonary tree would not be ideal targets for these agents. Two agents are currently in clinical development for use of treating arterial or venous thrombi with regional administration regimens. These agents differ from the previously mentioned PA's as they degrade fibrin directly.

#### **1.7.2.1 Alfimeprase**

Alfimeprase and fibrolase, are enzymes derived from the snake *Agkistrodon contortrix contortrix* (139, 140). Alfimeprase is the recombinant form of fibrolase that is almost identical in amino acid sequence except for several amino acids at the N-terminus. This change in sequence was designed to decrease chemical modifications that can occur during manufacturing of the protein and has no effect on efficacy. At the time of this writing there had not been many peer-reviewed articles on the efficacy and safety of alfimeprase. Therefore, it is

difficult to speculate on its true potential; however, the mechanism of this drug is unique. Similar to plasmin (discussed in the next section) alfineprase has a circulating inhibitor that rapidly neutralizes its activity in plasma. This inhibitor, A2M, is present in large amounts (approximately 2 mg/mL, 3  $\mu$ M) and traps the molecule in its clathrate cage to prevent uncontrolled systemic proteolysis. When applied regionally by a catheter, alfineprase can degrade fibrin when it overwhelms the local concentrations of A2M. While alfineprase is a zinc metalloproteinase its mechanism of fibrin degradation is similar to that of plasmin as both hydrolyze lys-X bonds (where X represents a different amino acid). The hypothesized function of fibrolase as a snake venom is to deplete fibrinogen within the snake's prey. While fibrin(ogen) is depleted it would allow an increase in the circulating concentrations of other toxins within the venom to act; all of this occurring without promoting hemorrhage. In addition to its fibrinolytic effects fibrolase also degrades bradykinin to kinin which has been shown to promote hypotension in fibrolase treated animals (141). This effect may be present in patients treated with alfineprase at doses that escape A2M inhibition. Pre-treating animals with captopril, an angiotensin converting enzyme inhibitor, could be used as a test to augment the kinin response. Fibrolase was shown in a preclinical dog model to lyse arterial thrombi (142) and alfineprase, has been studied in vitro and in vivo (143). Lysis experiments with human whole blood clots demonstrated that 2 mg of alfineprase could completely lyse 1 gram

of clot in three hours. These clots were approximately 90 minutes old and thus relatively new and not fully retracted. To demonstrate the in vivo potency of alfineprase a rat model was used with the endpoint of time whereby flow was restored. In these models, alfineprase (2 mg total) had a significantly decreased time to lysis of 6.3 +/- 1.6 minutes versus 33.5 +/- 4.1 minutes with 250 U/min of UK. In addition, at these doses the UK treated rats had a 32% increase in blood loss from incision sites compared to alfineprase treated rats (144). Significant modifications to the protein have been made in attempt to prevent A2M inhibition (via pegylation) as well as to increase specificity to fibrin (via RGD peptide linkage) (141). These modifications would increase efficacy during systemic administration, but may also decrease its therapeutic index. The non-RGD and non-pegylated form of alfineprase is currently in phase I-II trials for treatment of peripheral arterial occlusions (PAO).

### **1.7.2.2 Plasmin**

Plasmin, the ultimate enzyme in the physiologic degradation of fibrin, has been known since the early 1950's. This non-specific serine protease is rapidly inhibited by A2AP, and at slower rates by A2M and  $\alpha_1$ -antitrypsin. The conversion of plasminogen to plasmin can be accomplished through the plasminogen activators, e.g., streptokinase-plasminogen complex, t-PA, or UK, or by the contact activation protein kallekrein. The forms of plasminogen that

can activated are the glutamic acid and lysine forms (glu-plasminogen, lys-plasminogen, respectively); however, there are no appreciable concentrations of lys-plasminogen found in plasma (145). Lys-plasminogen is an intermediate structure of plasminogen that forms during the activation to plasmin. As the activators convert plasminogen to plasmin the molecule becomes autocatalytic and through limited proteolysis cleaves a 78-79 amino acid stretch of protein from its N-terminus, thus converting glu-plasminogen to the lys-plasmin(ogen). The terms "glu-" and "lys-" are named for the N-terminal amino acid of their respective forms of plasminogen (Figure 1.7.4.2).

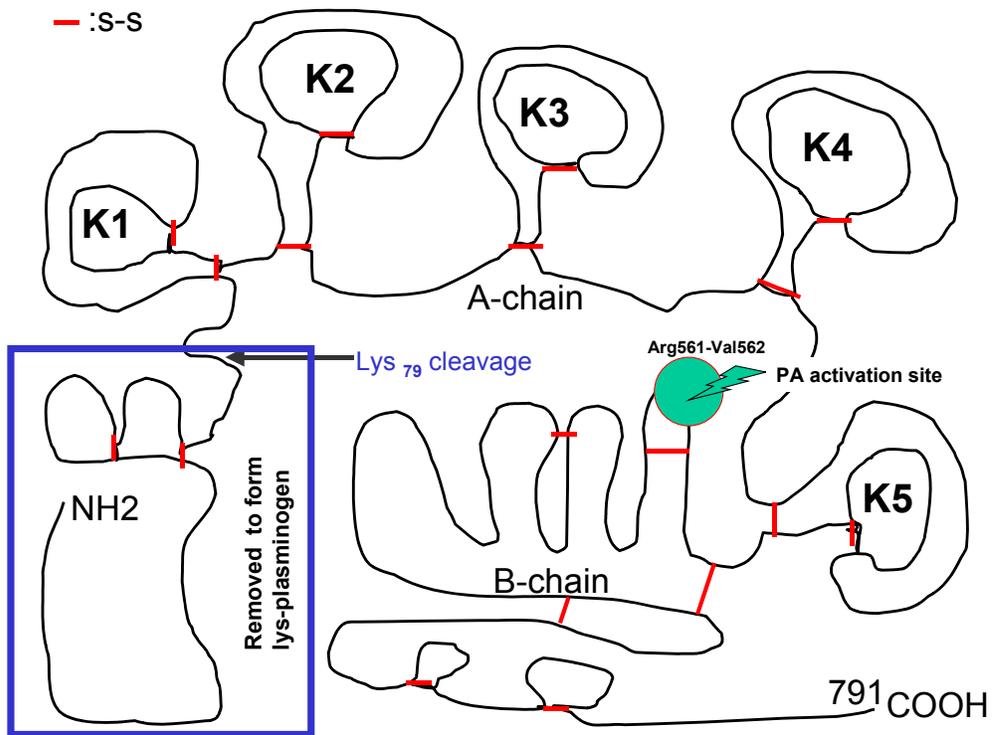


Figure 1.7.2.2 Plasminogen Molecule Structure

(adapted from (146)). Activation of glu-plasminogen to lys-plasminogen occurs when the Arg561-Val562 bond is cleaved by one of the plasminogen activators. The different activators have different prerequisites for activation, i.e., fibrin binding (for t-PA), or concurrent plasminogen binding (for SK) and accounts for

increase rates of activation. The resulting two-chain plasmin molecule consists of heavy (A) and light (B) chains connected via the interaction of disulfide bridges throughout the molecule. The activation to plasmin also removes a small 19 amino acid signal peptide from the aminoterminal end of lys-plasminogen; therefore, the entire lys-plasmin molecule is 712 amino acids and has a molecular weight of approximately 85 kDa.

The plasmin molecule has several structural motifs that impart function to the molecule. Upon conversion of glu-plasminogen to lys-plasmin the structure becomes more “open” and facilitates the binding to fibrin. Several kringle regions, so named for their similarity in appearance to a pastry, facilitate the binding to a variety of substrates, but it is Kringle 5 that is most important for fibrin binding. Kringles 1-4 can be formed through limited proteolysis by plasmin as degradation products and have been shown to inhibit cell migration. This last observation has resulted in research of the potential anticancer drug, angiostatin, which contains Kringles 1-4 (147); however, Kringle 5 has also shown potent antimigratory action on endothelial cells (148). The catalytic domain, located in the light chain, has homology to other serine proteases such as trypsin and contains the His-Asp-Ser catalytic triad.

The use of plasmin as a direct-acting fibrinolytic has been limited by plasmin's promiscuous proteolytic activity that will cannabilize itself at neutral pH. In addition, the activation of plasminogen requires an activator that must be removed prior to use so that interpretation on clot lysis is based on plasmin's effect and not the activator's. These criteria make it difficult to manufacture preparations that are stable for experimental work, thus previous in vitro and in vivo studies are difficult to interpret (149-151). More recently, a patent by Novokhatny, Jesmok, and Landskroner, et al.(152), demonstrated that when plasmin, formulated in a unique low pH non-buffered solution, is mixed with plasma or a buffer at neutral pH the protein regains activity. This formulation has enabled the development of a stable, lyophilized form of plasmin that can be reconstituted and used immediately. Like Alfimeprase, plasmin is being investigated for the treatment of vascular thrombo-occlusive diseases and for assisting surgical vitrectomy.

## **1.8 Coagulation Derangement and Resulting Diseases**

### **1.8.1 Hereditary Coagulopathies**

There are several hereditary disorders that result in hemorrhagic or thrombotic phenotypes that can be caused by diminished levels of a particular protein or loss of activity due to mutations. Most of these disorders can be linked to a

single protein; however, some disorders have multiple factors disrupted. The best described hemorrhagic disorders, and the factors affected, include FV, hemophilia A (FVIII), hemophilia B (FIX), hemophilia C (FXI), hypoprothrombinemia (thrombin), Stuart-Prower factor (FX), and dysfibrinogenemia (fibrin). In addition, platelet dysfunction can also result in bleeding and several hereditary defects include, Bernard-Soulier syndrome (GPIb), Glanzmann thrombasthenia (GPIIb/IIIa), storage pool diseases (ADP), von Willebrand's Disease (vWD, vWF), and Wiskott-Aldrich syndrome (cytoskeletal proteins and GPIa, GPIb). In addition, there are several additional inherited disorders that result in thrombocytopenia.

For the hereditary disorders that increase the risk of thrombosis the list is slightly shorter than the hemophilias. In addition, the phenotype rarely appears when there are not other hematologic disruptions and occur primarily in the venous circulation. This list includes deficiencies in ATIII, the Factor V Leiden mutation (FV), PC, PS, and prothrombin mutation G20210A. Just as in the bleeding phenotypes, there can also be multiple deficiencies within the thrombotic phenotypes. The overall presence in the general population of any of these disorders ranges from 0.02%-5%, with FV Leiden occurring primarily in the white population (153). In most of the thrombotic episodes there is oftentimes a triggering event, e.g., age, diabetes, smoking, pregnancy, that tips the balance

of normal coagulability to a hypercoagulable state. Thus, it is not surprising that the anatomical locations of thrombosis are in the low-flow venous systems.

### **1.8.2 Acquired Coagulopathies**

Increased bleeding due to an acquired condition can result from disease, injury, after major surgery, and can also be drug induced. Examples of each of these are cancer, congestive heart failure or pregnancy, traumatic shock resulting in disseminated intravascular coagulation (DIC), decreased mobility, knee and hip replacements, and warfarin--although this is considered the primary pharmacologic action of the drug. In addition, hypercoaguable states have been attributed to hormone replacement therapy (HRT), e.g., ethinylestradiol, and inflammatory bowel disease (IBD), and pregnancy. The mechanism of thrombosis for these is unclear, but for HRT it may be related to the increase lipid profile, and factor VII increase. For IBD, dysfunction may be related to APC resistance or a combination of several factors, including genetics, e.g, FV Leiden mutation (154, 155). For pregnancy, several factors increase the risk of thrombosis including formation of the antiphospholipid antibodies (anticardiolipin and lupus anticoagulant). These antibodies bind to proteins that normally interact with the phospholipid surfaces of proteins, the  $\beta_2$ -glycoprotein complexed to phospholipids, yet the exact mechanism of this interaction and how it leads to thrombosis is not known. Intuitively, it seems plausible that an

antibody binding to PL surfaces would result in a bleeding tendency. In fact, however, the result is usually thrombotic. In vitro tests usually demonstrate a prolongation in aPTT as the antibodies bind to the lipids within the system. However, in vivo, the thrombotic phenotype is thought to be due to cellular changes resulting in a thrombotic phenotype due to increased thrombin generation and prothrombin fragments, through the inhibition of anticoagulant processes, or from decreased clearance of phosphatidylserine expressing cells (156, 157). Patients having these coagulopathies may develop DVT and PE, but arterial thrombosis can also occur.

### **1.8.3 Deep Vein Thrombosis (DVT)**

DVT is characterized by the formation of a thrombus, typically originating behind one of the venous valves, and propagating both proximally and distally within a vein. The etiology of DVT can arise from any of the factors described above and usually presents itself unilaterally with increased tenderness along the affected limb and increased circumference compared to the contralateral limb. In contrast to arterial thrombi, formation of the venous thrombi is very different as endothelium denudation is uncommon, the clots are not platelet rich (the so-called “red thrombus”), and stasis due to right heart failure, vein compression, or direct injury may initiate thrombus formation. Furthermore, arterial occlusions affect approximately 7-12 million people in the US; whereas, for DVT there are

approximately 250,000-300,000 cases per year (158). Accurate diagnosis of DVT can occur with the use of imaging modalities such as magnetic resonance imaging (MR), computed tomography (CT), or Doppler ultrasound (US). In addition, diagnostic tests such as D-dimer have also been shown to be helpful in diagnosis of DVT. This test is not used universally as, although it has high sensitivity, it also has low predictive value since other diseases can cause elevations in D-dimer levels; therefore, it is used in conjunction with other diagnostic tests. Unlike the acute effects of an arterial clot that may result in ischemia to the affected limb, the venous clot does not represent a life-threatening or limb-threatening situation. Chronically, i.e., over the course of several days, there is the increased risk for thromboemboli and for phlegmasia curulea dolens. The latter occurs when the limb becomes edematous and stasis within the venous system prevents adequate circulation of blood resulting in mottling of the skin and, if not treated, can result in gangrene and limb amputation. While phlegmasia is not uncommon, the most critical sequela of DVT is the occurrence of PE. It is estimated that the number of PE occurring in DVT patients is approximately 30% (159). Thrombi from the large caliber veins, e.g, suprainguinal, can break off from a larger clot and travel through the right heart, pulmonary artery, and into the lung. Sufficiently large clot fragments, or repeated showering of clot fragments are able to increase pulmonary artery pressures, thereby decreasing venous return and diminishing gas exchange.

The response is not entirely mechanical as it has been shown that soluble mediators, primarily TXA<sub>2</sub>, also contribute to the increases in pulmonary pressures (160). Chronic emboli, even if they are small in diameter, may also lead to an inflammatory state in the peripheral pulmonary capillary beds.

In addition to the acute and chronic effects of VTE the chronic effects of having DVT can result in the post-thrombotic syndrome (PTS). A sequelae of DVT, PTS is characterized by pain, edema, and venous distension (ectasia). The exact cause is unknown but may relate to valvular damage and trauma from the resident thrombus (161). Treatment for PTS is to manage it before it occurs by early removal of the thrombus and use of compression stockings. Because PTS occurs several years post-removal of the thrombus its use as a therapeutic endpoint is difficult. In addition, secondary prophylaxis, i.e., anticoagulant therapy, may play a larger role than the acute removal of thrombus. Currently there are no FDA approved treatments for DVT, but current standard of care is via time-consuming infusions of t-PA, or UK with concomitant heparin therapy or heparin therapy alone over 24-48 hours, thus allowing for endogenous fibrinolytic mechanisms to resolve the DVT.

#### ***1.8.4 Hemodialysis Graft Occlusions***

Patients with end-stage renal disease undergo dialysis treatment approximately every three days to remove waste products from the circulating blood. Most of these patients either have a native arteriovenous fistula (AVF) or a synthetic graft made from expanded polytetrafluoroethylene (ePTFE) placed in the arm or upper extremity for percutaneous access. These fistulas and grafts are necessary as access points for dialysis and to decrease repeated damage to the vasculature during frequent needle insertions. Approximately 230,000 patients in the US undergo dialysis each year and many of these fistulas or grafts stenose or thrombose. In fact, the average lifetime of the synthetic grafts is less than 5 years and average patency rates range are 63-74% within 1 year and it is not unusual to remove several clots per year (162). Typically, flow in these grafts is maintained at greater than 800 ml/min; however, over time the flow diminishes due to compression effects, damage to the grafts, or from restenosis at the sites of surgical anastomoses. As flow diminishes a thrombus forms and the grafts occlude. Because of the necessity to remove thrombi for dialysis to occur, these patients are immediately sent to an interventional radiologist whereby the clots are removed either by mechanical, pharmacologic, or a combination of these methods. Currently, no drug is licensed for the specific removal of thrombi from dialysis grafts; however, both UK and t-PA have been used extensively as off-label regimens. Because of the nature of the occlusion it is difficult to determine the effectiveness of these treatments as no controlled

studies have been performed to determine patency rates. For example, the venous anastomoses is frequently occluded and requires angioplasty to distend this area for sufficient outflow. Therefore, simple removal of the clot is not the only obstruction to flow, in fact, the clot occurs secondary to the closure of the venous anastomoses. Also, simply removing the distal stenosis and platelet plug can result in vessel patency by “washing” thrombus away. Finally, accurate pre-treatment measurements of clot burden within the graft are difficult unless CT or MR imaging technologies are used, which is not often the case. Non-ionic iodinated contrast medium is frequently used, but again quantitation of clot burden is hindered by the 2D limitations of contrast angiography and contrast may obscure clot resolution. These features make accurate determination of clot dissolution by a lytic agent problematic.

An often underdiagnosed complication of hemodialysis graft occlusions are thromboemboli. During thrombolysis or stenosis removal the contents within the graft frequently move into the venous circulatory system and embolize to the lung. While the size and amount of clot burden released into the lung may vary greatly among patients, acute complications are either rare, or underdiagnosed (163).

## **1.9 Pulmonary Physiology and the Impact of Venous Thromboembolism**

In a large study of PE conducted in 1990, the Prospective Investigation of Pulmonary Embolism Diagnosis (PIOPED) study conducted in 1995 by Stein et al., it was discovered that death due to PE was only correctly diagnosed in less than 30% of patients (164, 165). Overall, death from PE was small, as only 22 of 51,645 patients had confirmed PE at the time of autopsy that also contributed to their death (0.04%); however, 122 patients were suspected of having PE (0.2%) at autopsy, but emboli could not be determined. Importantly, of the cases where autopsy confirmed PE caused or contributed to death, only 3 of 22 these patients were correctly diagnosed and treated. Furthermore, 14 of the 22 patients that had unsuspected PE that *directly* resulted in their death were dead within 2.5 hours. These statistics highlight the importance of diagnosing and treating a suspected PE rapidly, thus a discussion of pulmonary physiology and the pathologic effect venous emboli have on lung is prudent.

### **1.9.1 Lung Physiology**

The lung is a complex organ that is responsible for gas exchange during respiration, provides an immunologic function, and also acts as a filter that clears debris from the air and in the blood to prevent clots from entering the arterial circulation. Architecturally, the lung is composed of the trachea that branches off into the left and right lung via bronchi. These bronchi taper into

bronchioles, which then branch into the alveolar ducts and sacs. These alveoli are lined with capillaries that are ultimately responsible for transferring oxygen and CO<sub>2</sub> in and out of the circulation, respectively, through the process of diffusion through a single cell interface. This interface is the blood-gas barrier, also called the “respiratory zone.” As the airways make their branches from the trachea and through the respiratory zone the cross sectional area increases dramatically going from a few cm<sup>2</sup> to greater than 500 cm<sup>2</sup>. This large surface area imparts the lungs’ remarkable ability to compensate for most insults that create an increased demand on pulmonary function. In addition, more severe injuries are mitigated via the regulation of ventilation and gas exchange; which are complex responses to blood flow, temperature, blood pH, inspired air, atmospheric pressures, hemoglobin content, and lung pathology to name a few. Therefore, anything that can sufficiently block either the airway side of the lung or pulmonary circulation has the potential to alter gas exchange through the capillaries.

To our advantage, the lung has extremely large reserves to accommodate increased demand for oxygen to tissues during exercise. This feature of the lung is primarily due to large surface area of capillary alveoli and the unique properties of hemoglobin. Hemoglobin is a highly efficient distributor of oxygen that maintains 50% saturation even at low partial pressures of oxygen (27

mmHg) and unloads oxygen to tissues at very low  $PO_2$  (1-3 mmHg). This molecule is important because if we relied only on dissolved oxygen we would only have 0.3 ml  $O_2$ / 100mL of blood, and during exercise tissues would deplete the blood of oxygen. Hemoglobin allows the blood to carry more oxygen and, most importantly, creates a gradient that can deliver oxygen to tissues more efficiently—even under conditions of sustained exercise, or when a rapid burst of physical activity is required.

Because the lung is the only organ that receives all of the cardiac output, it plays a large role in regulating blood flow. There are several vasoactive substances that act on the lung that may also alter compliance of the airway and vasoconstriction of the pulmonary vascular system and can include ET-1, histamine, LTD4, NO, norepinephrine, PG, platelet-activating factor (PAF), serotonin, as well as TXA2. In addition, the lung is a metabolic organ, and is responsible for converting angiotensin I to angiotensin II via the angiotensin-converting enzyme (ACE) located in pulmonary endothelial cells. ACE also regulates the inactivation of bradykinin, therefore, angiotensin II is responsible for increasing blood pressure by increasing vascular resistance and bradykinin can promote vasodilation. In addition, the lung also produces PGA2, PGI2, TXA2, and LT, but it also is capable of eliminating all but PGI2 and PGA2. The primary mechanisms for removal of these vasoactive substances described

above are primarily by cellular uptake or by enzymatic degradation (166). In general, the primary substances and (receptors) mediating bronchoconstriction are: acetylcholine (M3), AT (AT-1), ET (ET<sub>B</sub>), histamine receptor-1 (H1), PAF (PAF), PG (D2 and F2 $\alpha$ ), serotonin (5HT2), TX receptor. For vasodilation, the major substances and (receptors) are: epinephrine ( $\beta_2$ ), NO (Guanylyl cyclase), PGE<sub>2</sub> (EP), and prostacyclin (IP) (167). Decreasing vascular tone is accomplished primarily through the use of  $\beta_2$  agonists because this receptor system not only increases cAMP, resulting in the phosphorylation and inactivation of myosin light chain kinase, but also hyperpolarizes the cells through the opening of K<sup>+</sup> channels.

During thromboembolism and dependent on the severity of the insult, the function of the lung coordinates changes with gas exchange, circulatory pressure, and the presence of the vasoactive substances described above. Most of the time, thromboembolism has very little clinical impact and the lung, as described above, can withstand large amounts of clot burden. However, if the embolism is large enough, blood may be blocked at the point of the pulmonary artery whereby no blood can move across the lung and death would be rapid without surgical intervention. More frequently, only portions of the lung may be blocked from flow or gas exchange and unless there is endogenous lysis of these clots the thrombi may grow and portions of lung function may be

decreased due to fibrosis. This is especially worrisome for patients with a hypercoagulable state such as cancer which can increase the relative risk of thromboembolism by three-fold (168), or patients who are heterozygous for Factor V Leiden and the prothrombin mutation G20210A mutations which can increase the relative risk greater than 4 fold (159). Therefore, it is not unusual for patients with malignancy associated with hypercoagulability to be administered LMWH and warfarin for periods of six months. While all venous thromboembolism may not be acutely fatal, there is the potential for severe complications and therefore should be treated prophylactically with anticoagulants or, if a thrombosis arises, with surgical intervention or catheter-directed thrombolysis.

The studies presented in the next chapters are studies that involve thrombosis or thrombolysis. The first chapter focuses on the role of plasmin as a direct-acting thrombolytic agent. This unique agent is capable of lysing thrombi faster and safer than current plasminogen activators and research was needed to determine what non-human species could be used experimentally to simulate human clots, as well as to accommodate large animal development. In addition, information regarding the administration regimen for plasmin was also needed. As may be deduced, the administration of any drug agent is linked to its clinical success not only in terms of efficacy and safety, but also in terms of physician or

patient compliance. In addition, the use of plasmin on the dissolution of clot burden on the venous side also has implications on pulmonary pressures; therefore, experiments were performed to examine the role of plasmin's ability to diminish pulmonary hypertension. The results of these studies help to define plasmin as a direct-acting thrombolytic and will be used as a bridge between experimental and clinical studies.

Subsequent chapters investigated the ability to detect FVIII at low levels in a hemophilic mouse using a clot detection device called ROTEG. The importance of this research is that many patients with hemophilia have a variety of bleeding phenotypes, even though their FVIII levels are well below what is needed for clotting in standard clot-based assays. This technique may be useful to stratify these individuals into FVIII levels that correspond to their phenotype. In addition, because of the sensitivity of this assay system, it may also be useful for pharmacokinetic studies where low levels of FVIII may be needed. This system utilizes whole blood and provides insight into the formation and character of fibrin clots over time.

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**2 Cross-Species Pharmacologic Evaluation of Plasmin as a Direct-Acting  
Thrombolytic Agent: Ex Vivo Evaluation for Large Animal Model  
Development**

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## Abstract

**Purpose:** Human plasma-derived plasmin has been developed for the treatment of thrombosed hemodialysis arteriovenous grafts and vascular occlusive diseases. To further investigate this drug in large animal models and derive preliminary dosing estimates, we compared plasmin's relative lytic potential in four species, including man. Our goal was to find which species' whole blood clots best compared to human clots in terms of lysis with plasmin. The results from these studies will serve to guide species selection for large animal experimentation.

**Materials and Methods:** Clotted blood from human, pig, sheep, and bovine subjects were treated with saline control, plasmin, or tissue plasminogen activator. Electron microscopy (EM) techniques were used to investigate the effects of clot size and fragmentation on plasmin lysis, the effects of intrathrombic infusion by injection of plasmin directly into whole blood clots, and species fibrin structural differences.

**Results:** Under static conditions, plasmin efficiently lysed clots from all species studied at an optimal dose of 4-5 mg per 4-5 grams of clot. With fragmented, human clots, plasmin (5mg)-induced lysis was  $80\% \pm 2\%$  at 60 minutes.

Porcine clots were more resistant to plasmin lysis compared with human, ovine

and bovine clots. Percent lysis at 60 minutes with plasmin for ovine clots was  $72\% \pm 3\%$  (4 mg dose), compared with  $50\% \pm 4\%$  for porcine clots (5 mg dose,  $p < 0.05$ ). EM of porcine clots showed a compact fibrin network that appeared more dense than that in human or sheep clots, and may account for the decreased lytic rate.

Conclusion: Human plasmin is an effective direct acting thrombolytic that is capable of lysing fibrin from several species. Ex vivo lysis studies were used to investigate the most appropriate large animal model that best approximates plasmin lysis with human clots under certain conditions. It was determined that ovine clots treated with plasmin more closely resemble the lysis observed with human clots.

## **2.1 Introduction**

The active serine protease plasmin, a potent fibrinolytic agent, has recently been purified in stable form from human plasma for evaluation in the treatment of thrombotic diseases (165). Unlike current thrombolytic agents (plasminogen activators) that convert plasminogen into active plasmin in the presence of fibrin, the biopharmaceutical development of plasmin circumvents this dependence on plasminogen in the vicinity of the thrombus. Because plasmin is the native physiologic fibrinolytic protein, regional delivery of active plasmin using a catheter-directed approach is a potentially viable method to rapidly degrade fibrin under conditions where plasminogen is not present, or is present at low concentrations (e.g., limited or no flow and fully retracted clots (166, 167)). In addition, since plasmin has a circulating inhibitor ( $\alpha_2$ -antiplasmin) present at high plasma concentrations, the safety profile of plasmin may be superior to all current thrombolytic agents. This occurs because any free plasmin travelling distal to the thrombi will be rapidly inactivated, provided the dose of plasmin does not exceed circulating  $\alpha_2$ -antiplasmin concentrations. This is in contrast to plasminogen activators that easily overwhelm their inhibitor, plasminogen activator inhibitor-1, and then travel and degrade distal thrombi. These distal thrombi may be providing normal hemostasis and can occur in the absence of a lytic state (168). Therefore, theoretically, the therapeutic index of locally

delivered plasmin may provide significant advantages when compared to current thrombolysis treatments for venous and arterial occlusions.

Thrombosed arteriovenous grafts represent a significant current medical problem afflicting patients with end-stage renal disease treated with dialysis. These synthetic grafts are vascular conduits are the lifelines for individuals undergoing hemodialysis, a necessary treatment occurring approximately every three days. A great deal is known about the pathophysiology and composition of these clots, and because they occur in a relatively fixed volume of synthetic graft (approximately 12 mL), meaningful dose-response relationships can be readily determined (169). The age and weight of these clots can be estimated at 1-3 days, because patients are dialysis treatment every three days, and the clot weight range is between 3-5 g, based on dimensions of typical synthetic grafts. Additionally, the entire interventional procedure occurs in a fixed space that has low or no blood flow over a relatively short period of time (30-60 minutes).

Because the clinical presentation of thrombosed arteriovenous grafts is well defined, it facilitated *ex vivo* experimental design for clot dissolution studies. We investigated the fibrinolytic effects of human plasmin by examining dose-response relationships across four species: pig, sheep, cow, and human. Porcine, ovine, and bovine were selected on the basis of body size that would

allow frequent blood draws, potential application for future development of large animal models, use of clinical-grade human catheters and synthetic hemodialysis grafts, and cross-reactivity with common human coagulation tests. In addition to evaluating dose-response relationships across several species, we also examined the effect of clot fragmentation on fibrinolytic rate because a number of mechanical devices are routinely used to enhance clot disaggregation in hemodialysis grafts (170, 171). Although specific delivery techniques were not examined, the impact of clot fragmentation on plasmin-induced clot lysis was investigated to determine optimal conditions for enhanced clot dissolution.

## **2.2 Materials and Methods**

### *Plasmin source:*

Human plasmin was used for all experiments (168). The inactive zymogen, glu-plasminogen, was purified from human plasma and converted to the active protease plasmin by the activator, streptokinase. Human plasmin was purified to a homogeneity of >95 % as estimated by non-reducing SDS-PAGE. After final purification to remove inactive protein and remaining impurities, plasmin was maintained at low pH to inhibit autodegradation. MALDI mass spectrometry and N-terminal amino acid sequencing confirmed the identity and integrity of the product used in pharmacology studies. Plasmin potency was measured using a direct chromogenic assay with substrate S2403, on the MLA 1600C analyzer. Potency was expressed as milligrams of active plasmin.

*Blood Collection:*

Human blood was drawn from healthy volunteers by a registered nurse at Bayer Corporation (Research Triangle Park, NC). The use of blood donors was approved by the IRB at North Carolina State University. Donors were asked to abstain from donating if taking aspirin or other anticoagulants. Porcine blood was obtained from a donor sow (30-45 kg) under anesthesia via a femoral artery catheter. Bovine and ovine samples were obtained from conscious animals via venipuncture. Clot structure was thought to be similar when blood was allowed to clot *ex vivo* when collected by venipuncture or via arterial catheter. All blood was collected in glass, anticoagulant-free, Vacutainer® tubes and incubated at 37°C before use 1-3 days after the blood draws. The incubation time was chosen to cover the range that may occur in a thrombosed hemodialysis grafts. All blood collections were in accordance with the Institutional Animal Care and Use Committee at the College of Veterinary Medicine at NCSU.

*Creation of Clot Fragments:*

To ensure uniform clot fragment size, clots were placed over a wire mesh screen (1mm pore) and manually fragmented for 10-60 seconds while visibly examining size to ensure homogenous fragmentation. For generating larger fragments for lysis, whole clots were cut into large pieces of equal size (0.5-1 gram) using tonometry scissors.

#### *Intrathrombic Injection:*

To simulate catheter-directed intrathrombic injection, whole human blood clots were removed from their clotting tubes, placed on a sieve, and injected with plasmin or recombinant tissue plasminogen activator (t-PA; Activase, Genentech) with use of a 27GA needle. Approximately 10-15 injections along the length of the clot were made. The total volume injected was 1.5 mL. The serum from the clotting tubes and any thrombolytic agent that spilled was collected into a tray below the sieve and was replaced during the incubation.

#### *Electron Microscopy of Thrombi:*

Whole blood was drawn from human (venous), sheep (arterial), or pigs (arterial) and immediately placed in a 3 mL Vacutainer brand sterile glass tube with no anticoagulation agent added. The tube was then placed in an incubator at 37 °C and allowed to fully contract over three days. The clot was then removed from the tube and cut saggitally in the midpoint of the clot's length. A small piece from the middle segment was then placed into Trump fixative (Electron Microscopy Science, Hatfield, PA) until EM processing was performed. All images were acquired on a JEOL JSM 35CF electron microscope.

#### *Ex Vivo Treatment Regimen:*

Clot fragments weighing 4-5 grams simulating a typical clot burden in hemodialysis arteriovenous grafts were added to tared 15 mL conical tubes, followed by the addition of 2-3 mL of serum 1-5 mg/mL plasmin solution. The remaining volume was filled to 12 mL with normal saline solution. The entire contents were then placed in a 37°C incubator. At 30 minutes, the tube was removed from the incubator and the entire contents of the tube were emptied onto a sieving screen to remove any fluid so only clot fragments remained on the screen. These remaining clot fragments were then removed from the screen with forceps, placed back into the same tared conical tube, and weighed on an analytical balance. These residual weights were calculated as a percent of the weight from the starting fragmented clots. This residual weight was expressed as percent lysis. The remaining solution containing partially fibrin degradation products, red cells, plasmin, serum and saline was combined with the remaining fragments and incubated for an additional 30 minutes under identical conditions. At the end of the second incubation, a 60-minute lysis was determined as described above for the 30-minute measurement.

### *Statistical Analysis*

All data were analyzed with SigmaStat (v. 2.03, SPSS, San Rafael, CA) by one-way analysis of variance and significance was determined at  $P < 0.05$ . The Tukey test was used for all pairwise comparisons. For comparisons within

doses, the Student's paired t test was used and P values of <0.05 were designated significant differences.

### **2.3 Results**

In human clots, plasmin induced a dose dependent fibrinolytic response over a concentration range of 1 mg to 10 mg (Figure 2.1-1). Compared with control, 1 mg plasmin did not result in a significant difference in lysis; however, the dose of 5 mg and 10 mg plasmin was significantly different from control and 1 mg plasmin at both 30 and 60 minutes ( $P < 0.05$ ). Under these static experimental conditions, (i.e., no flow), 5 mg of plasmin resulted in  $61\% \pm 3\%$  and  $80\% \pm 2\%$  lysis at 30 and 60 minutes ( $P < 0.05$ ), respectively, and 10 mg of plasmin resulted in lysis of  $71\% \pm 4\%$  and  $89\% \pm 2\%$  at 30 and 60 minutes, respectively (dose of plasmin at respective time intervals were not significantly different). The dose of 5 mg was considered the optimal dose because doubling the dose to 10 mg did not result in a significant increase in lysis.

Figure 2.1 shows the dose-response effects of plasmin under similar conditions on porcine, ovine, and bovine clots. For porcine clots (Figure 2.1-2), plasmin was dosed from 1 mg to 20 mg and a dose-dependency was observed. At 1 mg there was no significant difference as compared to control at both 30 minutes (lysis at  $8\% \pm 3\%$ ) and 60 minutes (lysis at  $7\% \pm 2\%$ ). Doses at 5 mg resulted in significant lysis of  $35\% \pm 3\%$  (30 minutes) and  $50\% \pm 4\%$  (60

minutes) compared to 1 mg ( $p < 0.05$ ). At 60 minutes, 10 mg plasmin resulted in significantly more ( $78\% \pm 4\%$ ) lysis versus 5 mg ( $50\% \pm 4\%$ ,  $P < 0.05$ ). At 5 mg and 10 mg plasmin, clot lysis was greater at 60 minutes as compared to 30 minutes ( $P < 0.05$ ) at the respective dose. Twenty milligrams plasmin did not significantly enhance the clot lysis observed at 10 mg for either time point.

For ovine treated clots (Figure 2.1-3), lysis results with plasmin at 1 mg was  $14\% \pm 2\%$  (30 minutes) and  $29\% \pm 3\%$  (60 minutes). Ovine clot lysis at 4 mg and 8 mg were significantly different from both control and 1 mg at 30 and 60 minutes ( $P < 0.05$ ), respectively. However, statistical significance was not reached when comparing lysis at 4 and 8 mg of plasmin at any time point. At 4 mg plasmin, clot lysis was greater ( $P < 0.05$ ) at 60 minutes as compared to 30 minutes.

For bovine clots (Figure 2.1-4), lysis rates at 2 mg was  $10\% \pm 2\%$  (30 minutes) and  $40\% \pm 2\%$  (60 minutes), which were not significantly different from control at 30 minutes; however, at 60 minutes, lysis was significantly enhanced ( $P < 0.05$ ). Bovine clot lysis at 4 mg and 8 mg was significantly different from both control and 2 mg at 30 and 60 minutes ( $P < 0.05$ ); however, statistical significance was not reached when comparing lysis at 4 mg and 8 mg of plasmin for any time point. Conversely, 4 mg plasmin induced greater ( $P < 0.05$ ) clot lysis at 60 minutes compared with 30 minutes. The percent lysis at each dose of plasmin

differed across species; however, the dose that produced maximum lysis at 60 minutes was similar in the ovine and bovine at approximately 4 mg.

When comparing human versus porcine clots in the presence of 5 mg plasmin (at 60 minutes), lysis rates were  $80\% \pm 2\%$  (human, Figure 2.1-1) and  $50\% \pm 4\%$  (porcine, Figure 2.1-2). In contrast, for bovine and ovine clots, 4 mg plasmin induced  $71 \pm 3\%$  and  $72 \pm 3\%$  lysis at 60 minutes, respectively. Clearly, porcine clots displayed greater resistance to plasmin-induced lysis as compared to human, ovine, and bovine clots. Although the 5 mg dose was not used in ovine and bovine clots, it is unlikely that this dose would have resulted in significantly more lysis as compared to 4 mg plasmin. This is because, at 8 mg plasmin, the lysis percentages for plasmin doses (for ovine and bovine clots) were virtually the same as that observed at the 4 mg dose. Comparing differences between human, ovine, and porcine clot lysis at 30 or 60 minutes, the porcine clots had significantly less lysis both time points ( $P=0.005$ ).

Figure 2.2 shows the relationship between clot fragment size and the extent of lysis with a fixed, saturating dose of plasmin (10 mg, estimated from dose-response) while utilizing the most resilient species tested (i.e., porcine) as defined by lysis and EM data (described later). Although firm swine clots with relatively less surface area (whole clots) can be lysed, the rate of lysis is

reduced as compared to clots where more surface area is exposed (i.e., cut clots). Across all doses tested, there were significant differences in lysis at 30 minutes ( $P=0.001$ ). Taken together, these experiments clearly demonstrate the dependence of lytic rate on fragment size with saturating doses of plasmin.

Catheter delivery of thrombolytic agents allows intrathrombic placement of the drug and avoidance of serum antiprotease inhibition. To examine plasmin's lytic effect in the absence of serum inhibitors, we administered saturating doses of plasmin (10 mg) and rt-PA (4 mg) as an intrathrombic injection to whole, nonfragmented human clots. We believe that 4 mg rt-PA is saturating as addition of 8 mg of t-PA did not result in significantly greater lysis (data not shown). Under these conditions, plasmin's fibrinolytic effect is significantly greater than rt-PA's at both 30 and 60 minutes (Figure 2.3). These experiments were performed in the presence of autologous human serum after the intrathrombic administration of plasmin or rt-PA. At 30 minutes, lysis was 16% $\pm$ 3% and 47%  $\pm$  6% for rt-PA and plasmin, respectively ( $P =0.001$ ). At 60 minutes, lysis was 30% $\pm$ 5 % and 58%  $\pm$  5 % for t-PA and plasmin, respectively, ( $p=0.001$ ).

We initially observed that porcine whole blood clots, as compared to human clots, were firmer to palpation, relatively resistant to manual compression, and

required more time to ensure homogenous fragmentation in this species. To investigate what may account for the differences in clot rigidity, as well as greater resistance to plasmin-induced lysis, we performed EM on porcine, human, and ovine clots. Figure 2.4 shows EM images for pig, human, and sheep clots formed under similar conditions. Networks of fibrin are observed on the surface on cross-sectional views. Fibrin networks appear to be more dense on pig as compared to human and sheep clots. These fibrin networks may account for some of the differences seen for dose-response studies with plasmin described above.

## **2.4 Discussion**

These *ex vivo* studies were initially designed to aid in development of a large animal model of thrombosed arteriovenous graft occlusion and provide a dose estimate for efficacy under static conditions. Initially, the pig appeared to be an appropriate model because of the opportunity to collect relatively large volumes of blood, cardiovascular similarities to humans, and applicability to humans of many coagulation tests in the pig (172). Interestingly, compared to human clots, all three animal species (porcine, ovine, bovine) had clot structures that were noticeably different from man in that they were more rigid. In particular, 1-3-day old porcine clots were remarkably tough and resilient to manual compression; whereas 1-3-day old human clots were easily disaggregated by application of

minor pressure. To investigate these observations we performed EM on the whole blood clots collected in the same manner as the lysis studies. Interestingly, the pig appeared to form a more dense network of fibrin layered on their surface as compared to the sheep and human clots. It has been previously demonstrated that plasmin-mediated fibrinolysis occurs slower with thin fibers compared with thick fibers, and is most likely related to prothrombin levels and the rate of thrombin generation as fibrin is being formed (173, 174). Therefore, our EM findings (Figure 2.4) offer a potential morphological basis for explaining the more rapid dissolution of clots formed within the human blood (Figure 2.1-1) compared with the pig (Figure 2.1-2). In addition, other factors such as thrombin activatable fibrinolysis inhibitor (175), FXIIIa concentration (176), or fibrin differences between species may also play a role. Because the blood, was collected and stored similarly regardless of species, the differences observed in clot morphology are due results of species differences. Any differences in lysis data may be caused by the inability to use plasmin from the species tested; however, the goal of the study was to determine the appropriate animal model to study the fibrinolytic effect of human plasmin on thrombi.

Plasmin, derived from fractionated human plasma, has been shown to be efficacious and safe in pre-clinical animal models of thrombosis and recurrent bleeding models (165, 168, 177). In addition, plasmin, the active fibrinolytic

enzyme responsible for fibrin clot degradation would be a unique agent for regional thrombolysis in the treatment of thrombosis because it bypasses the need for plasminogen that all plasminogen activators require for thrombolytic activity. In addition, plasmin's cognate inhibitor,  $\alpha_2$ -antiplasmin, rapidly neutralizes its activity; therefore, the expectation is that plasmin will prove to be extremely safe as a human therapeutic as long as doses used do not exceed the inhibitory capacity of  $\alpha_2$ -antiplasmin (178, 179).

The present studies summarize *ex vivo* experiments designed to assess plasmin's pharmacological activity under conditions that approximate the intended clinical indication, e.g., thrombosed arteriovenous grafts. In particular, this pathophysiologic indication is well characterized, can be readily accessed by catheter, and clots can be fragmented via mechanical devices or manual maneuvers. Because of the compliant graft material, expanded polytetrafluoroethylene, the graft can even be compressed by hand to induce clot fragmentation. The effects of compression were experimentally demonstrated in Figure 2.2, as increased fragmentation enhances the rate of plasmin-induced clot lysis. The occlusive thrombi that occlude the grafts are of a known age (1-3 days), a relatively known weight (4-5 grams) and occur in a fixed volume cylinder or graft (12-15 mL) (180-182). Therefore, given the well described characteristics of thrombosed hemodialysis arteriovenous grafts,

estimating minimally effective doses, optimal doses, and pharmacologic interactions are simplified.

All dose-response data were designed to evaluate the fibrinolytic effect of human plasmin on whole blood clots from different species under conditions that approximate the intended clinical indication of thrombosed arteriovenous grafts. When performing treatment, different pharmacomechanical techniques are used to fragment thrombi within the graft, but fragment size cannot be directly visualized, thus, fragment size may vary considerably. This difference in fragment size may have a significant effect on fibrinolytic rate with a given dose of plasmin; however, the doses used across species provide a rationale for lysis of clots of any clot fragment size. Thus, while heterogeneity within patient's grafts are a certainty, from the results of our experiments it is possible to estimate an optimal dose, and/or concentration.

It is unknown why the differences between porcine and human clot morphology occurs; however, the pig has many coagulation proteins, such as FV and FVIII, that have more activity per mL of plasma compared to man (183). In addition, differences in each species' FXIII level or activity, von Willebrand factor activity, or platelet activation may also be participating in events controlling clot morphology and/ or strength (184-186). Factors that influence the rate of

thrombin generation may have a significant impact on the clot structure (187). Our in vitro pharmacologic analysis, EM studies, and visual observation of clot structure suggest that it would be advantageous to use the ovine for large animal model development relative to the porcine, unless perhaps the porcine clots could be pharmacologically manipulated to make them more like human clots. Because of the body mass of the adult bovine species, and difficulties with surgical instrumentation and logistics of husbandry, the bovine would be less ideal than ovine.

The results of these ex vivo experiments demonstrate a clear dose-response with plasmin across all four species evaluated when fragment size was made uniform. Importantly, and related, we also demonstrate that the fibrinolytic effect of plasmin appear to be dependent on the fibrin surface area exposed on the clot and that the greater the degree of fragmentation, the greater the extent of lysis at saturating doses of plasmin. Although the lytic rate at a fixed dose of plasmin differed between each of the species, the dose at which maximum lysis occurred was similar for the ovine and bovine clots (4 mg). However, consistent with our qualitative observations that the pig clots were more resistant, a higher dose of plasmin (10 mg) was needed to maximize lysis for the pig (Figure 2.1-2). Under conditions of thrombosed hemodialysis grafts with known volumes, these doses will overwhelm the inhibitory effects of  $\alpha_2$ -antiplasmin that are present in

serum at concentrations of approximately 60  $\mu\text{g/mL}$  (188, 189). It is thought that  $\alpha_2$ -macroglobulin may also play a role in the inhibition of plasmin (190), but these doses of plasmin overwhelm that serum inhibitor as well.

The intrathrombic delivery technique, as we have applied it here, may not be applicable to current clinical treatment regimens; however, these experiments were performed to demonstrate the dependence of plasminogen activators on plasminogen and the role that inhibitors in serum have on plasmin. The rate of lysis for both agents did not increase appreciably after 30 minutes, which may be the result of absence of plasminogen substrate for rt-PA and autolysis of concentrated plasmin. Although increasing fragmentation will result in increased lytic rates, our data suggests that the utilized doses are saturating under these described experimental conditions, which provide estimates for the effective use of plasmin. In addition, we also provided insight into a delivery strategy that allows plasmin to escape plasmin inhibitors. The use of an intrathrombic instillation of plasmin would allow a high concentration of plasmin to be localized on the fibrin surface or interior of the clot, unopposed by inhibitors and not dependent on plasminogen concentration.

Potential limitations of these studies may include its applicability to thrombi on the arterial side that are formed under higher pressures or clots that have

remodeled. These clots may contain collagen and/or a greater platelet content and may be more resistant to plasmin; however, the same limitation would be present for plasminogen activators as well. In addition, although mechanical methods of clot removal may provide some advantages (191), these devices can be expensive, relatively invasive, and many interventionalists still prefer the “lyse and wait”, or “lyse and go” techniques. Plasmin, because of its superior efficacy with clots that exhibit diminished plasminogen content, and because of its potential safety profile, may provide a therapeutic and time-saving advantage when used in conjunction with a lyse and wait, lyse and go, or mechanical techniques.

The present experiments, in conjunction with previous work (167) with plasmin on human whole blood clots, demonstrate an increased efficacy for plasmin relative to rt-PA (Figure 2.3), a thrombolytic agent that is dependent on plasminogen for its therapeutic activity. The delivery and clot fragmentation techniques described herein provide information on how best to use plasmin for the dissolution of thrombi under conditions of limited or no flow. In addition, the dose-response across species with human plasmin was necessary to determine the best species to begin model development. We do not know whether plasmin derived from each respective species would exhibit different properties compared to human plasmin. It is possible that autologous plasmin within

species would behave differently, but it was imperative that we determine the species most appropriate for evaluation of human plasmin. A previous study by Bookstein et al. (192), utilized plasmin from porcine plasminogen activated by streptokinase. Results obtained from these experiments demonstrated, on a molar basis, that porcine plasmin was not as effective as rt-PA on human clots. Whether these results can be explained by porcine plasmin's lack of affinity for human fibrin, autolysis of plasmin upon reconstitution, or impurities in the plasmin formulation are unknown. It may also be that the effect of rt-PA may be overestimated since the exogenous addition of lys-plasminogen (species not defined) was added to the reaction mixture. While we believe that there is a finite rate of lysis that can be achieved, regardless of the amount of t-PA or plasmin that can be added to the system, the dose limitations of t-PA are more than that of plasmin. Activation of the systemic lytic state via plasminogen activator plasmin formation, and lysis of hemostatic plugs formed during normal hemostasis, occur at lower doses for rt-PA than for plasmin (193).

In summary, this in vitro characterization of plasmin's fibrinolytic activity under conditions which approximate the intended clinical indication demonstrate important dose and pharmacologic properties of this novel thrombolytic agent. Plasmin's fibrinolytic efficacy is dependent on dose and degree of clot fragmentation. Increased clot fragmentation increases substrate availability through increased surface area exposed. This is important because time to

restoration of flow is an important consideration for salvaging thrombosed limbs and maintaining patency in grafts of patients undergoing hemodialysis.

Experimentally, we have demonstrated that plasmin administered at 1 mg/gram of clot is sufficient for clot lysis. However, in human clinical medicine the vagaries associated with patient heterogeneity, delivery strategies, and the contribution of flow from small collateral vessels, make dose prediction challenging. Therefore, estimates of an efficacious dose for treating human thrombi may require larger amounts of plasmin.

In terms of a therapeutic index, previous studies in rabbits determined that doses of plasmin up to 6 mg/kg body weight did not consume sufficient clotting factors to impair clot formation, i.e., no bleeding or recurrent bleeding occurred (168). Because of this excellent safety profile, clots such as deep vein thromboses which may reach 50 grams could be lysed with 100 mg of plasmin (dose on a per kg body weight of 2-3 mg plasmin/kg) and no untoward bleeding effects would be anticipated. Therefore, plasmin administered at twice the amount described in these experiments (i.e., 2 mg/gram of clot) would theoretically provided effective lysis and a sufficient safety margin. In conclusion, the development of stable, purified plasmin that is active only at physiologic pH will be a unique agent for lysing venous or arterial thrombi because it provides a wide therapeutic margin.

## **2.5 Acknowledgements**

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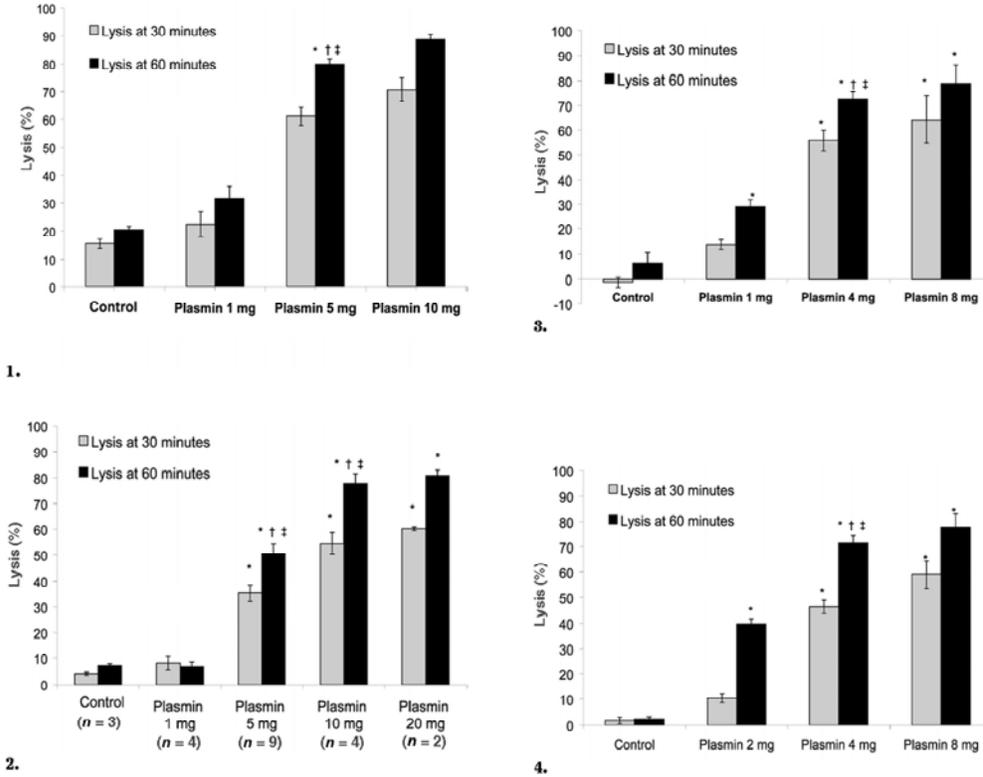


Figure 2.1 Effect of Plasmin on Human, Porcine, Ovine, and Bovine Clots

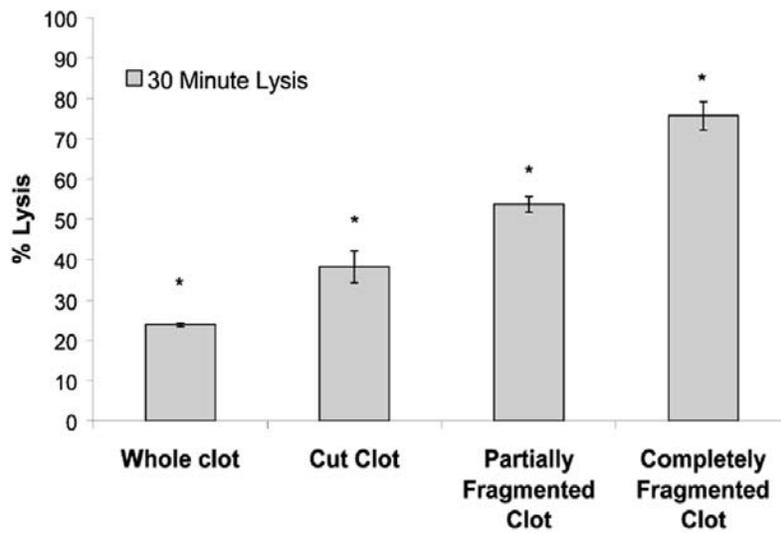
(1) Human clots aged for three days at 37 °C were treated with plasmin or control in the presence of serum in a total volume of 12 mL following fragmentation over meshed screen. To determine clot lysis, the clots were weighed after 30-minute or 60-minute incubations of clots with either plasmin or control. Lysis percentages were determined with the values of initial weights of the clots versus the weights at 30 and 60 minutes (N=3 per group). Plasmin dose response for (2) porcine, (3) ovine and (4) bovine clots. All clots were aged

for 3 days at 37 °C and treated with control or plasmin in the presence of serum after fragmentation over a meshed screen. To determine clot lysis, the clots were weighed after 30-minute or 60-minute incubations of clots with either plasmin or control. Lysis percentages were determined with the values of initial weights of the clots versus the weights at 30 and 60 minutes (N=3 per group except porcine groups).

\* Significantly different from control at respective time intervals,  $P < 0.05$ . †

‡ Significantly different from all lower doses at respective time intervals,  $P < 0.05$ .

‡ Significantly different from same dose at 30 minutes,  $P < 0.05$ .

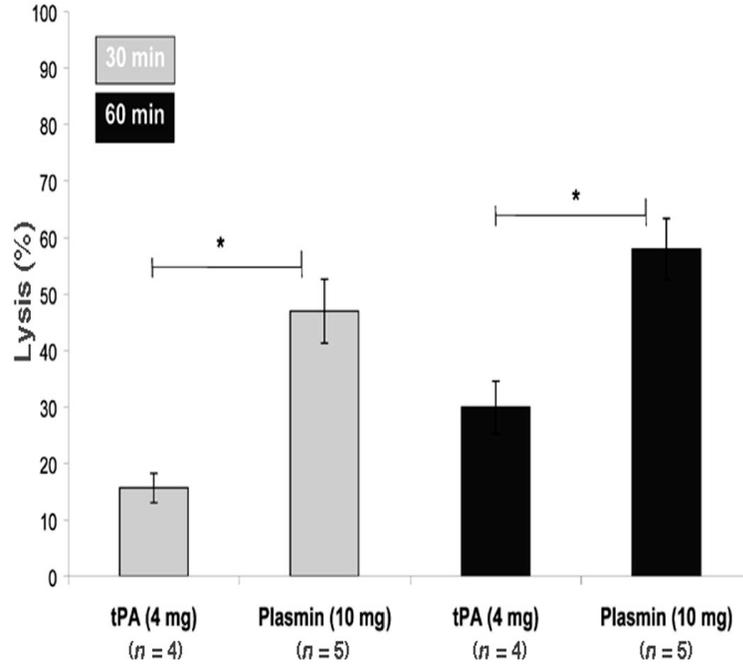


5.

Figure 2.2 Effect of Plasmin on Porcine Clots with Differing Sizes.

Porcine clots aged for three days at 37 °C were treated with plasmin in the presence of serum after either cutting the clots or fragmenting the clots to different sizes. To determine clot lysis, the clots were weighed following 30 minutes of incubation of clots with plasmin. Percent lysis were determined by using the values of initial weights of the clots versus the weights at 30 minutes.

\* Significantly different from each other.  $p=0.001$ , on analysis of variance,  $N=3$  per group.



6.

Figure 2.3 Effect of Intrathrombus Infusion of Plasmin versus rt-PA on Human Clots.

Human clots aged for three days at 37°C were treated with a saturating dose of tPA or plasmin in the presence of serum by injecting the clots with a 27 GA needle. To determine clot lysis, the clots were weighed following 30-minute or 60 minute incubations of plasmin or rt-PA. Percent lysis was determined by using the values of initial weights of the clots versus the weights at 30 and 60 minutes. These results demonstrate that intrathrombus administration of plasmin is significantly more effective than rt-PA at these doses. \* Significantly different,  $p=0.001$ , on analysis of variance with Tukey multiple comparison test.

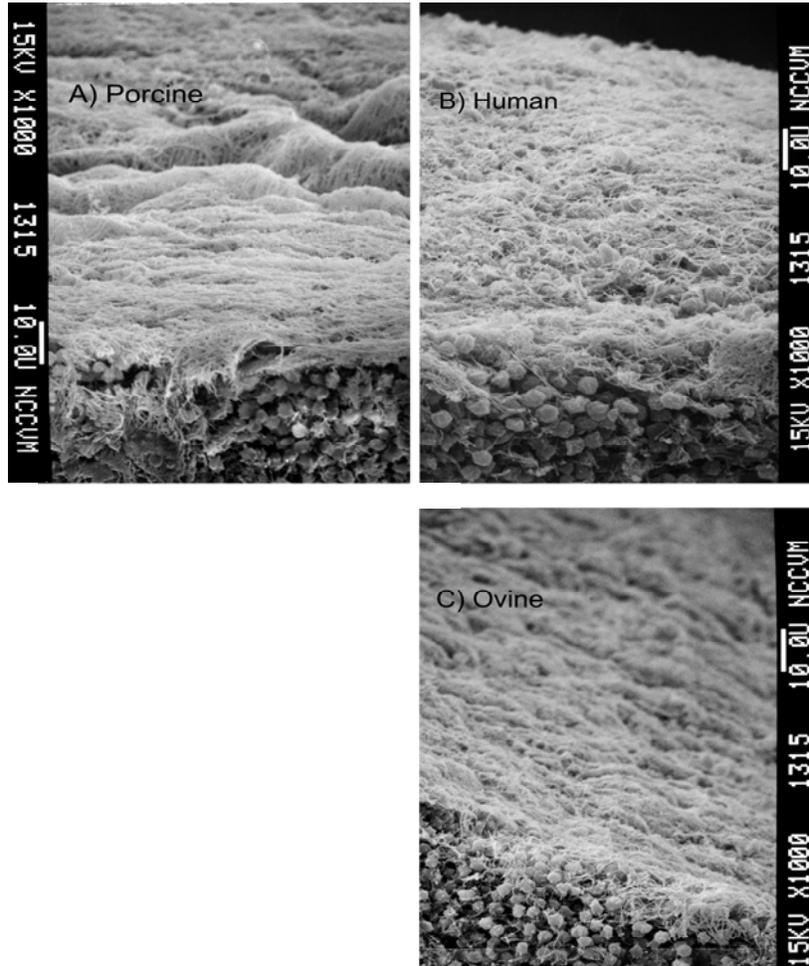


Figure 2.4 EM images of porcine, human, and sheep clots

Each clot was prepared by collecting each of the clots in glass blood collection tubes with no anticoagulant and left to clot over three days at 37°C. Clots were then cut, sagittally, with a scalpel and placed in fixative until prepared for EM imaging. Porcine, human, and ovine clots are labeled A,B,C, respectively.

Fibrin networks on the surface of the porcine (A), fixed whole blood clot, appear

to be more dense than the fibrin networks on the human (B) clot. The ovine (C) clot structure appears to have fibrin networks that are intermediate between human and porcine clots. Magnification for all images was 2,250X.

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**3 Thromboelastography Measurements of Whole Blood From Factor VIII  
Deficient Mice Supplemented with rFVIII**

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### **3.1 Abstract**

The rotational thromboelastography (ROTEG) assay system allows the real-time analysis of clot formation (fibrin formation) in a whole-blood assay format. The ROTEG system provides significant advantages over the current plasma-based assay systems as it includes the important interactions between cellular and plasmatic coagulation factors. We have employed the ROTEG system to characterize clot formation dynamics in factor VIII (FVIII) deficient mouse whole blood and examined the ability of recombinant FVIII (rFVIII) supplementation to restore the normal phenotype. The ability to generate a clear dose-response relationship by adding rFVIII to FVIII deficient murine whole blood (FVIII  $-/-$ ) demonstrates the feasibility of this approach. A dose response from 1 U to 0.00001 U/ml demonstrates the enhanced sensitivity of the ROTEG system. Further characterization of this experimental approach may provide a potential tool for comparing the activity of FVIII concentrates and/or evaluating FVIII mutants.

### **3.2 Introduction**

Use of plasma based coagulation assay systems, such as the prothrombin time (PT) and activated partial thromboplastin time (aPTT) tests, are limited in their ability to simulate the dynamics of clot formation because the tests are conducted in the absence of cell surfaces. While these tests have been the mainstays for clinicians, researchers, and manufacturers of purified factor concentrates to determine potency and coagulation factor deficiencies, these assays are inadequate to gain insight into the dynamics of clot formation *in vivo*. In particular, the important interaction of platelets and other cellular components in the coagulation process cannot be studied with the plasma based tests. While PT and aPTT tests both measure the formation of fibrin, whole blood systems containing platelet cell surfaces measure the dynamics of fibrin formation under conditions that more closely mimic *in vivo* coagulation. FVIIIa, functioning as a cofactor in the conversion of Factor X to Xa requires assembly on a phospholipid or cell membrane surface (11). The renewed interest in thromboelastography (TEG) systems, as more accurate ROTEG instruments have become available, has provided clinicians and researchers another tool to evaluate clotting processes in whole blood (194, 195). Recently, Ingerslev, et al. (196-198), have characterized differing severities of hemophilia A patients and patients treated with vitamin K inhibitors with the ROTEG assay. These studies highlight the ability of the ROTEG to detect differences between patients with

hereditary and acquired coagulation defects and described the intrinsic heterogeneity within the hemophilia population that complicates standardized Factor VIII dosing regimens. These studies also provide evidence of enhanced sensitivity for the measurement of various coagulation factors, including FVIII. To further extend this work we examined the thromboelastographic profile of Hemophiliac A mouse whole blood and its response to Factor VIII supplementation over a 100,000 fold range (1-0.00001 units/ml). The ability to generate a clear dose-response relationship over this range of Factor VIII doses demonstrates the novelty and enhanced sensitivity of this experimental approach.

### **3.3 *Materials and Animals***

Whole blood from FVIII deficient mice (exon 16 disrupted knockout mice) were purchased from the University of Pennsylvania and bred at Taconic Laboratories until they were delivered to the Biological Resource Facility at the North Carolina State University. Mice were bred from a C57BL/6 background to homozygosity and both male and female mice lack the FVIII gene (199). After mice were acclimatized for > 1 week they were euthanized with CO<sub>2</sub> gas. Immediately upon respiratory cessation, an abdominal incision was made and mice were bled (0.5-0.8 ml) from the inferior vena cava using a 25 gauge (GA) needle. Blood was immediately anticoagulated with 3.8% citrate in a 1:9 dilution of citrate to whole blood. For control mice we used normal C57BL/6 mice. All procedures

were in accordance with the NCSU Institution Animal Care and Use Committee (IACUC). To diminish inter-animal variability and to maximize the volume of blood obtained, blood samples were pooled, from three to seven animals, for thromboelastographic analysis and several pool of animals were used on different days.

#### Animal sampling

Within eight hours of sampling, the blood was assayed with ROTEG system with the following protocol: immediately before samples were tested, dilutions of a single lot of rFVIII (Refacto, Wyeth) were made in normal saline and added to aliquoted whole blood samples. The labeled units were used to calculate final concentrations. For each dilution, 3.3  $\mu$ l of rFVIII was added to 330  $\mu$ l of whole blood.

#### *ROTEG procedure*

Using the NATEG protocol as a guide (Pentapharm, Gmbh, Munich), the samples (300  $\mu$ l) were added to 20  $\mu$ l of 200 mM  $\text{CaCl}_2$  and immediately returned to the ROTEG instrument, which incubates the samples at 37°C. No exogenous tissue factor (TF) was added. ROTEG measures the dynamics of clot (fibrin) formation by measuring the restriction in movement of a rotating rod placed in a cup of clotting blood. A beam of light focused on the reflective

surface of the rod moves as the reflective surface rotates through 4° 45' over 10 seconds. As the clot forms, torque on the rod increases, the rod's movement is restricted, and light displacement is limited. From these changes in light displacement the following measurements can be obtained: CFT (clot formation time, k), CT (clotting time, r), MCF (maximum clot firmness), alpha angle (a measurement of the velocity of clot formation), and MCE which has been described elsewhere (198, 200). The tests were halted after the alpha angle was calculated, or two hours, whichever occurred first. Curves were subsequently analyzed using the CalcuRo software program provided with the ROTEG analyzer (Pentapharm, Gmbh, Munich), and raw data were imported into an EXCEL worksheet for analysis of r, k, and max parameters. Data were summarized as the mean  $\pm$  SEM of pooled blood samples from several different animals and pooled samples from different days.

### **3.4 Results**

#### *rFVIII addition to FVIII -/- mice and analyzed with ROTEG*

The thromboelastograph of hemophilic and normal mice were strikingly different (Figure 3.1) as can be seen by the amount of time required for the curves to widen (a measurement of clot formation). Supplementation of Factor VIII at 1 U/ml restored the hemophilic whole blood to a normal thromboelastograph phenotype (Figure 3.2). The effect of recombinant FVIII

supplementation on multiple thromboelastographic parameters was examined with 10-fold dilutions ranging from 1.0 to 0.00001 U/ml. The dose-response effect of rFVIII on MaxVel is shown in Figure 3.3 and demonstrates that FVIII is capable of altering the rate of fibrin formation in a dose-dependent fashion.

Mean values for the MaxVel ranged from  $33.0 \pm 6.5$  mm/min ( $19.8 \pm 3.9$  100\*mm/sec) for normal mice and  $2.3 \pm 0.3$  mm/min ( $1.4 \pm 0.2$  100\*mm/sec) for FVIII -/- mice administered 0.0001 U of rFVIII. The values within parentheses represent MaxVel curve values in units that Ingerslev et al.(201), have used to describe their MaxVel values. Calculated values describing the kinetics of clot (fibrin) formation are summarized in Table 1. Notably, the MaxVel parameter responds in a dose-responsive manner which may signify thrombin generation, while AUC, t-MaxVel, and t-AUC were not different across doses.

#### *Clot formation parameters upon addition of rFVIII to FVIII -/- mice*

The CFT (k) and the CT (r), which describe clot formation time and clotting time, respectively, also exhibited a clear dose-dependency (Figure 3.4). Over a dose range of 1.0- 0.0001 U/ml, there was a direct relationship of a decreasing alpha angle with decreasing doses of rFVIII, while with r and k the relationships were inversely related. Normal animals had alpha, k, and r values of  $81 \pm 1.7$ ,  $46.3 \pm 8.1$ , and  $269.8 \pm 39.6$  seconds, respectively. While the FVIII -/- mice had values of  $-1 \pm 0$ ,  $1325.7 \pm 104.4$ , and  $2174.7 \pm 183.3$  seconds, respectively. For the

rFVIII dosed animals the values for the alpha angle, k, and r values ranged from  $76.7 \pm 3.4$ ,  $67.0 \pm 15.4$ ,  $266.0 \pm 106.3$  at a dose 1U/ml to  $5.7 \pm 6.7$ ,  $855.7 \pm 31.9$ , and  $1767.3 \pm 63.9$  seconds at the lowest dose, 0.00001 U/ml.

Contribution of platelets on clot formation with FVIII -/- mice supplemented with rFVIII.

To examine the role of platelets on clot (fibrin) formation and perhaps account for the enhanced sensitivity to Factor VIII (0.00001 U/ml) observed in whole hemophilic blood, we centrifuged the mouse whole blood to create platelet poor plasma (PPP) (2400xG for 15 minutes). The thromboelastograph of PPP from FVIII -/- deficient mice treated with 0.01 U/ml of FVIII was compared to the whole blood samples treated with the same dose of Factor VIII (Figure 3.5). Values for r, k, and alpha angle for whole blood were  $675 \pm 74$  seconds,  $188 \pm 22$  seconds, and  $58 \pm 3$  degrees. For PPP the same parameters were  $1823 \pm 281$  seconds,  $2345 \pm 74$ , and no angle was calculated due to the extreme prolongation of the measurement. In addition, we also calculated the MCE which has been previously described as the elastic shear modulus (202), an index of clot strength. The MCE is calculated with the formula  $(100*MA)/(100-MA)$ . The absence of platelets decreased the MCE values over 100 fold with the same

dose of Factor VIII ( $342 \pm 37$  vs.  $11 \pm 2$ ), whole blood versus PPP as shown in Figure 3.6.

To further evaluate the temporal effect of rFVIII supplementation on clot (fibrin) formation, we examined the amplitude of the clot formation profile over the first 20 minutes. Output from the ROTEG instrument provided data on amplitude at 5, 10, 15, and 20 minutes post clot formation. Data from these time points are presented in Figure 3.7. Amplitudes at 5, 10, and 15 demonstrate a clear Factor VIII dose-dependent effect. The amplitude begins to plateau after the 15 minute measurement and may reflect that thrombin generation is diminishing.

### **3.5 Discussion**

The ROTEG coagulation analyzer was used in this study to examine clot (fibrin) formation using FVIII -/- mouse whole blood and to determine if this device was capable of distinguishing differences in clot formation dynamics as a function of Factor VIII doses. The studies reported here demonstrate a clear dose-response on multiple thromboelastographic parameters over a range of Factor VIII from 1 to 0.00001 IU/ml. These dose ranges are approximately 1000 times more sensitive than the aPTT or chromogenic assays. The ability of the ROTEG to measure fibrin formation in whole blood is particularly useful since some of the discrepancies between the plasma based assays appears to be related to

the source and substance of phospholipid (203). In addition, we believe that the use of whole blood increases the sensitivity to FVIII levels via its interaction with the normal platelet phospholipid surfaces it would encounter in vivo during tenase complex assembly (11). Indeed, our observations when whole blood is compared to platelet poor plasma (Figure 3.5 and 3.6) support this suggestion. We did not use tissue factor (TF) to initiate coagulation in an attempt to slow thrombin generation (and fibrin formation) in order to provide a more prolonged coagulation event to evaluate the effects of Factor VIII more directly. Without having tested TF in our system it is unknown whether the omission of TF achieved this effect, but clearly, dose-response relationships were demonstrated across a 100,000 fold dilution of Factor VIII. Because the catalytic efficiency of the tenase complex is increased approximately 100,000 fold by the addition of Factor VIII (11) we are encourage that we are measuring the biologic function of Factor VIII under these experimental conditions. We believe this is the first demonstration of this degree of sensitivity using hemophilic mouse whole blood.

The fibrin formation events captured by the ROTEG device in this study are real-time measurements of clot formation dynamics and include the clotting time, the clot formation time, maximum velocity of clot formation, and the maximum amplitude of the clot firmness after the fibrin clot has fully formed. These

measurements are dependent on the rate and/or the amount of thrombin generated/unit time. Not surprisingly, the clot time of approximately 240 seconds in normal mice correlates well with the CT of approximately 280 seconds seen with other ex vivo models of thrombin generation (204, 205). This time point corresponds to similar assays that detect clot formation as it is prolonged under conditions of clotting factor deficiencies, inhibitors, or low platelets. The ability to use whole blood from a homogeneous population of mice was advantageous in our studies since hemophilia in humans results in differing FVIII:C levels and the clotting phenotype is heterogeneous (198). Because FVIIIa can increase the activation of X to Xa, in the presence of FIX and phospholipid, by a factor of 100,000, it is not surprising that small amounts of FVIII were capable of clotting FVIII deficient murine blood (206).

Many previous studies have demonstrated the importance of platelets for the optimal generation of thrombin and subsequent fibrin formation. The assembly of the coagulation factors, especially the tenase complex, is necessary for generation of thrombin during the propagation phase of coagulation (207). Central to this event is FVIII which acts as template where factors assemble (208) after platelet. It has been estimated that approximately 400 FVIII binding sites exist on each platelet (209) and thrombin is one the most potent stimulators of platelets. The conversion of prothrombin to thrombin on the platelet surface is

clearly necessary for efficient and rapid clot formation. Our use of FVIII deficient whole blood with the ROTEG enabled us to examine the direct effects of rFVIII titration on fibrin formation and indirectly measure thrombin generation. Interestingly, Brummel, et al.(210) have demonstrated that less than 5% of the thrombin is generated before the clot has been formed, so only minute amounts of thrombin are necessary for clot formation. Recently, it has been shown by Allen et al.(9) using a cell based model system of coagulation, that the rate of thrombin generation and peak levels of thrombin are Factor VIII dependent; whereas the total amount of thrombin generated is not (9). These effects were also seen in our system, as the AUC and Max values (data not shown) did not change significantly; however, the amplitude (0-20 minutes) of clot formation was dependent on the amount of rFVIII added to our system (Figure 3.7). Thus, the rate of thrombin generation and the early amplitude of fibrin formation are most likely directly related. The assay system used in those studies (9) varied the levels of FVIII (from 0-200%) in a cell-based system while maintaining physiologic levels of other coagulation factor levels and coagulation was triggered by the addition of TF. Although our system did not use TF to trigger the coagulation reaction, a similar biologic function for Factor VIII appeared evident, i.e., modulating the rate and extent of thrombin formation and as a consequence the rate and extent of fibrin formation.

The mice used in these studies were knockout mice that lack the FVIII gene located on exon 16. Original characterization of the mouse demonstrated that FVIII activity was <1% of normal values for mice by using the aPTT methodology (211, 212). The mouse cDNA is approximately 84-93% identical to the A and C domains whereas the B domain is more heterogeneous (213). While this is not a natural model of hemophilia, the mouse displays the inability to clot from a traumatic injury, e.g., tail snipping, but they do not spontaneously bleed. In addition, female mice that lack FVIII are capable of having normal pregnancies which has been suggested to imply that mice have compensatory mechanisms to halt bleeding post-partum (212). Therefore, while these FVIII -/- mice have little, if any, FVIII activity it is not necessarily an exact model of human hemophilia; however, many characteristics of human hemophilia can be studied in this unique strain of mice, including clot formation and they provides a unique tool to examine the effect of FVIII deficiencies on multiple clotting parameters.

The ROTEG's utility resides in its ability to simultaneously measure clot dynamics and clotting parameters using whole blood. While this assay is not likely to replace standard assays (e.g., aPTT, PT) anytime soon, we do believe that the ROTEG system in combination with whole hemophilic mouse blood may be useful for some research and discovery activities. For example, this assay may be useful for examining the relationship between the rate of thrombin

generation and fibrin formation and fibrin structure. Furthermore, this assay system could be used to study coagulation factors, e.g., FIX, FX, FXIII, FVIII, with novel activity by adding them to a knockout mouse with the factor of interest deleted. The sensitivity gained by the combination of the ROTEG instrument and FVIII deficient mice whole blood provides another assay system to help describe the complex events of coagulation.

### **3.6 Acknowledgements**

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	<b>MaxVel (mm/min)</b>	<b>+/- SEM</b>	<b>MaxVel (100*mm/sec)</b>	<b>+/- SEM</b>
<b>FVIII +/+</b>	19.0	0.6	11.4	1.0
<b>FVIII -/-</b>	2.1	0.6	1.3	1.1
<b>1</b>	15.9	0.5	9.5	0.9
<b>0.1</b>	15.7	1.7	9.4	2.9
<b>0.01</b>	9.9	1.0	5.9	1.7
<b>0.001</b>	5.6	0.1	3.4	0.2
<b>0.0001</b>	2.3	0.2	1.4	0.3
<b>0.00001</b>	3.3	0.1	2.0	0.2

Figure 3.1 Summary of Major ROTEG Parameters after rFVIII Supplementation to FVIII Deficient Mice.

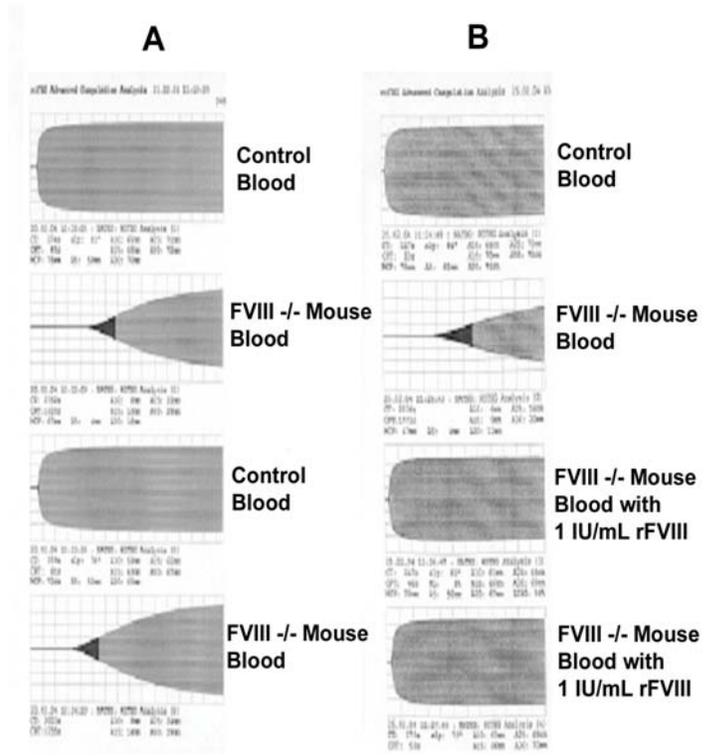


Figure 3.2 Thromboelastography measurements of normal and FVIII  $-/-$  mice. Whole blood from FVIII deficient animals and control C57Bl/6 mice were analyzed with ROTEG. The graphs demonstrate the prolonged clotting time and time to reach maximum clot firmness for FVIII deficient animals. The alpha angle, a determinant of clot formation velocity was only obtained for the control mice as the FVIII deficient mice did not reach the requisite amplitude (height of the curve). Figure A shows control mice and FVIII  $-/-$  (hemophilic) whole blood in duplicate. Figure B shows whole blood for control mice, FVIII  $-/-$  mice (hemophilic), and rFVIII supplemented FVIII  $-/-$  mice whole blood.

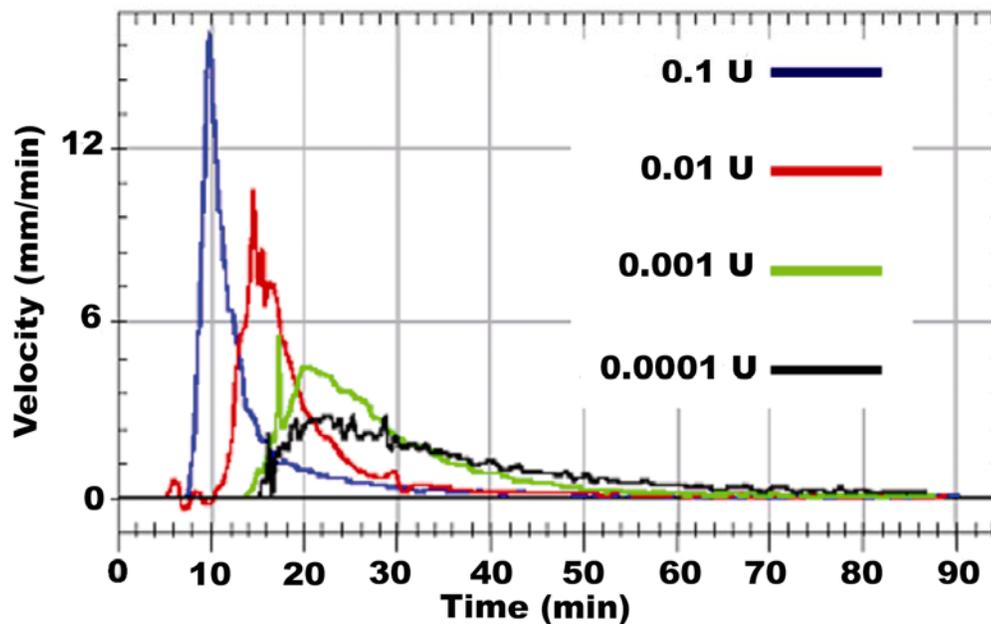


Figure 3.3 MaxVel Plots after rFVIII supplementation to FVIII deficient mice. Representative plots of a single pool of FVIII  $-/-$  mice and control mice. FVIII  $-/-$  mice were dosed with rFVIII (graph at right) and the compared to normal mice, FVIII  $-/-$  mice, or FVIII  $-/-$  mice supplemented with 1 U of rFVIII. Note vertical access for each graph are different to highlight the dynamic range of values for FVIII  $-/-$  mice supplemented with rFVIII versus control mice.

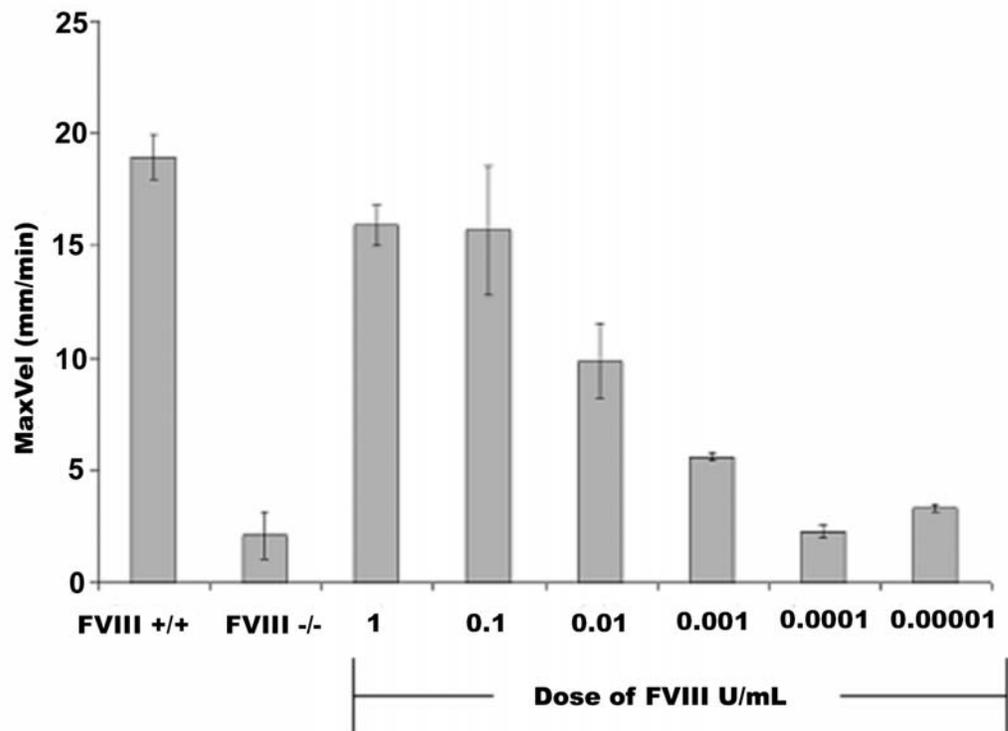


Figure 3.4 Effect of rFVIII supplementation to FVIII deficient mice on MaxVel. Whole blood from FVIII deficient animals were dosed with rFVIII (U/ml) over a 100,000 fold dilution and analyzed on the ROTEG after recalcification with 200 mM CaCl<sub>2</sub>. Pooled blood was used to diminish assay variability and several pools were used to aquire data over entire dose-relationship.

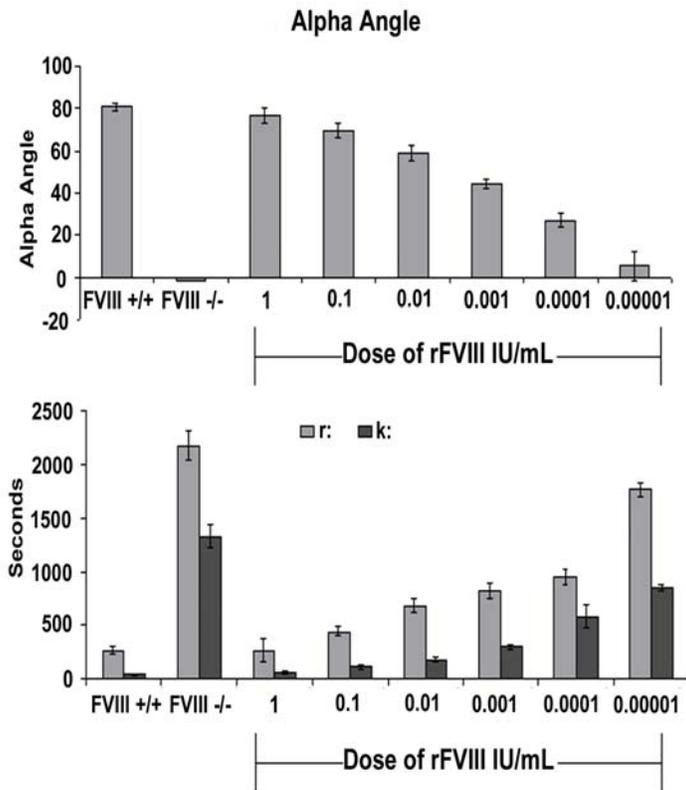


Figure 3.5 Effect of rFVIII supplementation on alpha angle, CT (r), and CFT (k) in the FVIII -/- mouse.

Whole blood from FVIII deficient animals were dosed with rFVIII (U/ml) over a 100,000 fold dilution and analyzed on the ROTEG after recalcification with 200 mM CaCl<sub>2</sub>. Pooled blood was used to diminish assay variability and several pools were used to aquire data over entire dose-dependency.

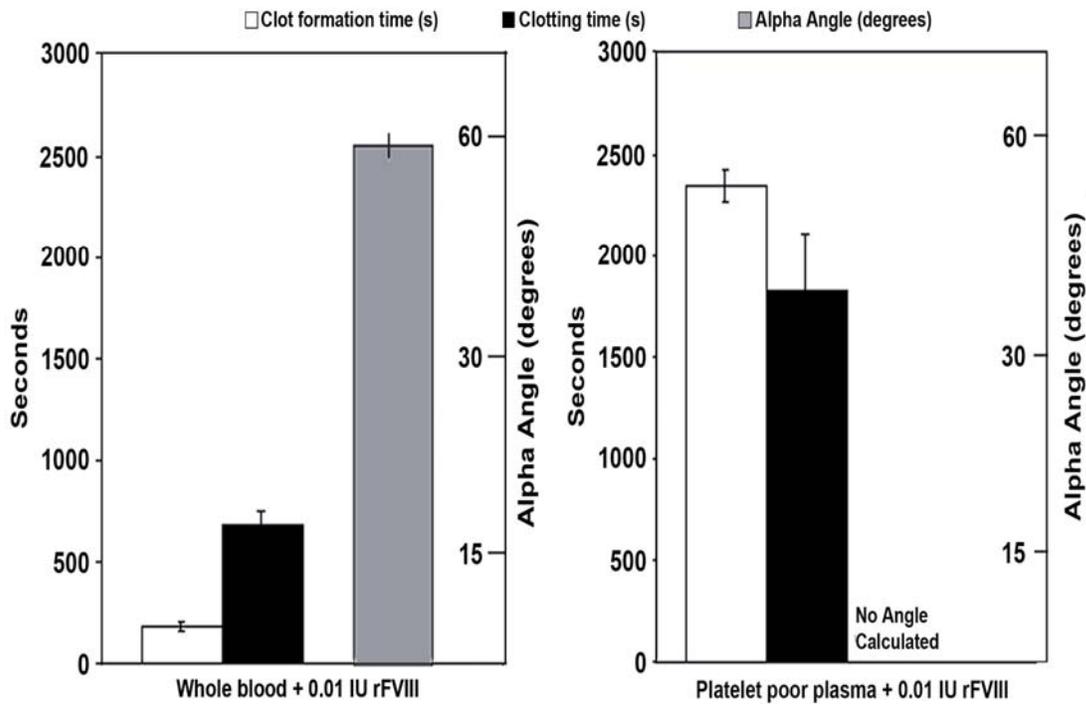


Figure 3.6 Differences between FVIII <sup>-/-</sup> mice whole blood or platelet poor plasma supplemented with rFVIII.

Pooled, whole blood from mice were analyzed with ROTEG and compared to pooled whole blood after centrifugation to create platelet poor plasma.

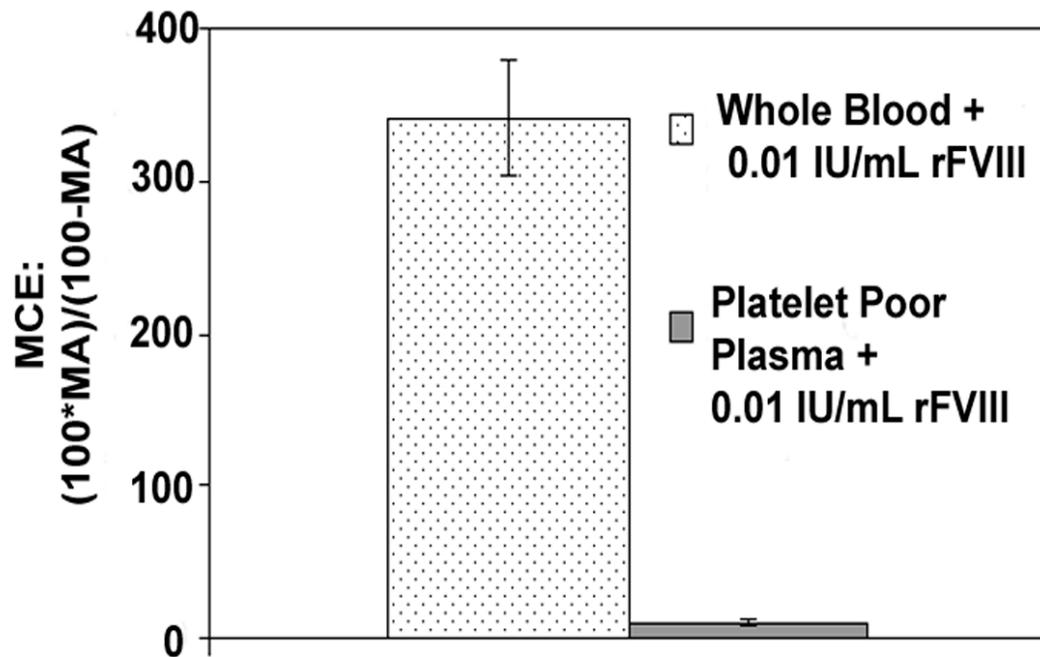


Figure 3.7 MCE values between FVIII -/- whole blood and platelet poor plasma supplemented with rFVIII.

MCE is a measurement of the shear elastic modulus of clots and is calculated from the maximum amplitude values (MA). Whole blood from mice were analyzed with ROTEG and compared to pooled whole blood after centrifugation to create platelet poor plasma.

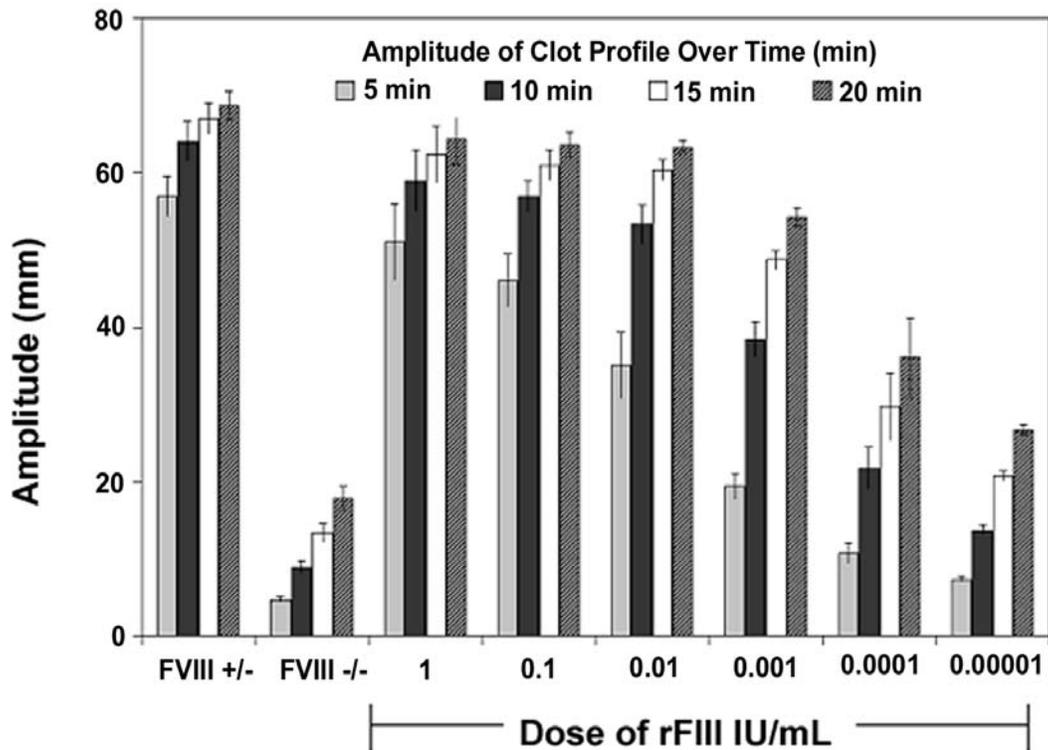


Figure 3.8 Effect on clot formation amplitude over time with rFVIII

supplementation to FVIII  $-/-$  mice and compared to normal.

Amplitude at 5, 10, 15, and 20 minutes were recorded and plotted against rFVIII concentrations. Amplitude measurements at 5 minutes behave in a dose-dependent manner and as time increases the dose-dependency becomes less significant, indicating potential plateau of thrombin generation.

#### **4 Enhanced Factor VIII Activity Measurements Using Roteg and Factor**

##### **VIII -/- Mice Whole Blood**

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To quantify the rate of fibrin formation after recombinant FVII (rFVIII) administration to FVIII-deficient mouse whole blood we have employed the rotational thromboelastography (ROTEG, Pentapharm) assay system. This system demonstrated greater sensitivity than traditional assay systems and allows the analysis of clot (fibrin) formation in real-time. This system used FVIII-deficient mouse whole blood and provides a more relevant fibrin formation assay system than plasma-based systems. In contrast to ROTEG, current assays used to measure FVIIIa-dependent fibrin clot formation, such as the activated partial thromboplastin time (aPTT), are less sensitive. Reasons for the decreased sensitivity are due, in part, to the absence of platelets that are necessary for optimal tenase and prothrombinase assembly. While there has always been interest in measuring low FVIIIa activity, i.e.,  $<0.001 \text{ U mL}^{-1}$ , the technology and methodology was not available. Thromboelastography (TEG) systems have been available for decades, however recent ROTEG technology has improved ease of use and reproducibility (1, 2). In order to evaluate the detection sensitivity of ROTEG at low levels of FVIII we examined the thromboelastographic profile of FVIII  $-/-$  mice (3) whole blood supplemented with rFVIII over a 100,000 fold concentration range. The ability to generate a clear dose-response relationship between Factor VIII levels of  $0.0001 \text{ U/mL}$  (0.001%)

to  $1 \text{ U mL}^{-1}$  (100%) and the rate of fibrin formation demonstrates the enhanced sensitivity of the approach.

Briefly, blood from mice was drawn from the descending vena cava under anesthesia and immediately anticoagulated with 3.8% citrate in a 1:9 dilution (citrate: blood). For comparison, blood from C57BL/6 mice (FVIII +/+ background strain), was used as control. Blood samples were pooled from three to five animals for analysis. Within eight hours of sampling the blood was assayed with ROTEG system using the NATEG protocol provided by the manufacturer (1, 2). Coagulation was allowed to proceed without the exogenous addition of tissue factor. Recombinant FVIII (Refacto, Wyeth) as each dilution was added in a total volume of  $3.3 \mu\text{L}$  to  $330 \mu\text{L}$  of whole blood. The coagulation reaction was started by the addition of  $20 \mu\text{L}$  of  $200 \text{ mM CaCl}_2$ . Using this protocol the following measurements were made: clotting time (CT), the time delay from start of sample run to point where trace is 1 mm wide; clot formation time (CFT), the time between the 1mm wide point to the 20 mm wide point; and alpha angle, which is an indirect measurement of the rate of clot formation (1, 2). The tests were halted after the alpha angle was calculated by the software program, or 2 hours, whichever happened first. Experiments consisted of drawing blood from three to five animals, pooling the blood, and adding dilutions of rFVIII. Each experiment was repeated three to four times on

different days with different mice. Data are summarized as mean  $\pm$  SEM of pooled blood. The effect of FVIII levels on these thromboelastographic parameters was examined with repeated 10 fold dilutions ranging from 1.0 to 0.00001 U mL<sup>-1</sup> final concentrations.

The dose-response effect of Factor VIII on the rate of clot formation (alpha angle) is shown in Figure 4.1A. Normal mice (FVIII +/+) had an alpha angle value of  $81 \pm 2$ , while Factor VIII deficient mice (FVIII -/-) did not generate an alpha angle over 2 hours. The addition of Factor VIII over a range of 1.0-0.00001 U mL<sup>-1</sup> elicited a clear dose response on clot (fibrin) formation in FVIII -/- deficient mouse whole blood. As the concentration of Factor VIII increased, the rate of clot (fibrin) formation increased in a uniform manner. Similarly, CT and CFT also exhibited a clear dose-dependency over a range of 1 to 0.00001 U mL<sup>-1</sup> of Factor VIII (Figure 4.1B). CT and CFT in normal (FVIII +/+) mice were  $270 \pm 40$  and  $46 \pm 8$  seconds, respectively. Whereas, in FVIII (-/-) mice, the values were  $2175 \pm 183$  and  $1326 \pm 104$  seconds. Factor VIII, at concentrations between 0.00001 to 1 U mL<sup>-1</sup>, restored CT and CFT towards normal values. As the concentration of Factor VIII increased the clotting time and clot formation time decreased in a uniform manner.

These data demonstrate that the ROTEG provides a new methodology to detect the biological activity of very low levels of Factor VIII in patients with hemophilia and may help to explain the wide variation of bleeding phenotype in severe hemophilia where levels are not easily measured below 1% or  $0.01 \text{ U mL}^{-1}$  (4, 5).

To examine the role of platelets on clot (fibrin) formation and perhaps account for the enhanced sensitivity of Factor VIII measurements observed in whole hemophilia blood, we centrifuged mouse whole blood to create platelet-poor plasma (PPP) (2400 xG for 15 minutes). The thromboelastograph profile of PPP from FVIII<sup>-/-</sup> deficient mice treated with  $0.01 \text{ U mL}^{-1}$  of FVIII was compared to the whole blood samples treated with same dose of Factor VIII. In the presence of FVIII<sup>-/-</sup> whole blood and  $0.01 \text{ U mL}^{-1}$  of Factor VIII the CT and CFT were  $675 \pm 74$  seconds and  $188 \pm 22$  seconds, respectively, and the alpha angle was  $58 \pm 3$  (Figures 4.1A, B). In the absence of platelets the CT and CFT were  $1823 \pm 281$  seconds and  $2345 \pm 74$  seconds, respectively, while the alpha angle was not detected over 2 hours. Thus, in the absence of platelets the activity of Factor VIII on the formation of fibrin is profoundly impaired.

These data demonstrate that the presence of normal cellular elements (platelets) increase the sensitivity of Factor VIII measurements by several orders of magnitude. Because Factor VIIIa increases the activation of Factor (F)X to

FXa, in the presence of FIX and platelet surface phospholipid by a factor of 100,000 (4), it should not be surprising that a clear Factor VIII dose response can be demonstrated over a similar 100,000 fold range when clot (fibrin) formation is measured in FVIII deficient murine whole blood. These preliminary studies demonstrate a Factor VIII dose-response on the dynamics of clot (fibrin) formation in whole blood over a range of 1.0 to 0.00001 U mL<sup>-1</sup> (100-0.001%).

The enhanced sensitivity to very low levels of Factor VIII is most likely related to the provision of normal platelet surfaces and an instrument (ROTEG) capable of measuring small changes in the temporal formation of fibrin. These dose ranges are approximately 100-1000 times more sensitive than standard Factor VIII detection assays and may provide an approach to evaluate variable bleeding phenotypes in severe hemophilic patients (5, 6).

#### **4.1 Acknowledgments**

Dr. Mohan Pamarthi for mice bleeding assistance and Mr. John Bromirski for assistance with ROTEG sampling.

#### **4.2 References**

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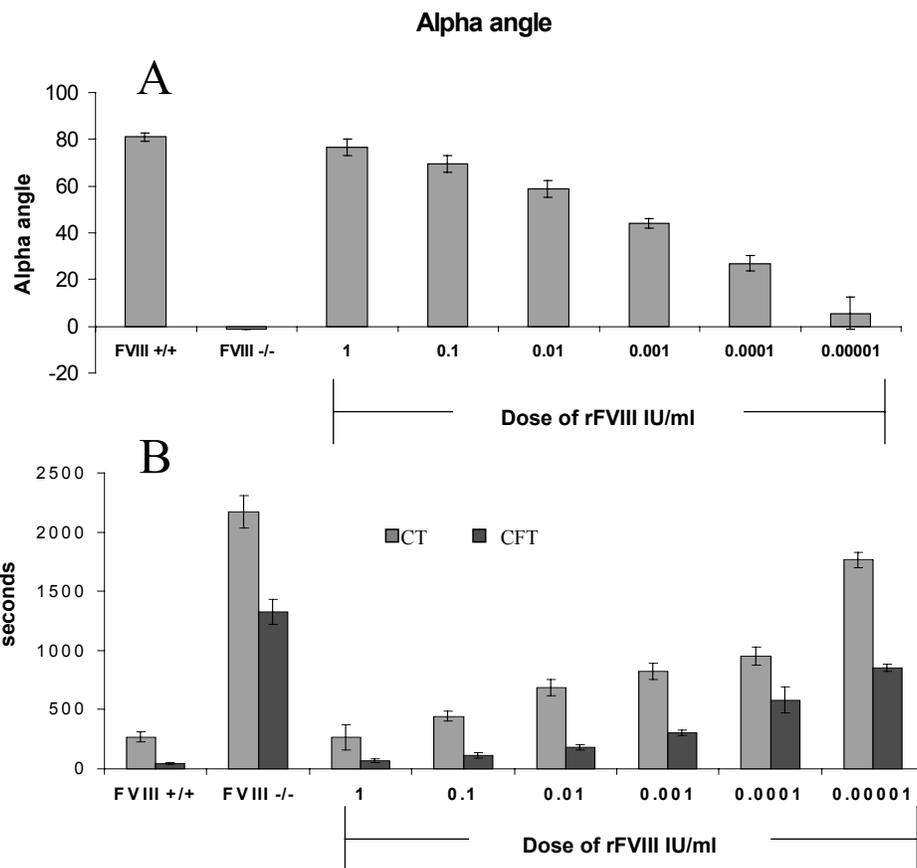


Figure 4.1 Effect of rFVIII supplementation on alpha angle clotting time (CT) and clot formation time (CFT) in the FVIII  $-/-$  mouse.

Effect of rFVIII supplementation on alpha angle **(A)**, clotting time (CT) and clot formation time (CFT) **(B)** in the FVIII  $-/-$  mouse. Whole blood from FVIII deficient animals were dosed with rFVIII ( $\text{U mL}^{-1}$ ) over a 100,000-fold dilution and analyzed on the ROTEG after recalcification with 200 mM  $\text{CaCl}_2$ . Pooled blood was used to diminish assay variability and several pools were used to acquire data over entire dose-dependency.

## **5 Summary and Future Studies**

### **5.1 Summary**

The work presented within this dissertation addressed a detailed overview of the complex interactions that occur within the processes of thrombosis and thrombolysis and the ability to manipulate these processes pharmacologically. A simplistic view of the clotting of blood and lysing thrombi is unnecessary and prevents an understanding of the nuances contained within these highly complex and physiologically important events. In addition, the experimental studies presented within the previous chapters focused on the use of plasmin as a direct acting thrombolytic for regional thrombolysis and the ability of the ROTEG, in combination with a FVIII deficient mouse, to evaluate the dynamics of FVIII-dependent clot formation.

The plasmin experiments focused on methods to choose an appropriate species to begin large animal model development work with plasmin. These studies were to serve as the groundwork for developing large animal models of diseases such as DVT, PAO, and stroke. The results of these experiments highlighted not only that plasmin is an effective fibrinolytic compared to its rival, rt-PA, but also that there were distinct differences in the architecture of experimentally formed clots between pig and human. The porcine clots were relatively resistant

to thrombolysis compared to human, ovine, and bovine clots; therefore, because of this observation and other physical characteristics of this animal, the ovine was ultimately chosen as the most appropriate species to use for comparative thrombolysis studies.

The experimental mouse model of hemophilia is a FVIII deficient mouse that has many of the characteristics of human hemophilia. This mouse has proven to be a valuable research tool for examining the specific role of FVIII in context with cellular and plasma constituents, as well as the role of changes in vasculature tone (e.g, flow and vasoconstriction) that occur when blood clots are trying to form. Because of the usefulness of this animal model we attempted to determine whether a coagulation monitoring device, ROTEG, could provide insight into clot formation upon supplementation of this mouse's whole blood with rFVIII. Surprisingly, the ROTEG device was capable of detecting very small amounts of rFVIII. These results were most likely due to both the uniqueness of the FVIII interaction with the mouse platelet, or the presence of platelets. This latter observation was tested by eliminating platelets from the reaction mixture which attenuated the clot formation profile. Together these results indicate that the rate of thrombin generation increases with increasing concentrations of FVIII.

## **5.2 Direction of future studies**

### **5.2.1 Plasmin administration**

Plasmin has the potential to become a useful therapeutic for the treatment of several thrombotic disorders where catheter-directed thrombolysis can be achieved. However, to implement this drug into a clinical setting more needs to be known about how to best deliver plasmin within the confines of the biochemical properties of the drug itself, the inhibitory capacity of normal plasma, and the logistics of clinical medicine. This last feature can be the most challenging aspect of implementing the drug into routine clinical practice because of the constraints of time, money, and availability of qualified clinical personnel. To address these concerns more studies are needed to answer the question of, "how is plasmin best delivered?" We have begun to address this question by designing an in vitro apparatus that allows the placement of human clinical grade catheters into clotted sheep blood contained within vascular grafts. This device allows the administration of plasmin into four separate grafts by means of manual injections or via an infusion pump. Preliminary data have been generated that investigate some of these questions.

#### **5.2.1.1 Methods**

Clot Formation:

Blood from sedated sheep was used for all clots tested. Briefly, each animal was sedated with medetomidine hydrochloride (15 mcg/kg) via an intravenous injection into the jugular vein. For blood collections, an 18GA needle was inserted into a clean, well-sheared neck. Immediately after the 30 mL of blood was collected, it was gently added to the graft. Reversal of medetomidine sedation was accomplished with atipamezole hydrochloride (75 mcg/kg). Clamps were placed on the grafts' ends to trap the blood within a 40 cm length. The graft was then wrapped in ultrasound gel and Saran Wrap to prevent the clots from drying and then incubated at 37°C for approximately 6 to 8 hours. After this time period the grafts were drained to determine any serum expressed. If sufficient amount of serum (>5 mL) was liberated from the contracting clot an additional blood draw was performed on the same sheep and an equal volume of serum lost was replaced with blood. The clots were then placed back into the incubator as described above and incubated overnight.

Graft Apparatus (Figure 5.1):

To allow the application of plasmin with different infusion regimens we designed a chamber (TAP Plastics, San Leandro, CA) that allowed the attachment of expandable polytetrafluoroethylene (ePTFE) grafts. The grafts (50 cm x 1.0 cm) were attached to fitted adapters with one end connected to a 5 Fr. introducer

sheath port (proximal end) and the other end connected to polyethylene (PE) tubing that was used as an outflow to collect samples (distal end). The distal ends of the grafts were clamped after repeat bolus dosing to prevent fluid from leaking out; whereas, for the continuous infusion the distal end tubing was unobstructed to prevent excessive generation of pressures within the grafts. The grafts were stacked in a 2x2 fashion within the chamber and kept at 37°C using recirculating pumps that pumped deionized water into the chamber. The apparatus was covered with SaranWrap to retain heat within the system.

For all infusions the Cragg-McNamara infusion catheters (Ev3 Inc., Plymouth, MN. 5 Fr, 100cm, and 40 cm fenestration infusion length) were used. After the grafts were connected to the adapters the catheters were inserted and approximately 60 ml of contrast agent (250 mg/ml, Omnipaque (Iohexol) Amersham Health, Princeton, NJ) diluted 1:10 in saline was slowly infused through the catheters. The contrast agent was used for planned studies that will evaluate clot morphology and volume using computed tomography. Following the administration of contrast, plasmin or saline was added using the regimens outlined in Figure 5.2. All infusion regimens examined a total dose of 100 mg of plasmin with concentration and administration methods as variables. Four different methods were tested: (A) 75 ml continuous infusion over 4 hours, (B) two pulse-spray infusions totaling 30 ml followed by a 45 ml continuous infusion,

(C) a 15 ml pulse-spray infusion followed by three 20 ml pulse-spray infusions, and finally, (D) five 5 ml pulse-spray infusions to test the effect of higher concentrations of plasmin. Sampling of effluent occurred immediately following the completion of each bolus, or in the case of the continuous infusions, after each hour.

### 5.2.1.2 Results

The effects of plasmin on lysis using differing infusion regimens are shown in Figure 5.3. If no treatment was performed on clots the weight of the clots after approximately 32 hours was 16.0 g +/- 2.0g. Saline treated clots, regardless of treatment resulted in 16 +/- 0.5 g; therefore, all saline data were pooled.

Percent clot lysis was determined by the following calculation:

$$\% \text{ Lysis} = 100 - 100 \times [\text{treatment/pooled saline treatment group}]$$

For changes in  $\alpha_2$ -antiplasmin, FVIII, and fibrinogen, the assays were compared to a human standard; thus, the sheep values are represented as a percentage of the human values. Final values are presented as a percentage of each animal's baseline level, which was based on the average of two independent measurements immediately before the plasmin infusion.

For the repeat bolus infusions at 5 mg/mL the clot weight was 6.5 g +/- 1.1g and lysis was 59.6% +/- 5.2%. For the repeat bolus infusions at a lower

concentration of 1.33 mg/mL the clot weight and lysis were 6.8 g +/- 0.8g and 57.7% +/- 4.7%, respectively. Bolus pulse spray infusions followed by a continuous infusion resulted in a clot weight of 3.6g +/- 0.8g and lysis of 77.5 % +/- 5.0%. Using only the continuous infusion alone resulted in clot weight and lysis of 8.5 +/- 1.3 g and 53.3 % +/- 7.9%, respectively.

Between treatment comparisons were also evaluated for statistical differences. Clots treated with saline alone or without any treatment were statistically different from all other treatment regimens ( $P < 0.00001$ ). The pulse spray followed by continuous infusion treatment was statistically different compared to both the pulse spray treated group at 5 mg/mL ( $P = 0.035$ ) and the pulse spray treated group at 1.33 mg/mL ( $P = 0.016$ ). The comparison between the 5 mg/mL and 1.33 mg/mL pulse spray only treatments groups were not statistically different from each other ( $P = 0.79$ ). In addition, the continuous infusion alone was not statistically different from the pulse spray by the continuous infusion regimen ( $P = 0.008$ ).

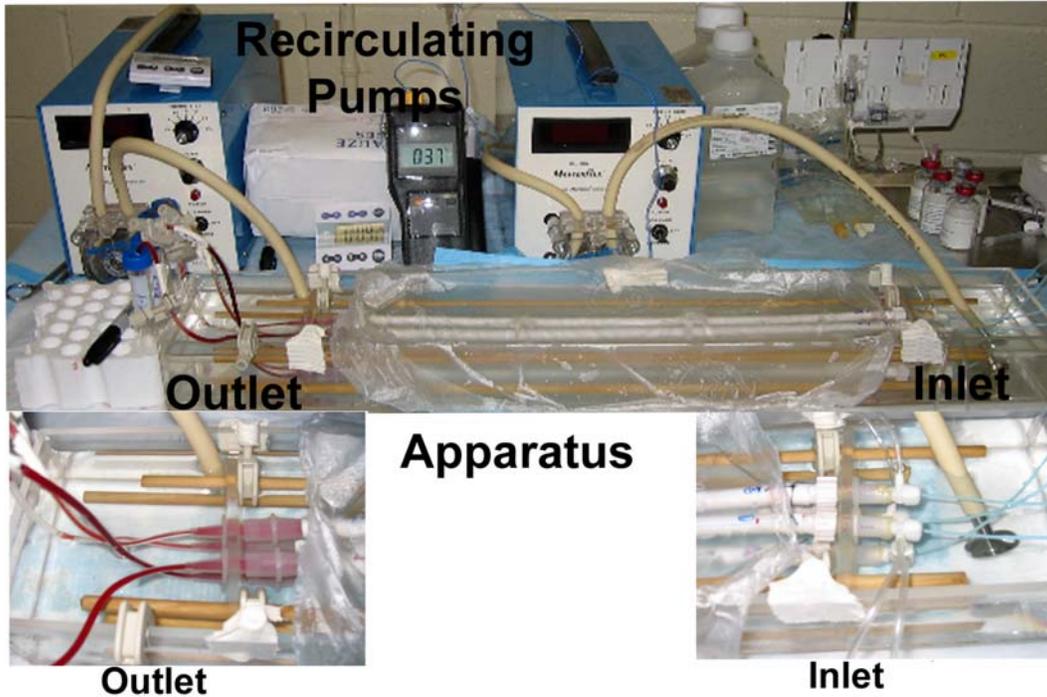


Figure 5.1 Experimental infusion apparatus.

This infusion apparatus allows for the introduction of clinical catheters to be placed along the clot for administration of test articles. Clots are formed in the ePTFE grafts overnight in an incubator and then connected to adapters before the beginning of the experiment. The grafts are stacked 2x2 in close proximity to allow for analysis by computed tomography. The grafts are maintained at 37°C through the use of a recirculating water bath that is connected to peristaltic pumps. Effluent from the infusions can be collected in tubes placed at the outflow.

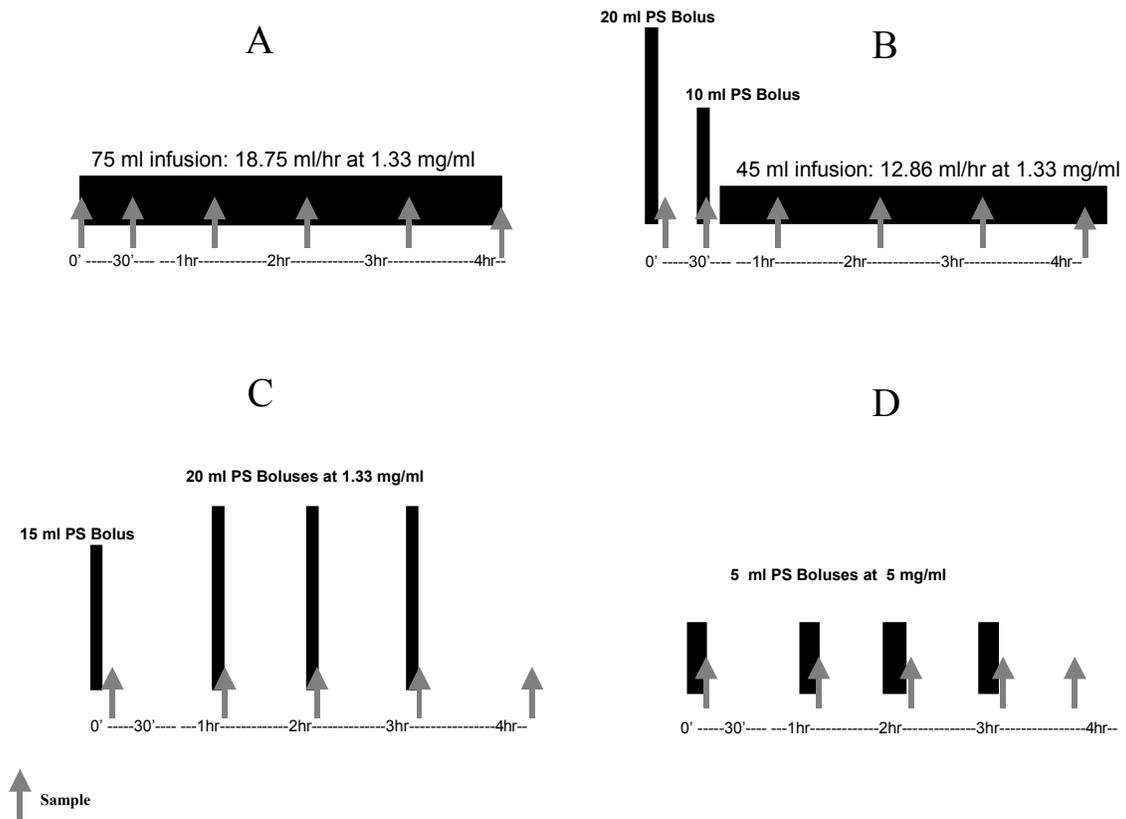


Figure 5.2. Dosing regimens for plasmin infusions.

Four dosing regimens were tested to determine which was most efficacious within the constraints of the experimental design. All doses were 100 mg. A) Continuous infusion of dose in 75 ml of saline over 4 hours. B) Two pulse-spray infusions, the first 20 ml and the second 10 ml, followed by a continuous infusion of 45 ml over 4 hours. C) Repeat pulse-spray infusions. The first 15 ml spray was followed by 20 mL pulse-sprays at each hour over four hours. D) High concentration pulse-spray. Repeat pulse spray of five 5mg/ml plasmin solution.

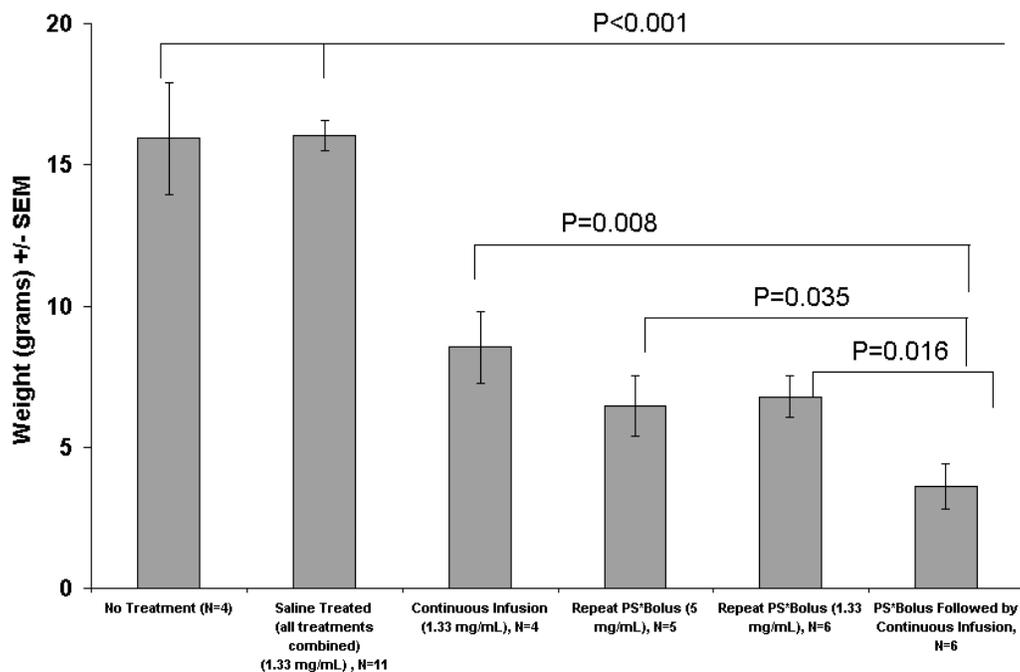


Figure 5.3. Effect of a fixed dose of plasmin on unfragmented clot lysis with differing infusion regimens.

Administration regimens described in Figure 5.2 were evaluated for dissolution of thrombi. Whole blood clots from sheep were incubated for 24 hours in ePTFE grafts at 37C and then treated with plasmin. Immediately after the infusion regimen was complete the clots were removed from their grafts and weighed. Lysis was compared to saline treated clots to determine the percentage of clot lysed.

### **5.2.1.3 Summary**

These experiments highlight the ability of plasmin to effectively lyse fibrin under several conditions that would be reasonable for clinicians to implement and provided insight into what the most effective treatment regimen might be for plasmin. The ability of a pulse spray administration at the beginning of treatment followed by a continuous infusion is a relatively easy administration protocol to implement. It was unknown which treatment modality would be the most effective at the onset of these experiments and therefore these data provide the groundwork to explore why these results occurred and the ability to test new combinations. A limitation to these experiments was that they were performed in the absence of flowing blood and the results could be very different in this situation. Blood flow not only contains inhibitors to plasmin, but can also dilute drug concentration at the site of action. Therefore, future studies should test the effect of plasmin under the conditions when flow is present. While one would assume that since the vessel is occluded there should be an absence of flow; this would only be true at the commencement of thrombolysis. During the course of treatment it is possible that channels of flow could be established which could limit plasmin's effectiveness. It is unknown what the impact of this scenario would be in terms of lysis. These studies are not easily accomplished in an in vitro setting since anticoagulated blood or plasma would have to be

used, this blood (plasma) would most likely have to be recirculated, and the dilution of plasmin into the plasma volume (which contains plasmin inhibitors) is difficult to simulate. It would, however, be possible to devise an extracorporeal circuit within an anesthetized sheep to form clots of varying ages and then administer plasmin into the region of the thrombosis. This experiment would allow the measurement of lysis and allow for sampling of blood for coagulation protein measurements. In addition, a chronic model of thrombosis should be performed to determine the ability of plasmin to degrade older, more established thrombi. The clots formed for all of the current work ranged from 1-3 days; however, most clots that are treated in human clinical medicine can be several weeks old. It is tempting to speculate that plasmin would have a greater therapeutic advantage on these older clots, as compared to plasminogen activators, because these clots lack plasminogen; however, without performing these necessary experiments one can only speculate.

## **5.2.2            *Role of clot burden on pulmonary hypertension***

### **5.2.2.1        Introduction**

Despite novel pharmacologic and mechanical methodologies for removing thrombi from veins and artificial arterio-venous anastomoses, there are still many instances when large thrombi are released to the lung. Although these

clot fragments have the potential to negatively affect cardiopulmonary parameters, the amount of debris needed to cause these effects is poorly understood. In addition, pharmaco-mechanical methodologies are not typically evaluated with cardiopulmonary effects as endpoints. Surrogate endpoints of clot lysis, such as restoration of flow, are measured. These measurements may not capture the impact of clot lysis where large fragments break off and travel to the lung—even though flow had been restored. To begin to answer these questions we treated thrombi with plasmin, rt-PA, or heparin and then embolized the remaining clot fragments into an anesthetized sheep to determine the effect of clot burden on pulmonary hypertension.

#### **5.2.2.2 Methods**

##### *Animal, surgical, and catheter placement methods*

Sheep (Kathadin, 30-40 Kg) were anesthetized with ketamine/xylazine, intubated, and further anesthetized with isoflurane while ventilating at 10-12 mL/kg. The animal was placed in dorsal recumbancy and a catheter (7Fr, Cook, Check-flo Performer Introducer) was placed, percutaneously, into the left femoral artery for the purpose of blood pressure monitoring and blood draws. A second catheter (7Fr, Cook, Check-flo Performer Introducer) was also placed in the left jugular vein for administration of fluids (lactated Ringer's) and for positioning of the pulmonary artery thermodilution catheter (PA) (Abbott, 7 Fr, 110cm).

After catheter insertion, 150 ml of blood were drawn into separate 30 cc plastic syringes and allowed to incubate at 37°C for 2 hours, and allowed to clot and retract. The animal was then placed in sternal recumbancy to prevent atelectasis over the course of the experiment.

Before placement of the PA catheter the pressure readings were zeroed while holding the catheter in position where the heart beat was strongest using a stethoscope. The catheter was then inserted into left jugular vein and advanced into the pulmonary artery while viewing right heart pressure changes. A pulmonary capillary wedge pressure (“wedge”) was formed to confirm proper placement of the catheter.

To allow clot fragment embolization a small 3 cm skin incision was made with a cautery blade above the right jugular vein. Using blunt dissection the jugular vein was exposed and 1 cm diameter x 6 cm silastic tube was inserted. The tubing was modified with connectors to allow fluid administration to maintain patency. This tubing can be disassembled to allow a modified syringe (described below) to be connected which then acts as the delivery device for thrombi.

### *Treatment Regimen*

Clots were treated for 60 minutes with a clinical dose of heparin (500 IU), a saturating dose of rtPA, 4 mg, or Plasmin at a dose of 2 mg/gm clot at 0 and then again at 30 minutes. All clots weighed approximately 10 g. Clots were weighed at 30 and 60 minutes post administration of test agent on an analytical balance. All clots were washed thoroughly with saline before embolization.

### *Clot Fragmentation Technique*

Previous studies have demonstrated that clot fragmentation impacts rate of clot lysis. To form reproducible clot fragments a 30 cc syringe was modified by drilling the hub to a 0.7 cm diameter opening. Aged clots were placed in this syringe and forced through the opening three times over a meshed filter to allow serum to pass through into a reservoir below. Not all clots used for lysis experiments were embolized into sheep.

### *Data Analysis*

WINDAQ software (DATAQ Instruments Inc., Akron, OH) was used to capture pulmonary arterial pressures (PAP) and MAP. For MPAP the WINDAQ software was used to capture the mean of several time points before and after the clot infusions since the rise in MPAP was oftentimes too sudden to capture with hand recordings. The data are expressed as mean net increases from pre to

post clot infusions +/- SEM. Statistical analysis used Student's t-test with  $P < 0.05$  considered significant.

### **5.2.2.3 Results**

Clots were sized to simulate the diameter of lower limb thrombi that could occur in human veins or arteries. Lysis of clots with an optimal dose of plasmin of 2 mg/gm clot at 30 minutes was 43% +/- 5%. At 60 minutes there was an increase in lysis to 74% +/- 1%. For heparin treated clots at 30 and 60 minutes the lysis was 10% +/- 2% and 16 +/- 2%, respectively. For rt-PA the dose of 4 mg was assumed to be saturating as the lysis at 60 minutes (34% +/- 3%) was greater than lysis at 60 minutes at the higher dose of 8 mg/kg (20% +/- 2%). It is unknown why there was less lysis at the higher dose, but it may be because any plasmin generated could not bind to fibrin binding sites, since they were currently bound by the saturating doses of rt-PA.

For the thromboemboli experiments there was a significant increase in the mean pulmonary arterial pressure following the embolization of the clot fragments treated with heparin or rt-PA compared to plasmin. For heparin, rt-PA and plasmin treated thrombi the changes in pulmonary pressures were 9.4 +/- 3.2 mmHg, 4.0 +/- 1.2 mmHg, and 1.8 +/- 0.5 mmHg, respectively. Both the rt-PA and plasmin treatments resulted in pressures that were significantly different

from heparin treatment ( $P < 0.05$ ), and the pressure change after plasmin treatment was statistically different from the pressures after treating the clots with rt-PA,  $P = 0.45$ .

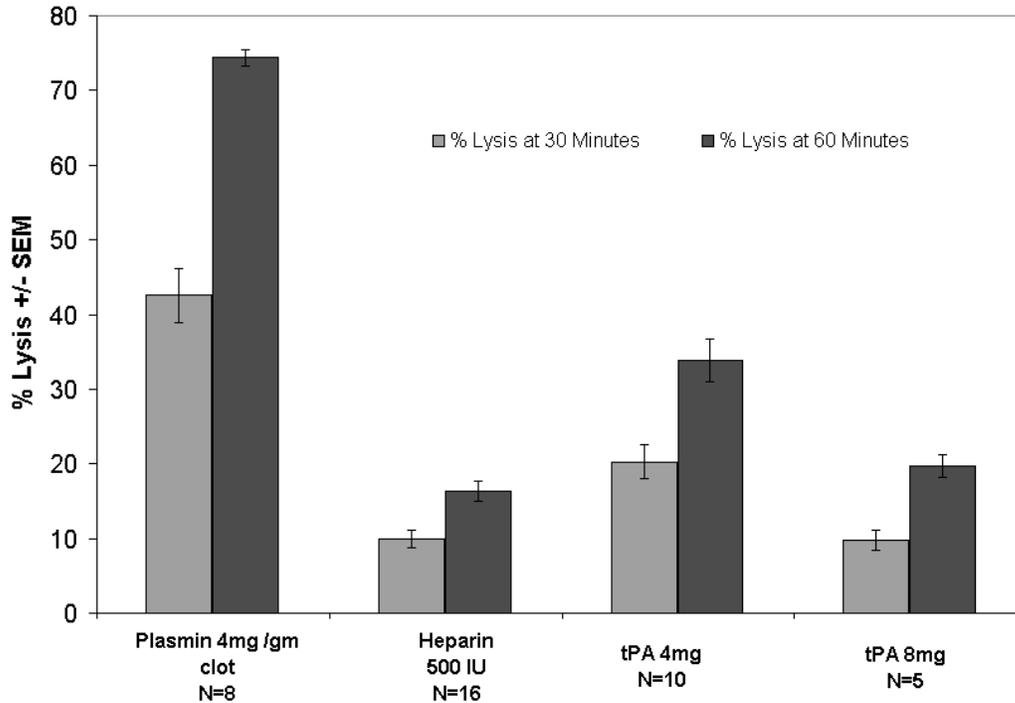


Figure 5.4 Lysis of Sized (0.7cm) sheep clots with plasmin, heparin, or rtPA.

Sheep clots weighing 10g were sized in a syringe to approximately 0.7cm in diameter. The clots were then treated with test article and clot lysis was monitored by weighing clots remaining on a sieve at 0, 30, and 60 minutes. Percent lysis was based on the amount of clot remaining from initial.

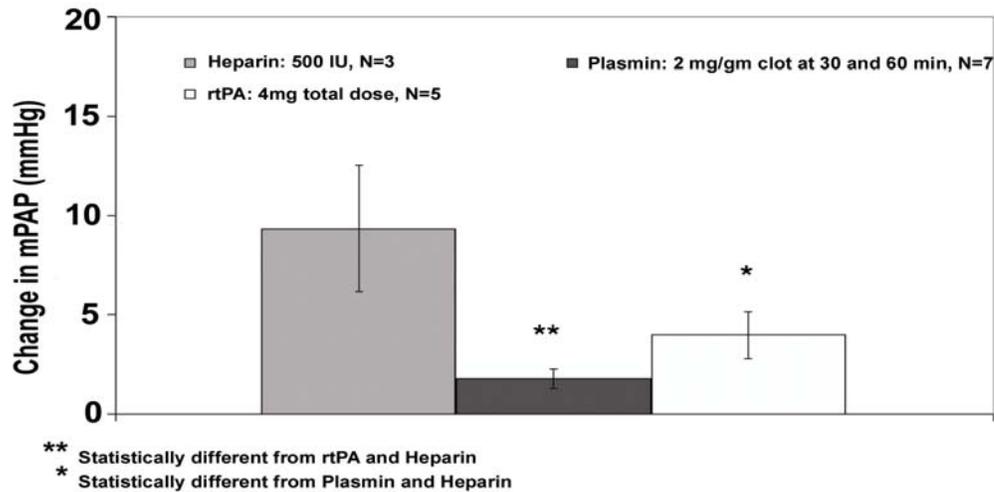


Figure 5.5 Change in mean pulmonary arterial with differing fragment size. Clots were lysed ex vivo with heparin, plasmin, or rt-PA for 60 minutes, the residual clot was thoroughly rinsed with saline, and then embolized through a venous conduit placed in the jugular vein of a sheep. This graph represents the result of one embolization procedure per sheep. A Swan-Ganz pulmonary arterial pressure catheter was placed in the contralateral jugular vein and advanced into the pulmonary artery. The catheter was then used to monitor pre and post pulmonary pressures. Differences between pre and post embolization pressures were compared.

#### **5.2.2.4 Summary**

These preliminary studies were designed to investigate the role of acute pulmonary emboli in sheep. All clots were lysed in a test tube outside the animal, washed thoroughly, and then injected into the animal. TPA and plasmin were used as agents to decrease clot size enzymatically, and were not administered directly into the animal. Typically, the sheep is sensitive to insults to the lung as the pulmonary circulation has a larger population of resident macrophages than man or other non-hoofed animals. Therefore, this model has the potential to be a sensitive method of detecting pulmonary insults. Because it is difficult to experimentally ascertain the effect of small thrombi in man, the sheep may prove to be a surrogate for these studies. The studies describe the ability to correlate relatively modest differences in clot burdens with an increase in pulmonary pressure. While, the effects in humans can vary depending upon the vascular mediators released, and the effects can also be independent of clot mass, the sheep appears to be susceptible to clot mass—at least with sizes of clots tested. It is important to note that none of the clots in graph 5.5 would be considered pulmonary hypertension. We attempted to inject multiple clots sequentially and the results, while not shown, demonstrated that the response to increased clot burden was not recalcitrant up to a mass of approximately 35-40 grams. However, it is unknown if the previously embolized thrombi did indeed exert an effect, thus making any interpretation complicated. Future studies

should investigate whether increases in TXA2 or other vasoactive amines contribute to this response and what other cardiopulmonary effects may also be affected by clot burden, e.g., arterial CO<sub>2</sub>, blood pH, and cardiac output.

### **5.2.3            *Future ROTEG studies***

Many additional studies are needed to further explore the usefulness of the ROTEG to quantitate fibrin formation. In particular, a thorough validation of the assay procedure needs to occur to define the accuracy and precision of this instrument at lower levels of FVIII supplementation in mouse whole blood. In addition, the role of TF has not been tested and it may be possible to augment the response through the addition of small amounts of TF, or by increasing the sensitivity through the inhibition of TF by using a monoclonal antibody to mouse TF. The ability of small amounts of TF to trigger the coagulation response in the mouse is an important variable when working at vanishingly small quantities of FVIII. In addition, this technique could be used to identify populations within hemophilia patients that have different bleeding phenotypes. It may be possible to further characterize patients with severe to moderate hemophilia using a ROTEG profile that could refine their FVIII dosing regimens. This could help patients to both minimize the number of injections they need per week (currently 2-3 times per week) and decrease their dosages which would have dramatic financial implications for these patients. In addition, the use of the ROTEG could

be used to investigate mouse models in which coagulation factors are knocked-out, knocked-in, inhibited (knocked-down), or mutated to increased activity or persistence in plasma. For example, clot structure in a Factor XIII knock-out or knock-down mouse model could be investigated to determine the impact of this protein on clot formation. In addition, mutated proteins such as a persistent or more active FVIII molecule could be administered to FVIII  $-/-$  mice and then clot formation could be explored temporally to determine the differences in the clearance between mutated and non-mutated FVIII proteins. The ability to measure low levels of FVIII enables this last experiment to be plausible.