A Quantitative and Mechanistic Assessment of Activated Thrombin-Activatable Fibrinolysis Inhibitor and its Role in Pathological Bleeding and Thrombosis

by

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Abstract

The coagulation and fibrinolytic systems are linked by the thrombin-thrombomodulin complex which regulates each system through activation of protein C and TAFI, respectively. We have used novel assays and techniques to study the enzymology and biochemistry of TAFI and TAFIa, to measure TAFI activation in hemophilia A and protein C deficiency and to determine if enhancing TAFI activation can improve hemostasis in hemophilic plasma and whole blood.

We show that TAFIa not TAFI attenuates fibrinolysis in vitro and this is supported by a relatively high catalytic efficiency ($16.41 \mu M^{-1}s^{-1}$) of plasminogen binding site removal from fibrin degradation products (FDPs) by TAFIa. Since the catalytic efficiency of TAFIa in removing these sites is ~60-fold higher than that for inflammatory mediators such as bradykinin it is likely that FDPs are a physiological substrate of TAFIa. The high catalytic efficiency is primarily a result of a low $K_m$ which can be explained by a novel mechanism where TAFIa forms a binary complex with plasminogen and is recruited to the surface of FDPs. The low $K_m$ also suggests that TAFIa would effectively cleave lysines from FDPs during the early stages of fibrinolysis (i.e. at low concentrations of FDPs).

Since individuals with hemophilia suffer from premature fibrinolysis as a result of insufficient TAFI activation we quantified TAFI activation in whole blood from hemophilic subjects. Both the rate of activation and the area under the TAFI activation time course (termed TAFIa potential) was determined to be reduced in hemophilia A and the TAFIa potential was significantly and inversely correlated with the clinical bleeding
phenotype. Using a novel therapeutic strategy, we used soluble thrombomodulin to increase TAFI activation which improved the clot lysis time in factor VIII deficient human plasma and hemophilic dog plasma as well as hemophilic dog blood.

Finally, we briefly show in a biochemical case study that TAFI activation is enhanced in protein C deficiency and when afflicted individuals are placed on Warfarin anticoagulant therapy, TAFI activation is reduced. Since TAFIa stabilizes blood clots, this suggests that reducing TAFI activation or inhibiting TAFIa may help restore blood flow in vessels with pathological thrombosis.
Co-Authorship

Chapter 2: Ms. Paula Kim helped develop the TAFIa assay used in this chapter and Dr. Michael Nesheim offered experimental advice, helped write this chapter and was the primary editor.

Chapter 3: Dr. Nesheim offered experimental advice and contributed to the data analysis by developing the mathematical models. He also helped write this chapter and was the primary editor. Paul Cook provided essential materials that allowed us to complete the project.

Chapter 4: Dr. Michael Nesheim offered experimental advice, contributed to the data analysis, helped write this chapter and was the primary editor.

Chapter 5: Dr. Georges Rivard organized the study and provided clinical samples to be assayed for TAFIa. Dr. Nesheim helped write and edit this chapter. Dr. Kathleen Brummel-Ziedins organized the study, provided clinical samples, contributed to the data analysis, helped write this chapter and was the primary editor.

Chapter 6: Dr. Karl-Uwe Petersen provided Solulin and advice pertaining to the use of Solulin. Dr. David Lillicrap provided access to whole blood from the hemophilic dog colony and Dr. Nesheim offered experimental advice, contributed to the data analysis, helped write this chapter and was the primary editor.

Chapter 7: Dr. Edwin Bovill provided clinical samples to be assayed for TAFIa. Dr. Nesheim helped write and edit this chapter. Dr. Kathleen Brummel-Ziedins organized the study, provided clinical samples, contributed to the data analysis, helped write this chapter and was the primary editor.
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<table>
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<th>Description</th>
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<tr>
<td>5IAF</td>
<td>5-idoacetamidofluorescein</td>
</tr>
<tr>
<td>AAFR</td>
<td>N-(4-methoxyphenylazoformyl)-Arg-OH</td>
</tr>
<tr>
<td>aPCC</td>
<td>Activated prothombinase complex concentrate</td>
</tr>
<tr>
<td>AUEC</td>
<td>Area under the elasticity curve</td>
</tr>
<tr>
<td>BU</td>
<td>Bethesda Units</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CPU</td>
<td>Carboxypeptidase U</td>
</tr>
<tr>
<td>ε-ACA</td>
<td>ε-aminocaproic acid</td>
</tr>
<tr>
<td>FDP</td>
<td>High molecular weight fibrin degradation products, an analogue of plasmin modified fibrin</td>
</tr>
<tr>
<td>FEIBA</td>
<td>Factor eight bypassing activity</td>
</tr>
<tr>
<td>fpA</td>
<td>Fibrinopeptide A</td>
</tr>
<tr>
<td>fpB</td>
<td>Fibrinopeptide B</td>
</tr>
<tr>
<td>FVIII</td>
<td>Factor VIII</td>
</tr>
<tr>
<td>fVIII-DP</td>
<td>fVIII-deficient plasma</td>
</tr>
<tr>
<td>fX</td>
<td>Factor X</td>
</tr>
<tr>
<td>fXa</td>
<td>Factor Xa</td>
</tr>
<tr>
<td>Glu-Pg</td>
<td>Native plasminogen</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N’-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>IIa</td>
<td>Thrombin</td>
</tr>
<tr>
<td>k_cat</td>
<td>Maximum rate of enzyme catalysis per unit enzyme</td>
</tr>
<tr>
<td>K_d</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>K_m</td>
<td>Michaelis-Menten constant, equal to one-half of maximal rate</td>
</tr>
<tr>
<td>Lys-Pg</td>
<td>Plasmin cleaved (K77/78) native plasminogen</td>
</tr>
<tr>
<td>NP</td>
<td>Normal plasmin</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor, type 1</td>
</tr>
<tr>
<td>PC</td>
<td>Protein C</td>
</tr>
<tr>
<td>PCPS</td>
<td>Phosphatidylcholine, phosphatidylserine</td>
</tr>
<tr>
<td>Pn</td>
<td>Plasmin</td>
</tr>
<tr>
<td>PPAck, FPRck</td>
<td>D-Phe-Pro-Arg chloromethyl ketone</td>
</tr>
<tr>
<td>PTCI</td>
<td>Potato Tuber Carboxypeptidase Inhibitor (TAFIa inhibitor)</td>
</tr>
<tr>
<td>QSY</td>
<td>QSY9 C5-maleimide</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SERPINS</td>
<td>Serine Protease Inhibitors</td>
</tr>
<tr>
<td>sTM</td>
<td>Truncated soluble thrombomodulin with M388L mutation</td>
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<tr>
<td>TAFI</td>
<td>Thrombin Activatable Fibrinolysis Inhibitor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>TAFIa</td>
<td>Activated thrombin activatable fibrinolysis inhibitor</td>
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<tr>
<td>TAT</td>
<td>Thrombin-antithrombin</td>
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<tr>
<td>TDP</td>
<td>TAFI-deficient plasma</td>
</tr>
<tr>
<td>TP</td>
<td>TAFIa potential</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue-type plasminogen activator</td>
</tr>
<tr>
<td>VFKck</td>
<td>D-Val-Phe-Lys chloromethyl ketone</td>
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Chapter 1
Introduction

The coagulation and fibrinolytic systems lie dormant in the blood as a collection of zymogens, substrates, cofactors and inhibitors until such a time that they are activated. The complex mechanisms that control hemostasis are at work when an injury to the vasculature occurs. When this happens, the coagulation pathway is activated and, as a result, activated platelets and fibrin form a tight cross-linked mesh that prevents blood flow out of the vessel. In order for this process to be efficient, an acute localized response is required upon injury. While the coagulation system exists to form a blood clot and prevent blood loss, the fibrinolytic system exists to degrade blood clots and ensure adequate blood flow during vessel repair. Fibrinolysis, like coagulation, is highly regulated and localized. In essence these two systems work in cooperation to maintain hemostasis at the site of injury while ensuring fluidity of the blood elsewhere.

Coagulation

Coagulation can be initiated through the tissue factor pathway or the contact pathway. Figure 1-1 shows that the tissue factor and contact pathways contain a common complex called the prothrombinase complex which activates prothrombin to thrombin.
Figure 1-1. The coagulation cascade
Coagulation can be initiated either by the tissue factor pathway (yellow) or the contact pathway (grey). Once coagulation is initiated, thrombin (IIa) is generated through the prothrombinase complex (blue) which is common to both pathways.

Tissue Factor Pathway

Activation of the tissue factor pathway (also known as the extrinsic coagulation cascade) is assumed to be the physiological trigger for blood coagulation (1). When a vessel is injured, membrane bound tissue factor is exposed. It binds to fVII/fVIIa and acts as a cofactor in activating fIX and fX (2). Activated fX can cleave prothrombin to form thrombin at a very low rate or become the enzymatic component of the prothrombinase complex which can generate thrombin much more efficiently.
Contact Pathway

The contribution of the contact pathway in physiological hemostasis is not well understood; however, when activated, the procoagulant activities of the coagulation system are increased (3). The contact pathway is so named because it is activated when blood comes into contact with an artificial surface such as glass or plastic (4). Through the sequential activation of several zymogens (fXII, fXI, fIX and fX) prothrombin is ultimately activated via the prothrombinase complex. Although the physiological contribution of the contact pathway to coagulation remains elusive, recent data suggest that it plays some role in physiological blood coagulation, since mice deficient in FXII have been shown to be protected against both stroke (5) and arterial thrombosis (6).

Prothrombinase and Thrombin

The tissue factor and contact pathways become part of a common pathway upon activation of fX by intrinsic or extrinsic tenase. Activated fX (fXa) is the enzymatic component of the prothrombinase complex. The prothombinase complex is the complex containing fXa and the cofactor fVa that assembles on a procoagulant phospholipid surface via interactions involving calcium ions. The prothrombinase complex activates prothrombin to thrombin (IIa) 300,000-fold more efficiently that fXa alone (7). The relative inefficiency of fXa in activating prothrombin (7) results in very little thrombin being generated via the tissue factor pathway. The low level of thrombin generated via this pathway; however, is sufficient to enhance thrombin generation by establishing positive feedback loops. Thrombin generation is upregulated though the activation of
platelets (8) which results in exposure of procoagulant phospholipid (a component of intrinsic and extrinsic tenase and prothrombinase) and the sequential activation of procofactors fV and fVIII to fVa and FVIIIa (9) which are cofactors of the prothrombinase complex and intrinsic tenase, respectively (3). Thrombin has also been shown to activate fXI which also contributes to enhanced thrombin generation and coagulant activity (10). Thrombin generation via the tissue factor pathway occurs in the initiation phase of coagulation and positive feedback results in coagulation entering the propagation phase. Following the propagation phase, thrombin generation enters the termination phase. The termination phase is also induced by the action of thrombin. Thrombin activates protein C and the catalytic efficiency of this reaction is over 1000-fold higher when thrombin is bound to a cofactor called thrombomodulin (11). Activated protein C (aPC) down-regulates thrombin generation by cleaving fVa and fVIIIa thus making them inactive (12-14). Once thrombin generation has been down-regulated by aPC, the rate of thrombin inhibition by serine protease inhibitors (SERPINS) exceeds that of thrombin generation and thrombin activity is lost. Regardless of the many roles of thrombin, perhaps, none are more important to hemostasis than the proteolytic cleavage of fibrinopeptides A (fpA) and B (fpB) from fibrinogen (15). Removal of fpA and fpB results in spontaneous association of the cleaved monomers to form an insoluble polymer called fibrin and this fibrin mesh constitutes the major protein component of blood clots.
**Fibrinolysis**

Plasminogen is activated by tPA to form the serine protease plasmin that is responsible for degrading fibrin clots. Figure 1-2 shows that fibrin and modified-fibrin act as cofactors for tPA-mediated plasminogen activation.

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**Figure 1-2. Plasminogen activation and fibrinolysis.**

Tissue-type plasminogen activator (tPA) activates plasminogen (Pg) to plasmin (Pn). This reaction is increased 1000-fold in the presence of fibrin. After plasmin is generated it cleaves fibrin and creates C-terminal lysine and arginine residues. The exposed C-terminal lysine residues tightly bind fibrin and as a result the cofactor activity is 3-fold higher than intact fibrin. Finally, TAFIa down-regulates fibrinolysis by removing these C-terminal lysine residues which causes a large reduction in the cofactor activity of fibrin for tPA-mediated plasminogen activation.
Fibrin(ogen)

One of the key events in coagulation is cleavage of fibrinogen by thrombin to form fibrin. This event is both the final step in coagulation and the first step in fibrinolysis. Fibrinogen is a homodimer consisting of 2 Aα, 2 Bβ and 2 γ chains (15). These chains are covalently attached by a complex series of disulfide bonds. When fibrin is formed the primary substrate of the fibrinolytic system is generated and fibrinolysis is initiated. Fibrin is eventually solubilized by plasmin and blood fluidity is restored.

Plasmin(ogen) and plasmin-modified fibrin

The fibrinolytic system has not yet been formally separated into initiation, propagation and termination phases like thrombin generation has been; however, similarly structured events likely occur. Tissue-type plasminogen activator (tPA) is secreted from endothelial cells during clot formation. TPA, as the name suggests, activates plasminogen (Pg) to form the serine protease plasmin (Pn) which is responsible for solubilizing the fibrin clot. Activation of plasminogen by tPA is enhanced 1000-fold in the presence of fibrin (16). Since very little free tPA exists in plasma in the absence of fibrin (17), this fibrin-dependent step in plasminogen activation can be referred to as the initiation phase of fibrinolysis. In the initiation phase, the plasmin generated cleaves fibrin after select lysine and arginine residues. Plasminogen and tPA have low and high affinity lysine binding sites in their kringle domains (18-20) and exposure of C-terminal lysine residues by plasmin results in a 3-fold increase in catalytic efficiency of tPA-dependent plasminogen
activation compared to in the presence of intact fibrin (21). At this stage, if not for TAFIa, fibrinolysis would enter the propagation phase.

**TAFI and TAFIa-modified fibrin**

The coagulation and fibrinolytic systems are linked via the thrombin-thrombomodulin (IIa-TM) complex (22). As mentioned, IIa-TM effectively activates protein C to down-regulate coagulation. Fibrinolysis is also down-regulated by the IIa-TM complex through the activation of TAFI (Figure 1-3). The TAFI gene, named \( CPB2 \) is located on chromosome 13 at position 13q14.11 (23). It is expressed in the liver, contains 11 exons and spans approximately 48 kb of genomic DNA (24). It has been speculated that single nucleotide polymorphisms in the 3’-untranslated region of \( CPB2 \) makes the resultant mRNA instable which may affect the plasma concentration of TAFI (25). It has also been suggested that TAFI expression is at least in part mediated by the cAMP/protein kinase A pathway (26).
Figure 1-3. Coagulation and fibrinolysis are linked through the thrombin-thrombomodulin complex. The IIa-TM complex efficiently activates by protein C and TAFI which down-regulate coagulation (II → IIa) and fibrinolysis (Pg → Pn), respectively.

Activated TAFI (TAFIa) is a carboxypeptidase B-like enzyme whose primary known physiological substrate is plasmin-modified fibrin (27). Recently, this has become a subject of debate since TAFIa also effectively cleaves and inactivates inflammatory mediators such as bradykinin, complement C5a, C3a and osteopontin (28). In removing the C-terminal lysine and arginine residues from Pn-modified fibrin TAFIa decreases the cofactor activity of fibrin by >97% (21) and in doing so prevents fibrinolysis from entering the propagation phase. Since TAFIa-modified fibrin does retain some cofactor activity for tPA-dependent plasminogen activation, plasmin continues to be generated albeit at a much slower rate (21). After thrombin generation ceases, TAFI activation is also greatly reduced. One of the unique attributes of TAFIa is its relatively short half-life
of 8 (29) or 16 (30) minutes at 37°C depending on the isoform. Recently, the mechanism of TAFIa inactivation was clarified using the crystal structure of TAFI (31). TAFI contains a highly dynamic flap region consisting of residues 296-350 which contain residues of the activate site wall. Removal of the activation peptide of TAFI results in increased mobility of this dynamic flap region. Once the activation peptide is removed, the region is destabilized and TAFIa decays.

The inception of the propagation phase of fibrinolysis has not been defined but it is likely a result of two key events: generation of Lys-Pg and spontaneous decay of TAFIa. Lys-Pg is generated by cleavage of native Pg (Glu-Pg) at lysine 77/78 by plasmin. This is a significant step in fibrinolysis since Lys-Pg is a 20-fold better substrate for tPA than Glu-Pg (32) and it can effectively bind and use TAFIa-modified fibrin as a cofactor (32) which allows it to avoid regulation by TAFIa. As the concentration of TAFIa decreases due to spontaneous decay, more plasmin modified fibrin is available and Glu-Pg is also effectively activated by tPA. These two processes, Lys-Pg generation and TAFIa decay, likely contribute to fibrinolysis entering the propagation phase. The termination phase of fibrinolysis occurs when plasminogen activation ceases. This phase potentially involves many processes. Both plasmin and tPA are effectively inhibited by the plasma SERPINS, α2-antiplasmin and plasminogen activator inhibitor, type 1 (PAI-1), respectively. So as the rate or plasmin generation or tPA release falls below the rate of inhibition, the steady state concentration of the respective enzymes is quickly reduced. Other processes that are likely involved, include solubilization of the fibrin cofactor for plasminogen activation and a secondary burst of TAFI activation which is mediated by
plasmin (33,34) that would further diminish the cofactor activity of soluble fibrin degradation products.

**Pathology of improperly regulated hemostasis**

In incidences when hemostasis is not properly balanced during injury, hemorrhage or thrombosis occurs. While excessive bleeding or clotting is generally thought to be a result of insufficient or excessive coagulation, respectively, it is more likely a complex function of inadequacies of both coagulation and fibrinolytic systems. Since the coagulation pathways are linked to the fibrinolytic system, any distortion of the coagulant system is likely mirrored or amplified by the fibrinolytic system.

**Hemophilia A and Factor VIII**

Hemophilia A is an extensively studied coagulation disorder that affects approximately 1 in 5000 males (35). In hemophilia A the gene that codes for factor (f)VIII, the cofactor of the intrinsic tenase complex, is mutated resulting in expression of protein that has reduced procoagulant activity. Hemophilia A is further classified as being mild, moderate or severe depending on the percentage of fVIII procoagulant activity (fVIII:C). An individual is diagnosed with mild hemophilia when the fVIII:C is 5-40% of normal. Moderate hemophiliacs have a fVIII:C between 1-5% and individuals with severe hemophilia have fVIII:C <1% (36). The clinical severity of the disease is usually a reflection of the level of fVIII:C; however, this is not always the case. Some severe hemophiliacs rarely have clinically severe bleeding episodes whereas some moderate hemophiliacs have severe bleeds while others do not (37). This variability in the bleeding
phenotype suggests that coagulation or fibrinolytic factors other than fVIII contribute to the bleeding tendency of moderate and severe hemophiliacs. It has recently been hypothesized that bleeding in hemophilia may be due to unregulated fibrinolysis in addition to insufficient coagulation (38,39).

**Thrombosis**

A large proportion of the population is at risk of developing a pathological thrombus whether in the form of a stroke, venous thromboembolism, or arterial thrombosis (40). Generally speaking, unwanted clots form as a result of unregulated or excessive thrombin generation (41) which causes an increase in TAFI activation. Excessive TAFI activation would attenuate fibrinolysis and consequently prevent the restoration of blood flow in a timely manner. Since TAFI is also activated by plasmin, recanalization of an occluded vessel with thrombolytic therapeutics such as Activase (tPA) may not be optimized. Recently, Brouns *et al.* (42) demonstrated that high levels of TAFIa are generated during both intravenous and intraarterial infusion of a thrombolytic in patients with ischemic stroke. This small study shows that the maximal level of TAFIa achieved is inversely correlated the recanalization rate and suggest that a TAFIa inhibitor would decrease that time required to restore blood flow. Whether TAFI activation contributes to the clinical outcome of pathological thrombosis remains to be seen but recent evidence suggests that TAFI(a) may have predictive value and could potentially influence the efficacy of treatment.
Current Studies

The current studies were undertaken to study the nature of the link between coagulation and fibrinolysis, particularly as it pertains to the biochemistry and enzymology of TAFI and TAFIa, to measure TAFI activation in hemophilia A and protein C deficiency, and to investigate how bleeding in hemophilia A may be controlled by suppressing fibrinolysis.

Hypotheses

The hypotheses that guided these studies were as follows:

1. TAFI is activated even in the absence of soluble thrombomodulin and this activated TAFI attenuates fibrinolysis.

2. TAFIa efficiently cleaves lysine residues and plasminogen binding sites from fibrin degradation products with high catalytic efficiency.

3. TAFI activation can be enhanced with soluble thrombomodulin and this has a positive influence on the clot lysis time in hemophilic plasma.

4. TAFI activation is reduced in whole blood from subjects with hemophilia A and is correlated with the clinical bleeding phenotype in this population.

5. Soluble thrombomodulin may be used to increase the clot lysis time in hemophilic dog blood.

6. TAFI activation is increased in whole blood from subjects with congenital protein C deficiency and reduced in subjects on Warfarin anti-coagulation.
Aims/ Objectives

The aims/ objectives of the current studies are as follows:

1. To determine whether TAFI or TAFIa is the physiological attenuator of fibrinolysis

2. To determine the kinetics of TAFIa in removing plasminogen binding sites and cleaving C-terminal lysine residues from fibrin degradation products

3. To determine the impact of fVIII on TAFI activation and if TAFI activation and the clot lysis time may be increased in hemophilic plasma supplemented with soluble thrombomodulin

4. To determine the rate and extent of TAFI activation in whole blood from healthy and hemophilic subjects and correlate measures of TAFI activation to the clinical bleeding phenotype

5. To determine if soluble thrombomodulin may be used to improve hemostasis in whole blood from hemophiliic dogs

6. To determine the time course of TAFI activation in whole blood from subjects with congenital protein C deficiency.

To accomplish these objectives we have shown that (1) TAFI zymogen does not play a significant role in the attenuation of fibrinolysis; (2) and the kinetics of TAFIa are such that very little active enzyme is required to attenuate plasminogen activation. We also show that (3) the rate of TAFI activation and the TAFIa potential is reduced in
hemophilia A whole blood and the TAFIa potential is significantly and inversely correlated with bleeding phenotype. Soluble thrombomodulin may be used to enhance TAFI activation in order to prolong fibrinolysis in (4) fVIII-deficient plasma clots and (5) hemophilic canine whole blood. We also briefly show that (6) TAFI activation in a protein C deficient kindred that are predisposed to thrombosis, have slightly increased TAFI activation compared to healthy individuals and that Warfarin, a vitamin K antagonist used to down-regulate coagulation, decreases TAFI activation to levels observed in an individual with a protein C mutation and no history of thrombosis.
Chapter 2

Thrombin-activatable fibrinolysis inhibitor zymogen does not play a significant role in the attenuation of fibrinolysis
Abstract

Activated thrombin-activatable fibrinolysis inhibitor (TAFIa) plays a significant role in the prolongation of fibrinolysis. During fibrinolysis, plasminogen is activated to plasmin, which lyases a clot by cleaving fibrin after selected arginine and lysine residues. TAFIa attenuates fibrinolysis by removing the exposed C-terminal lysine residues. It was recently reported that TAFI zymogen possesses sufficient carboxypeptidase activity to attenuate fibrinolysis through a mechanism similar to TAFIa. Here, we show with a recently developed TAFIa assay that when thrombin is used to clot TAFI-deficient plasma supplemented with TAFI, there is some TAFI activation. The extent of activation was dependent upon the concentration of zymogen present in the plasma, and lysis times were prolonged by TAFIa in a concentration-dependent manner. Potato tuber carboxypeptidase inhibitor, an inhibitor of TAFIa but not TAFI, abolished the prolongation of lysis in TAFI-deficient plasma supplemented with TAFI zymogen. In addition, TAFIa but not TAFI catalyzed release of plasminogen bound to soluble fibrin degradation products. The data presented confirm that TAFI zymogen is effective in cleaving a small substrate but does not play a role in the attenuation of fibrinolysis because of its inability to cleave plasmin-modified fibrin degradation products.
Introduction

Thrombin-Activatable Fibrinolysis Inhibitor (TAFI) is a 60-kDa carboxypeptidase-like protein that circulates in plasma at a concentration of ~75 nM (43). TAFI (also known as procarboxypeptidase U, plasma carboxypeptidase B, and carboxypeptidase R) was discovered independently by several groups (44-48), and its role in fibrinolysis was subsequently characterized (44). Thrombin in complex with thrombomodulin activates TAFI with a catalytic efficiency of 1.2 μM⁻¹s⁻¹, which is ~1250-fold greater than that with thrombin alone (22). Plasmin has also been shown to activate TAFI (0.008 μM⁻¹s⁻¹), and the second-order rate constant of this reaction increases by 16-fold in the presence of unfractionated heparin (33).

Activated TAFI (TAFIa) plays a significant role in attenuating fibrinolysis. During fibrinolysis, plasminogen is activated to plasmin, which can then solubilize the fibrin clot by cleaving fibrin after specific arginine and lysine residues (49). TAFIa attenuates fibrinolysis by removing the exposed C-terminal lysine residues on fibrin (48,50), thereby decreasing the tissue-type plasminogen activator (tPA) cofactor activity of plasmin-modified fibrin (21). Removal of these C-terminal lysine residues suppresses Pg activation (27,51) and down regulates the conversion of Glu-Pg to Lys-Pg. This is significant since Lys-Pg is a 20-fold better substrate for tPA than Glu-Pg (32). TAFIa is intrinsically unstable, with its inactivation being highly temperature-dependent (30). The existence of a TAFIa concentration threshold has been demonstrated, such that TAFIa at a concentration above the threshold inhibits fibrinolysis. Once TAFIa is thermally
inactivated and its level falls below the threshold lysis enters the propagation phase (52), and fibrin is quickly solubilized (52-54).

TAFI zymogen also has some carboxypeptidase activity. Previously, the carboxypeptidase activity of TAFI zymogen has been assigned to small synthetic substrates such as hippuryl-linked amino acids (55,56) and N-(4-methoxyphenylazoformyl)-Lys-OH (57). Recently, TAFI zymogen has been shown to have carboxypeptidase activity toward the tetrapeptide PFGK and larger fibrin peptides (55). Like TAFI, procarboxypeptidase A exhibits carboxypeptidase activity toward short peptides (58), but neither has been shown to cleave macromolecules.

Valnickova et al. (55) reported recently that the zymogen TAFI, acting as a carboxypeptidase, has the ability to suppress fibrinolysis (55). Willemse et al. (59) measured the generation of TAFIa activity during the clotting of human platelet-poor plasma and found that when clotting was initiated with thrombin plus calcium or calcium alone, TAFIa activity increased. They also confirmed that when coagulation was initiated with calcium, lysis was prolonged by the addition of TAFI in a concentration-dependent manner; however, when clotting was initiated with batroxobin, an enzyme that clots fibrinogen but does not activate TAFI, the prolongation of lysis was abolished. Consequently, the authors suggested that any prolongation of lysis is due to TAFIa generated upon the addition of thrombin plus calcium or calcium alone. Valnickova et al. (60) have rebutted these conclusions by pointing out that the carboxypeptidase activity assay used by Willemse et al. (59) is not specific for TAFIa and that clotting induced with batroxobin is not an optimal model because the mechanical properties, clot
Experimental Procedures

Materials – Newborn calf serum, Dulbecco's modified Eagle's medium/nutrient mixture F-12 (1:1), Opti-MEM, and penicillin/streptomycin/Fungizone mixture were obtained from Invitrogen. N,N-Dimethylformamide, 2-mercaptoethanol, and εACA acid were obtained from Fisher, Bio-Rad, and Sigma, respectively. Potato tuber carboxypeptidase inhibitor (PTCI), Phe-Pro-Arg-chloromethyl ketone, and Val-Phe-Lys-chloromethyl ketone were obtained from Calbiochem. Ancrod was obtained from Roche Applied Science GmbH (Mannheim, Germany). N-(4-Methoxyphenylazoformyl)-Arg-OH (AAFR) was purchased from Bachem Biosciences, Inc. (King of Prussia, PA). Baby hamster kidney cells and the mammalian expression vector pNUT were graciously provided by Dr. Ross MacGillivray (University of British Columbia). Recombinant human soluble thrombomodulin was a kind gift from Dr. Oliver Kops (Paion GmbH, Aachen, Germany). Methotrexate (Mayne Pharma Inc., Montreal, Quebec, Canada) and Activase (tPA) were obtained from Kingston General Hospital (Kingston, Ontario, morphology, and protein composition differ from those of clotting induced with thrombin.

This study was undertaken to determine whether TAFI zymogen is antifibrinolytic or whether thrombin at a low level (5 nM) activates TAFI to a degree sufficient to attenuate lysis. A new assay specific for TAFIa (61) was used to measure TAFIa levels in human normal platelet-poor plasma supplemented with TAFI (66–1000 nM final concentrations) and clotted with thrombin plus calcium. The results indicate that TAFI zymogen does not attenuate fibrinolysis.
Canada). The cysteine-specific fluorescent probe 5-iodoacetamidofluorescein and the cysteine-specific quencher QSY® 9 C₅-maleimide were obtained from Invitrogen. 5-iodoacetamidofluorescein-labeled plasminogen (5IAF-Pg) (62), QSY 9 C₅-maleimide-conjugated high molecular mass fibrin degradation products (QSY-FDPs) (61,63), thrombin (63), recombinant human TAFI (29), TAFIa standards (64), TAFIa (29), and TAFI-deficient (64) and barium-absorbed (65) plasmas were prepared as described previously. The buffer used in all experiments (unless stated otherwise) was 0.2-μm filtered 20 mM HEPES, 150 mM NaCl, and 0.01% (v/v) Tween 80, pH 7.4 (HEPES-buffered saline). All plasmas used were dialyzed extensively against HEPES-buffered saline.

Clot Lysis Assays – TAFI-deficient plasma (TDP; 1:3 dilution) supplemented with 0–133 nM TAFI was clotted with 5 nM thrombin and 10 mM CaCl₂ and lysed with 1 nM tPA in the presence or absence of 10 μM PTCI (final concentrations). Coagulation and lysis were monitored turbidometrically at 37°C and 405 nm using a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA), and lysis times were determined. Lysis time is defined as the time required, after clot initiation, to decrease the absorbance to one-half of the plateau value achieved after clotting.

AAFR Cleavage by TAFI and TAFIa – A solution (80 μL) containing AAFR in the presence or absence of PTCI was added to wells of a 96-well plate, and the absorbance (349 nm) was monitored continually (1-min intervals) at room temperature using the
SpectraMax Plus plate reader. Between readings, TAFI or TAFIa (40 μL) was added to each well containing AAFR (±PTCI). In each experiment, the final concentrations were 120 μM AAFR, 0–20 μM PTCI, and 133 nM TAFI or 300 pM TAFIa.

**Preparation of Samples for Assay of TAFIa** – A 25 μL mixture containing thrombin, CaCl₂, and tPA was added to a number of 1.5 mL Eppendorf tubes. A 50 μL aliquot comprising 25 μL of TDP and 25 μL of recombinant TAFI (prewarmed to 37°C) was added to each tube with mixing, and the solutions were immediately incubated at 37°C. The final concentrations were 5 nM thrombin, 10 mM CaCl₂, 1 nM tPA, 66–1000 nM TAFI, and a 1:3 dilution of TDP. Ancrod (0.52 units/mL final concentration) instead of thrombin was used to initiate clotting in some experiments. Parallel clots were made in 96-well plates, and the absorbance (405 nm) was monitored at 37°C using the SpectraMax Plus plate reader to determine the time courses of clotting and subsequent lysis. All 96-well plates were blocked with 1% Tween 80 for >1 h, washed with deionized distilled water, and dried before use. Clotting and fibrinolysis were arrested at various time points using 100 μM Phe-Pro-Arg-chloromethyl ketone (1 μL). Clots were vortexed for 10 s and centrifuged at 13,000 × g for 1 min at room temperature, and the supernatant was removed and immediately placed on ice. Samples were diluted 1:5 and 1:25 using TDP and assayed for TAFIa.

**Effect of TAFI Zymogen on the Assay for TAFIa** – TDP was supplemented with 500 nM TAFI zymogen, and the plasma was assayed for TAFIa activity as described (61).
Assay of TAFIa – QSY-FDPs were prepared and characterized essentially as described (61). 80 μL of a solution of 1.25 μM QSY-FDPs and 62.5 nM 5IAF-Pg in HEPES-buffered saline was added to each well of an opaque 96-well plate and allowed to equilibrate at room temperature while continually (1-min intervals) monitoring the intensity with excitation and emission wavelengths of 480 and 520 nm, respectively, with a 495-nm emission cutoff filter using a SpectraMax Gemini XS fluorescence plate reader (Molecular Devices). TAFIa standards or samples (20 μL) then were added to the 96-well plate using a multichannel pipette between readings. Initial rates were measured for each standard, and a standard curve was generated. The initial rate of each sample was measured, and the standard curve was used to determine the TAFIa concentration.

Results and Discussion

Effect of PTCI on tPA-induced Lysis – Clotting and lysis were initiated in TDP supplemented with 0–133 nM TAFI in the presence or absence of 10μM PTCI. Clotting was initiated with 5nM thrombin plus 10 mM CaCl₂, and lysis was initiated with 1 nM tPA. Clot lysis profiles are presented in Figure 2-1. In the absence of PTCI, increasing TAFI zymogen levels resulted in an increase in the lysis time, as reported previously by Valnickova et al. (55). This suggests that the addition of TAFI to TDP prolongs lysis. However, PTCI (10μM) completely abolished this prolongation of lysis, giving a lysis time equal to that found in TDP without added TAFI (24 min).
Figure 2-1. The lysis time is dependent upon the concentration of TAFI zymogen. Increasing the TAFI zymogen concentration in TDP resulted in an increase in the lysis time when 1 nM tPA was used to trigger lysis. This effect was nullified by PTCI. The lysis time in TDP was the same in the presence or absence of 10 μM PTCI. As TAFI zymogen was added to TDP, the lysis time increased in the absence of PTCI. The lysis time in the presence of PTCI did not change regardless of the amount of TAFI zymogen added. As a reference, the clot lysis profile of TDP in the presence of PTCI is shown in each panel (— — —).
PTCI Inhibits TAFIa but Not TAFI – To determine whether the prolonged lysis seen in Figure 2-1 was due to TAFI zymogen or TAFIa, the carboxypeptidase substrate AAFR, was used to determine the effect of PTCI on the activities of TAFI and TAFIa. PTCI (0–20μM) was titrated into AAFR (120μM) (data not shown), and the absorbance was monitored continually (1-min intervals). Once a base line had been established, TAFI zymogen (133nM) or TAFIa (300pM) was added. PTCI at a concentration of 10μM was sufficient to completely inhibit the cleavage of AAFR by TAFIa (Figure 2-2A), but it had no inhibitory effect on TAFI zymogen (Figure 2-2B). The curvature in the progress curves for hydrolysis of AAFR is due to the depletion of substrate over time. Because ~450-fold more TAFI was used compared with TAFIa, the higher rate of AAFR cleavage by TAFI was expected. Because TAFIa is inhibited by PTCI but TAFI zymogen is not, any prolongation of lysis in the presence of PTCI would be due strictly to TAFI zymogen in the plasma. In the presence of PTCI, no prolongation of lysis by TAFI was detected (Figure 2-1). These observations indicate the enzyme TAFIa (not the zymogen TAFI) attenuates clot lysis.
Figure 2-2. PTCI completely inhibits TAFIa activity but has no effect on the carboxypeptidase activity of TAFI.

TAFIa (300pM; panel A) or TAFI (133nM; panel B) was added to AAFR (120μM) in the presence (—) or absence (——) of 10μM PTCI to determine the effect of PTCI on the carboxypeptidase activity of the enzyme and zymogen.
**TAFI Zymogen Does Not Cleave Soluble Fibrin Degradation Products** – The standard curve for our assay of TAFIa is generated by measuring the initial rate (over ~10 min) of 5IAF-Pg dissociation from soluble fibrin degradation products (QSY-FDPs) in the presence of known concentrations of TAFIa (61). TDP supplemented with 200pM TAFIa showed an increase in fluorescence, suggesting cleavage of lysines on QSY-FDPs and subsequent dissociation of 5IAF-Pg. When TDP was supplemented with 500nM TAFI zymogen and assayed for TAFIa, the rate of 5IAF-Pg dissociation was not significantly different from that in TDP (Figure 2-3), indicating that TAFI zymogen is not able to cleave the macromolecular substrate QSY-FDPs, thus demonstrating that our assay is specific for TAFIa.
Figure 2-3. Our assay for TAFIa is not sensitive to TAFI zymogen. TDP supplemented with 200pM TAFIa (20 μL) was added to an 80-μL solution containing 1.25μM QSY-FDPs and 62.5nM 5IAF-Pg. Upon the addition of 200pM TAFIa, an increase in fluorescence was observed (●). When TDP was supplemented with 500nM TAFI zymogen (▲), the extent of fluorescence increase was not significantly different from that in TDP (■).

TAFIa Concentration Time Courses during tPA-induced Clot Lysis – TAFI zymogen at various concentrations was added to TDP to determine its effect on lysis when clotting was induced with 5nM thrombin plus 10mM CaCl₂ and lysis was induced with 1nM tPA. A recently described assay specific for TAFIa (61) was utilized to confirm that TAFIa was indeed present. In experiments designed to duplicate those of Valnickova et al. (55), it was demonstrated that the amount of TAFI activated was dependent upon the concentration of TAFI zymogen in the plasma (Figure 2-4). In the absence of TAFI, the lysis time was ~24 min. As the concentration of TAFI was increased, the lysis time also
increased. When 66nM TAFI was added, the lysis time doubled to 50 min, and 110pM TAFIa was measured at its peak. At the highest TAFI concentration (1000nM), the lysis time was 6-fold longer (155 min), and 525pM TAFIa was measured at its peak (Figure 2-4). Similar trends were observed when the plasma was supplemented with 133 and 500nM TAFI. In all cases, TAFIa levels peaked between 20 and 40 min after coagulation was initiated, and the peak levels decreased as the concentration of TAFI zymogen decreased.
Figure 2-4. TAFI activation increases as the concentration of TAFI zymogen increases.
The time course of TAFI activation was measured in plasma clotted with 5nM thrombin and 10mM CaCl₂ and lysed with 1nM tPA. TAFIa concentrations were reported over time in TDP supplemented with 66–1000nM TAFI zymogen (•). Turbidity was monitored continuously at 405nm (—) to determine the timing of lysis. TDP (1:3 dilution) had a lysis time of 22.6 min. As TDP was supplemented with TAFI, lysis times increased, as did the peak concentration of TAFIa. When TDP was supplemented with 1000nM TAFI the lysis time increased to 155 min, and TAFIa levels peaked at 525pM.
Valnickova et al. (55) showed that when soluble thrombomodulin was included during the clotting and lysis of normal plasma, the lysis time was extended by >3-fold compared with that in the absence of soluble thrombomodulin. When PTCI was added with soluble thrombomodulin to inhibit TAFIa, the lysis time was the same as in the absence of soluble thrombomodulin, an observation from which Valnickova et al. inferred that TAFI is not activated in the absence of soluble thrombomodulin. However, TAFI activation was observed in plasma supplemented with TAFI, as shown in Figure 2-4, and attenuation of lysis can be attributed specifically to TAFIa. The activation of TAFI by thrombin has a relatively high $K_m$ compared with its plasma concentration (22), which implies that the addition of TAFI zymogen would increase the rate of TAFI activation in a concentration-dependent manner. As a result, as the TAFI concentration is increased, the TAFIa concentration increases, and the extent to which lysis is prolonged increases.

Because TAFI activation occurs during the propagation phase of coagulation, TAFI-deficient, barium citrate-adsorbed plasma was supplemented with 500nM TAFI to determine the extent of TAFI activation in plasma lacking an intrinsic coagulation pathway. When clotting was initiated with 5nM thrombin plus 10mM CaCl$_2$ and lysis was induced with 1nM tPA, the lysis time was 30.5 min, and TAFIa appeared transiently at 5 min and again at 40 min (Figure 2-5A). This is consistent with the timing of both thrombin addition and subsequent plasmin production. This demonstrates that even in plasma lacking an intrinsic coagulation pathway, 5nM thrombin is sufficient to activate some TAFI and to prolong lysis in the absence of soluble thrombomodulin. Clots formed in TAFI-deficient, barium citrate-adsorbed plasma with no added TAFI lysed in 19.3 min.
under the same conditions. When clotting was induced in TAFI-deficient, barium citrate-
adsorbed plasma with 500nM TAFI and 0.52 units/mL ancrod in place of thrombin plus
10mM CaCl₂ in the presence of 1nM tPA, the lysis time was 13.3 min, and the TAFIα
level peaked at 207pM 10 min after the clot had fully lysed (Figure 2-5B). This peak was
probably due to TAFI activation by plasmin. The extent of TAFI activation post-lysis was
similar to that observed when coagulation was induced with thrombin. When ancrod
(instead of thrombin) was used to initiate coagulation, the TAFIα peak that corresponded
with thrombin addition was absent, whereas the secondary peak due to TAFI activation
by plasmin remained (Figure 2-5B). The lysis time was much shorter than that observed
when clotting was initiated with thrombin (13.3 min compared with 30.5 min). Clotting
induced with ancrod in the presence of 500nM TAFI had shorter lysis times compared
with clotting initiated with thrombin in the absence of TAFI, which may be due to
differences in clot structure (60).
Figure 2-5. Plasma lacking an intrinsic coagulation pathway can yield TAFIa when low levels (5nM) of thrombin are added.

In all experiments, lysis was initiated by the addition of 1 nM tPA. With thrombin (A), TAFIa levels (●) peaked at 125 and 187 pM after 5 and 40 min, respectively. The lysis time was 30.5 min in TAFI-deficient, barium-absorbed plasma supplemented with 500 nM TAFI (—) compared with 19.3 min in TAFI-deficient, barium-absorbed plasma with no added TAFI (——). Ancrod was also used to clot TAFI-deficient, barium-absorbed plasma (B). There was very little TAFI activated until after the clot had lysed. TAFIa levels peaked at 207 pM at 30 min (•), whereas the lysis time was 13.3 min (——). The thrombin-induced clotting and subsequent lysis of TAFI-deficient, barium-absorbed plasma (no added TAFI) are shown as a reference (——).
In summary, our results show that whereas TAFI zymogen catalyzes cleavage of a small substrate, it does not catalyze cleavage of plasmin-modified fibrin (fibrin degradation products). Consequently, TAFI zymogen is not antifibrinolytic. The fact that increased TAFI zymogen added at time 0 results in increased lysis times is due to increased levels of TAFIa appearing as a consequence of activation by thrombin used to form the clot.

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Chapter 3

The kinetics of TAFIa cleavage of lysine residues of fibrin degradation products and removal of plasminogen binding sites
Abstract

Partial digestion of fibrin by plasmin results in exposure of C-terminal lysine residues. These lysines act as binding sites for both plasminogen and tissue-type plasminogen activator which has the effect of increasing the catalytic efficiency of plasminogen activation by approximately 3000-fold compared to tPA alone. It is generally accepted that TAFIa attenuates fibrinolysis by removing these C-terminal lysine residues thus reducing the catalytic efficiency of plasminogen activation by tPA by approximately 97%. The aim of these studies was to determine the kinetics of lysine cleavage from fibrin degradation products by TAFIa and to determine the kinetics of loss of plasminogen binding sites. Here, we show that the $k_{cat}$ and $K_m$ of Glu-Pg binding site removal was 2.34 s$^{-1}$ and 142.6nM, respectively, implying a catalytic efficiency of 16.41 $\mu$M$^{-1}$s$^{-1}$. The corresponding values of Lys-Pg binding site removal were 0.888 s$^{-1}$ and 94nM, respectively, implying a catalytic efficiency of 9.45 $\mu$M$^{-1}$s$^{-1}$. We also show that the catalytic efficiency of lysine cleavage, whether involved in plasminogen binding or not, is 1.32 $\mu$M$^{-1}$s$^{-1}$ and this increases to 3.83 and 3.35 $\mu$M$^{-1}$s$^{-1}$ in the presence of Glu- or Lys-Pg, respectively. The catalytic efficiency of plasminogen binding site removal by TAFIa is the highest of any reaction reported to date and suggests that the primary physiological substrate for TAFIa is plasmin-modified fibrin. In addition, the plasminogen dependent decrease in the $K_m$ associated with lysine cleavage suggests that an interaction between TAFIa and plasminogen comprises a component of the reaction mechanism.
**Introduction**

The fibrinolytic system is initiated upon formation of an insoluble fibrin clot at a site of injury. Initially, native or Glu-Plasminogen (Glu-Pg) weakly binds intact fibrin and is subsequently activated to plasmin (Pn) by tissues-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). Plasmin has two major functions in fibrinolysis. Plasmin directly degrades fibrin by cleaving after specific lysine and arginine residues thus creating soluble fibrin degradation products (FDP) and solubilizing the clot. Plasmin also enhances fibrinolysis by converting Glu-Pg to Lys-Pg (66). Both Glu-Pg and Lys-Pg can bind intact fibrin but with very different affinities. Lys-Pg binds intact fibrin with a $K_d$ of 1.2μM and Glu-Pg binds with a $K_d$ of 30μM (62). This process is tightly regulated in order to preserve the clot and prevent blood loss.

TAFIa (activated thrombin-activatable fibrinolysis inhibitor also known as carboxypeptidase U [CPU]) is central in regulating plasminogen activation and therefore fibrinolysis. TAFIa is the active form of the plasma zymogen TAFI which is activated by thrombin, plasmin or the thrombin-thrombomodulin complex (22,33,48). TAFIa is a plasma carboxypeptidase B-like enzyme that removes C-terminal lysine and arginine residues from plasmin-modified fibrin in order to suppress plasminogen activation and clot dissolution (27). Since thrombin clots fibrinogen and then attenuates fibrin degradation by activating TAFI, TAFI represents a link between coagulation and fibrinolysis (22). TAFIa regulates tPA dependent fibrinolysis half maximally at 1nM which is only 1-2% of the plasma TAFI zymogen pool (22) suggesting that modest activation of TAFI can have a profound effect on inhibition of fibrinolysis. TAFIa has no
known physiological inhibitor but is regulated by its own instability (29,31,67). It has been suggested that TAFIa inhibits fibrinolysis by a threshold mechanism (52,53). In this model, TAFIa completely inhibits fibrinolysis when its concentration is above the critical threshold; however, as TAFIa decays it falls below the threshold and fibrinolysis continues.

The aim of the present study is to quantitatively analyze the mechanism by which TAFIa regulates fibrinolysis. The primary function of TAFIa in regulating fibrinolysis is to remove plasminogen binding sites from plasmin-modified fibrin thus attenuating plasminogen activation and fibrinolysis. Recently, it was demonstrated that TAFI zymogen has carboxypeptidase activity toward synthetic fibrin peptides (55) which can be attributed to the positioning of the activation peptide over the active site (31). These large synthetic fibrin peptides (1400 or 2600 Da) are quite small compared to the smallest fibrin degradation product (DDE; 250,000 Da) and interestingly, TAFI zymogen does not seem to show relevant carboxypeptidase activity toward FDP (68). This discrepancy between fibrin peptides and FDPs as substrates for TAFI and TAFIa has prompted the development of a physiologically relevant model to aid in determination of the kinetics of TAFIa. Here, we treat fluorescent plasminogen bound in equilibrium with fibrin degradation products (an analogue of plasmin-modified fibrin(21,63)) with TAFIa to determine the kinetics of plasminogen binding site removal in this physiologically relevant system. In addition, we have measured the rate of lysine cleavage from FDPs using an NADH linked reaction.
Materials and Methods

Materials – Fibrinogen was prepared as previously described (63) with one exception: the solution was made to 1.2% PEG-8000 instead of 2% PEG-8000 by the addition of 40% (w/v) PEG-8000 in water, subsequent to β-alanine precipitation. This change in protocol allowed for a greater yield of fibrinogen. QSY-FDPs (fibrin degradation products that are covalently attached to the quencher, QSY9 C5-maleimide) preparation and TAFI isolation and activation were conducted as previously described (30). Since such low concentrations of TAFIa were used in experiments, TAFI was activated in the presence of 1mg/mL bovine serum albumin to prevent non-specific binding to plastics. Recombinant human Pg (S741C) and the fluorescein derivative (5IAF-Glu-Pg) were prepared as described by Horrevoets et al. (62). 5IAF-Lys-Pg was prepared by treating 5IAF-Glu-Pg with 200nM plasmin in the presence of 5mM ε-ACA for 3 hours at room temperature. Plasmin was subsequently removed using a 1mL benzamide sepharose 6B column (GE Healthcare Biosciences, Baie d'Urfé, PQ). Residual plasmin activity (<1% as determined using the chromogenic substrate, S-2251 [Diapharma, West Chester, OH]) was inhibited with 10μM D-Val-Phe-Lys-chloromethyl ketone (Calbiochem, San Diego, CA). Human Glu-Pg was isolated and Lys-Pg was prepared (69) as previously described. Saccharopine dehydrogenase (SDH) was isolated as previously described (70). Solulin was a generous gift from Dr. Achim Schuttler, Paion, GmbH (Aachen, Germany). QSY9 C5-maleimide and 5-iodoamidofluorescein were purchased from Invitrogen Canada Inc. (Burlington, ON). Plasmin was purchased from Haematologic Technologies Inc. (Essex Junction, VT,
USA). The buffer used in all experiments was HEPES buffered saline (20mM HEPES, 200mM NaCl, pH 7.4). All other reagents were of analytical quality.

The binding of 5IAF-Glu- or 5IAF-Lys-Pg to QSY-FDPs – QSY-FDPs (95μL, 0 – 2μM final concentration) were added to wells of an opaque 96-well plate and monitored continually at 1-minute intervals by fluorescence in a Gemini SpectraMax XS (Molecular Devices, Sunnyvale, CA) with emission and excitation wavelengths of 480nm and 520nm, respectively, employing a cutoff filter of 515nm. After the signal stabilized, 5μL of 1μM 5IAF-Glu- or 5IAF-Lys-Pg (50nM final) was added and mixed with the QSY-FDPs. Again, after the signal stabilized, 1μL 1M εACA was added to each well and mixed. The stable fluorescence signals obtained with only QSY-FDP (S_{QSY-FDP}), after the addition of 5IAF-Pg (S_{5IAF-Pg}) and after the addition of εACA (S_{εACA}) were used to determine the K_{d} between 5IAF-Pg and QSY-FDPs. Binding curves were generated based on the extent to which fluorescence decreased in the presence of TAFIa. Because the absorbance spectrum of QSY-FDP overlaps the excitation spectrum of 5IAF-Pg, an internal filter effect is present. The magnitude of this (FF) is calculated from equation (1).

\[
FF = \frac{S_{εACA} - S_{QSY-FDP}}{S_{5IAF-Pg0}}
\]  

(1)

S_{εACA} is the fluorescence in the presence of ε-ACA and QSY-FDP, where S_{QSY-FDP} is the blank in the absence of 5IAF-Pg and S_{5IAF-Pg0} is the fluorescence signal in presence of ε-
ACA but in the absence of QSY-FDP. The fluorescence of 5IAF-Pg, in the presence of QSY-FDP and in the absence of ε-ACA, corrected for the internal filter effect is given by equation (2).

\[
S_{Pg} = \frac{S_{5IAF-Pg} - S_{QSY-FDP}}{FF} \tag{2}
\]

Similar experiments were conducted with TAFIa treated QSY-FDP. In these experiments, 20nM TAFIa (final concentration) was added to each well with QSY-FDP for 30-minutes at room temperature. After this incubation period, 5IAF-Pg and ε-ACA were added as described above.

The binding of 5IAF-Glu- or 5IAF-Lys-Pg to TAFIa – The dissociation constant for TAFIa and Glu-Pg or Lys-Pg were determined. Briefly, TAFI was activated by incubating 1µM TAFI with 25nM thrombin, 100nM Solulin and 5mM CaCl₂ (100µL final volume) for 15 minutes at room temperature (30). After TAFI activation thrombin was inhibited using Phe-Pro-Arg-chloromethyl ketone. A 90µL solution containing TAFIa (0 – 2.5µM final concentration) was added to wells of an opaque 96-well plate and monitored by fluorescence as described above. After the signal stabilized, 10µL of a solution containing 500nM 5IAF-Glu or 5IAF-Lys-Pg was added to each well.
Kinetics of plasminogen binding site removal by TAFIa – 80µL of a solution containing QSY-FDP (0 – 1µM final concentration) and 50nM 5IAF-Glu- or Lys-Pg was added to wells of an opaque 96-well plate and monitored continually at 1-minute intervals by fluorescence in a Gemini SpectraMax XS (Molecular Devices, Sunnyvale, CA) with emission and excitation wavelengths of 480nm and 520nm, respectively, employing a cutoff filter of 515nm. After the signal stabilized, TAFIa (20µL) in 1mg/mL bovine serum albumin was added to each well. The initial rate of fluorescence intensity increase for each reaction was recorded and a model was used to convert the rate of fluorescence increase into the rate of Pg binding site removal. The model includes two distinct binding sites on QSY-FDP (C-terminal and internal lysines), only one of which is susceptible to removal by TAFIa (C-terminal lysine).

Model for the Rate of Plasminogen Binding Site Removal by TAFIa – In order to determine the rate of plasminogen binding site removal by TAFIa we developed a model which allowed for the conversion of a rate of fluorescence change associated with plasminogen dissociation from FDPs to a rate of plasminogen binding site removal. In this model, plasminogen can bind either a C-terminal lysine (F₁) or an internal lysine (F₂) as depicted in equations 1 and 2. The dissociation constants are K₁ and K₂, respectively. TAFIa removes Pg binding sites over time and this is represented by an increase in fluorescence.

\[ F₁ + P \xrightleftharpoons[K₁]{K} F₁P \]  

(3)
\[ F_2 + P \xrightleftharpoons{K_2} F_2P \]  

(4)

As shown in equation (5), only F₁ may be cleaved by TAFIa.

\[ F_1P \xrightarrow{TAFIa} F_1' + P \]  

(5)

The total plasminogen concentration may be written as a sum of free and bound plasminogen.

\[ [P]_o = [P] + [F_1P] + [F_2P] \]  

(6)

Total plasminogen may be rewritten as

\[ [P]_o = [P] \cdot \left[ 1 + \frac{[F_1]}{K_1} + \frac{[F_2]}{K_2} \right] \]  

(7)

Since the plasminogen used in these experiments is covalently attached to fluorescein, the total fluorescence signal (S) is a sum of the signals of free (sₚ) and bound plasminogen (s_{F₁P}, s_{F₂P}). sₚ, s_{F₁P} and s_{F₂P} are signal coefficients for the three respective 5IAF-Pg species.

\[ S = s_p \cdot [P] + s_{F₁P} \cdot [F_1P] + s_{F₂P} \cdot [F_2P] \]  

(8)

Replacing [F₁P] and [F₂P] yields

\[ S = s_p \cdot [P] + s_{F₁P} \cdot \frac{[F_1][P]}{K_1} + s_{F₂P} \cdot \frac{[F_2][P]}{K_2} \]  

(9)

Since the FDPs have a quencher moiety attached that absorbs light at the excitation wavelength, this concentration dependent absorbance must be accounted for by correcting for the internal filter effect which is accomplished by multiplying by \( e^{-i[FDP]} \), where \( i \) is the internal filter constant.
\[ S = [P] \left( s_p + s_{F_1} p \cdot \frac{[F_1]}{K_1} + s_{F_2} p \cdot \frac{[F_2]}{K_2} \right) e^{-i[FDP]} \]  

(10)

Substituting for \([P]\) using equation (7) yields

\[ S = [P_o] \left( s_p + s_{F_1} p \cdot \frac{[F_1]}{K_1} + s_{F_2} p \cdot \frac{[F_2]}{K_2} \right) \left( 1 + \frac{[F_1]}{K_1} + \frac{[F_2]}{K_2} \right)^{-1} e^{-i[FDP]} \]  

(11)

Since \(S\) and \(F_1\) change over time when TAFIa is present, the time derivative may be used to describe the rate of signal change \(\left( \frac{dS}{dt} \right)\) and the rate of \(F_1\) cleavage by TAFIa \(\left( \frac{d[F_1]}{dt} \right)\).

\[
\frac{ds}{dt} = [P_o] e^{-i[FDP]} \cdot \left[ \frac{s_{F_1} p \cdot \frac{d[F_1]}{dt}}{K_1} \right] \left[ 1 + \frac{[F_1]}{K_1} + \frac{[F_2]}{K_2} \right] - \left[ s_p + s_{F_1} p \cdot \frac{[F_1]}{K_1} + s_{F_2} p \cdot \frac{[F_2]}{K_2} \right] \left[ 1 + \frac{[F_1]}{K_1} + \frac{[F_2]}{K_2} \right]^2 \left[ 1 + \frac{[F_1]}{K_1} \right] \left[ 1 + \frac{[F_2]}{K_2} \right]
\]  

(12)

The rate of signal change may be simplified using algebraic manipulation to yield

\[
\frac{ds}{dt} = [P_o] e^{-i[FDP]} \cdot \frac{d[F_1]}{dt} \cdot \frac{1}{K_1} \cdot \left[ s_{F_1} p + s_{F_1} p \cdot \frac{[F_2]}{K_2} - s_p - s_{F_2} p \cdot \frac{[F_2]}{K_2} \right] \left[ 1 + \frac{[F_1]}{K_1} + \frac{[F_2]}{K_2} \right]^2 \left[ 1 + \frac{[F_1]}{K_1} \right] \left[ 1 + \frac{[F_2]}{K_2} \right]
\]  

(13)

By isolating \(\left( \frac{d[F_1]}{dt} \right)\) we are able to write an expression for the rate of \(F_1\) removal by TAFIa in terms of the rate of fluorescence change \(\left( \frac{dS}{dt} \right)\).
\[ \frac{d[F_1]}{dt} = \frac{dS}{dt} \frac{1}{S_0} e^{(\epsilon_{FDP})k_1} \cdot \left( 1 + \frac{[FDP]}{K_1} + \frac{[FDP]}{K_2} \right)^2 \cdot \left( \frac{s_p - 1 + (s_p - s_{p_f})}{K_2} \right) \]

(14)

where \( s_{p_1} = \frac{S_{F1P}}{s_p} \), \( s_{p_f} = \frac{S_{F2P}}{s_p} \), and \( S_o = s_p \cdot [P_o] \) and \( i \) was determined experimentally using the data presented in Figure 3-1 and equations (1 and 2). Equation 15 and 16, apply to prior to and after treatment of QSY-FDPs with TAFIa, respectively, and were used to determine the best values of the parameters \( s_{p_1}, s_{p_2}, K_1 \) and \( K_2 \) by non-linear regression analysis utilizing SYSTAT 4 (SPSS Inc., Chicago, IL).

Prior to adding TAFIa, \( S = S_{\text{initial}} \) (before TAFIa)

\[ S_{\text{initial}} = [S_o] \cdot \left( \frac{1 + \frac{[F_1]}{K_1} + \frac{[F_2]}{K_2}}{1 + \frac{[F_1]}{K_1} + \frac{[F_2]}{K_2}} \right) \]

(15)

When the reaction is complete (after TAFIa), so that \( [F_1] = 0 \), the final signal \( (S_f) \) is given by

\[ S_f = [S_o] \cdot \left( \frac{1 + \frac{[F_2]}{K_2}}{1 + \frac{[F_2]}{K_2}} \right) \]

(16)

Using the values for \( i, s_{p_1}, s_{p_2}, K_1 \) and \( K_2 \) and the measured rate of signal change, \( \frac{dS}{dt} \), equation (14) can be used to calculate the rate of Pg binding site removal by TAFIa. The data generated were fit using the Michaelis-Menten model of enzyme kinetics.
Kinetics of lysine cleavage by TAFIa – Lysine cleavage by TAFIa was measured using similar methods to those described by Wang et al. (27) and Schneider et al. (30). The main difference here is that lysine cleavage by TAFIa was measured in real time by monitoring the rate of fluorescence change associated with the oxidation of NADH. A solution (50μL) of FDPs (0 – 10μM final) was added to wells of an opaque 96-well plate and monitored continually at 1-minute intervals by fluorescence in a Gemini SpectraMax XS (Molecular Devices, Sunnyvale, CA) with emission and excitation wavelengths of 340nm and 450nm, respectively, employing a cutoff filter of 435nm. Subsequently, the reaction was initiated by the addition of a 50μL solution containing 2mM α-ketoglutarate (αKG), 40μM NADH, 10μM Glu- or Lys-Pg (or no plasminogen), 0.01U/mL saccharopine dehydrogenase (SDH) and 2nM TAFIa. Lysine cleavage by TAFIa was measured by the fluorescence change associated with NADH oxidation during SDH mediated saccharopine formation. The initial rate for each reaction was recorded and converted to nM lysine/s using a series of lysine standards. The data generated were fit using the Michaelis-Menten model of enzyme kinetics.

Results

The binding of 5IAF-Glu- or 5IAF-Lys-Pg to QSY-FDPs – 5IAF-Glu-Pg (fluorescent native plasminogen) binds to QSY-FDP with a Kd of 176nM. When QSY-FDP are treated with TAFIa the Kd increases to 1.06μM (Figure 3-1, panel A). It appears that 5IAF-Glu-Pg has the ability to weakly bind TAFIa-treated QSY-FDP; however, the capacity is greatly reduced compared to untreated QSY-FDP. Figure 3-1, panel B shows that when
similar experiments were conducted with 5IAF-Lys-Pg (fluorescent plasmin-cleaved plasminogen) similar binding constants were obtained ($K_d = 92\, \text{nM}$; $K_d (+\text{TAFIa}) = 1.55\, \mu\text{M}$). The increase in $K_d$ upon treatment of the QSY-FDPs with TAFIa is similar to that observed with 5IAF-Glu-Pg; however, the capacity of the FDPs to bind 5IAF-Lys-Pg is relatively unchanged. The relative capacity of the QSY-FDPs to bind 5IAF-Glu- or Lys-Pg can be determined by analyzing the relative fluorescence. A decrease in fluorescence is associated with more 5IAF-Pg bound to the QSY-FDPs. When fitting the data to a binding equation the maximal percent quench in fluorescence on untreated FDPs is approximately 50 and 55% for 5IAF-Glu- and 5IAF-Lys-Pg, respectively. Treatment of the FDPs with TAFIa removes plasminogen binding sites and causes a lower maximal quench of fluorescence. TAFIa-treated QSY-FDPs maximally quench 5IAF-Glu-Pg fluorescence by 15% and 5IAF-Lys-Pg fluorescence by 35%.
Figure 3-1. Binding of 5IAF-Glu-Pg (panel A) or 5IAF-Lys-Pg (panel B) to untreated (●) or TAFIa-treated (○) QSY-FDPs. 5IAF-Glu- and 5IAF-Lys-Pg bind to QSY-FDPs with K_d values of approximately 175nM and 90nM, respectively. Upon treatment with TAFIa, the K_d values are increased to 1µM and 1.55µM, respectively. In addition, to this increase in K_d, it appears that less 5IAF-Glu- or 5IAF-Lys-Pg binds the TAFIa-treated QSY-FDPs compared to the untreated QSY-FDPs. The data presented here is for one preparation of QSY-FDPs but is consistent with data from all other preparations.
The binding of 5IAF-Glu- or 5IAF-Lys-Pg to TAFIa – To determine if TAFIa forms a complex with 5IAF-Glu- or 5IAF-Lys-Pg, TAFIa was incubated with each 5IAF-Pg variant and binding was assessed by a quench in fluorescence (Figure 3-2). Our data show that TAFIa binds Glu-Pg with a $K_d$ of approximately 900nM and Lys-Pg with a $K_d$ of approximately 425nM.

![Figure 3-2. Binding of TAFIa to 5IAF-Glu- or 5IAF-Lys-Pg.](image)

Binding of TAFIa to 5IAF-Glu- (●) or 5IAF-Lys-Pg (○) was determined by fluorescence. The $K_d$ of the interaction with TAFIa was determined to be approximately 900nM for 5IAF-Glu-Pg and approximately 425nM for 5IAF-Lys-Pg. The data represent an average of at least 2 experiments.

Kinetics of plasminogen binding site removal by TAFIa – The calculated rate of 5IAF-Glu-Pg binding site removal by TAFIa was determined at various QSY-FDP concentrations (0 – 1μM) (Figure 3-3, panel A). The data are hyperbolic in nature and when fitted using the Michaelis-Menten model of enzyme kinetics the $k_{cat}$ and $K_m$ of Glu-Pg binding site removal was 2.34 s$^{-1}$ and 142.6nM, respectively, implying a catalytic
efficiency of 16.41 $\mu$M$^{-1}$s$^{-1}$. The rate is sensitive to the TAFIa concentration with all TAFIa concentrations (50, 75 and 100pM) yielding similar kinetic parameters. Similar experiments were conducted to determine the kinetics of 5IAF-Lys-Pg binding site removal by TAFIa. When the data were fitted using the Michaelis-Menten model of enzyme kinetics the $k_{cat}$ and $K_m$ of Lys-Pg binding site removal was 0.888 s$^{-1}$ and 94nM, respectively, implying a catalytic efficiency of 9.45 $\mu$M$^{-1}$s$^{-1}$ (Figure 3-3, panel B). Like with Glu-Pg, the rate is sensitive to the TAFIa concentration with all TAFIa concentrations (50, 75 and 100pM) yielding similar kinetic parameters. 5IAF-Glu- and 5IAF-Lys-Pg binding site removal conformed well to the Michaelis-Menten model with $R^2$ values of 0.93 and 0.92, respectively. The kinetics of TAFIa on Glu- and Lys-Pg binding site removal are summarized in Table 3-1.
Figure 3-3. The kinetics of TAFIa in removing Glu- or Lys-Pg binding sites from QSY-FDPs.

The kinetics of 5IAF-Glu-Pg binding site removal by TAFIa were fitted using the Michaelis-Menten model of enzyme kinetics. Panel A shows that the $k_{cat}$ and $K_m$ of Glu-Pg binding site removal were 2.34 s$^{-1}$ and 142.6nM, respectively, implying a catalytic efficiency of 16.41 μM$^{-1}$s$^{-1}$. When fitting the kinetics of 5IAF-Lys-Pg binding site removal by TAFIa to the Michaelis-Menten model the $k_{cat}$ and $K_m$ were determined to be 0.888 s$^{-1}$ and 94nM, respectively, which implies a catalytic efficiency of 9.45 μM$^{-1}$s$^{-1}$. All experiments were performed at least 3 times and the data represent the mean ± SE of these experiments.
Table 3-1. A summary of the kinetics of Glu- and Lys-Pg binding site removal by TAFIa

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (µM⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-Pg</td>
<td>2.35</td>
<td>0.143</td>
<td>16.41</td>
</tr>
<tr>
<td>Lys-Pg</td>
<td>0.888</td>
<td>0.094</td>
<td>9.43</td>
</tr>
</tbody>
</table>

Kinetics of lysine cleavage by TAFIa – In order to determine the kinetics of cleaving all lysines from FDPs and not just those involved in plasminogen binding, we used an enzyme (SDH) that catalyzes the formation of saccharopine from lysine and α-ketoglutarate via an NADH-linked reaction. Since the SDH-dependant reaction was designed to be much faster than the TAFIa reaction, the kinetics of lysine cleavage by TAFIa was monitored by following the fluorescence of NADH oxidation. As shown in Figure 3-4, this method yielded a $k_{cat}$ of 3.79 s⁻¹ and a $K_m$ of 2.87 µM in the absence of plasminogen which implies a catalytic efficiency of 1.32 µM⁻¹s⁻¹ when fit to the Michaelis-Menten model. Upon the addition of Glu- or Lys-Pg, the catalytic efficiency was increased by 2.5 and 2.9-fold, respectively to 3.83 and 3.35 µM⁻¹s⁻¹. This is primarily a result of a change in the $K_m$ which decreased from 2.87µM in the absence of plasminogen to 0.95µM and 0.84µM in the presence of Glu- or Lys-Pg, respectively. This suggests that plasminogen augments the binding of TAFIa to FDPs and this has a positive effect on the catalytic efficiency. The Michaelis-Menten model provided a good fit for all data with $R^2$ values >0.98. The kinetic parameters associated with lysine cleavage by TAFIa are summarized in Table 3-2.
Figure 3-4. Effect of Glu- or Lys-Pg on lysine cleavage by TAFIa. The kinetics of lysine cleavage by TAFIa were analyzed in the presence of 5µM Glu- (●) or 5µM Lys-Pg (■) or in the absence of plasminogen (▲). The $k_{cat}$ did not vary to any great extent whether plasminogen was added or not; however, the $K_m$ was decreased from 2.87µM to 0.95 and 0.84µM in the presence of Glu- or Lys-Pg, respectively. This resulted in an increase in the catalytic efficiency from 1.32µM$^{-1}$s$^{-1}$ in the absence of plasminogen to 3.83µM$^{-1}$s$^{-1}$ and 3.35µM$^{-1}$s$^{-1}$ in the presence of Glu- or Lys-Pg, respectively. All experiments were performed at least 2 times and the data represent the mean of these experiments.

Table 3-2. A summary of the kinetics of lysine cleavage by TAFIa

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (µM$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Glu-Pg</td>
<td>3.66</td>
<td>0.95</td>
<td>3.83</td>
</tr>
<tr>
<td>+Lys-Pg</td>
<td>2.82</td>
<td>0.84</td>
<td>3.35</td>
</tr>
<tr>
<td>No Pg</td>
<td>3.79</td>
<td>2.87</td>
<td>1.32</td>
</tr>
</tbody>
</table>
Discussion

The apparent discrepancy between the kinetics of plasminogen binding site removal and lysine cleavage by TAFIa suggests that more than one class of C-terminal lysine exists. These different classes of lysine residues may differ in their ability to bind plasminogen and/or TAFIa which would greatly influence the kinetics of TAFIa. For example, exposed C-terminal lysines at a site where all 3 chains of fibrin have been cleaved by plasmin may be more accessible than nicked fibrin (only 1 or 2 cleaved chains) and as a result more readily cleaved by TAFIa. The higher catalytic efficiency of Glu-Pg binding site removal (16.41 s⁻¹µM⁻¹) compared to lysine cleavage (3.84 s⁻¹µM⁻¹ with 5µM Glu-Pg) is largely due to a change in the Kₘ (0.143µM vs. 1µM, respectively) which suggests that TAFIa or TAFIa-Pg binds some lysines more tightly than others. The kₘₐₜ(app) remains relatively unchanged when comparing the kinetics of plasminogen binding site removal to lysine cleavage (2.35 s⁻¹ vs. 3.66 s⁻¹ for Glu-Pg, respectively).

A limitation of the fluorescence method for measuring plasminogen binding site removal by TAFIa is that the kinetics are heavily weighted toward the class of lysines that tightly bind plasminogen. In the alternate method that does not discriminate between the different classes of C-terminal lysines the kinetics are likely a better approximation of the average kₘₐₜ and Kₘ of lysine cleavage by TAFIa. The kinetics of plasminogen binding site removal is more relevant to the generally accepted physiological role of TAFIa as an inhibitor of fibrinolysis. In the early stages of fibrinolysis the concentration of C-terminal lysines would be relatively low compared to the plasma fibrinogen concentration. A Kₘ of 0.143µM suggests that TAFIa would efficiently eliminate Glu-Pg
binding sites even in the early stages of fibrinolysis. It is generally accepted that fibrinolysis enters the propagation phase (49) after TAFIa decays below a certain threshold (52,53). Once TAFIa concentrations fall below this threshold, Lys-Pg is formed and can be activated by tPA with a 20-fold higher catalytic efficiency (32) compared to Glu-Pg. Our data suggest that in addition to the threshold theory, the decreased catalytic efficiency for Lys-Pg binding site removal (9.43 s⁻¹µM⁻¹) may play a role to fibrinolysis entering the propagation phase. Although the catalytic efficiency is still relatively high compared to other substrates of TAFIa (28), a large proportion of TAFIa would have decayed before significant accumulation of Lys-Pg.

In the current study, we show that 5IAF-Glu-Pg binds TAFIa-treated QSY-FDPs with a $K_d$ of 1.06µM. This value suggests that 5IAF-Glu-Pg binds TAFIa-treated QSY-FDPs with a much higher affinity than intact fibrin (~30µM) (62); however, our interpretation of the data differs from that used by Horrevoets et al. The assumption made by Horrevoets et al. in order to arrive at a $K_d$ of ~30µM is that intact fibrin has the same capacity for binding both Glu- and Lys-Pg. Since Glu-Pg often exists in a closed conformation (71) that may prevent it from binding to fibrin we have interpreted our data without this assumption. If we were to incorporate this assumption, the affinity of 5IAF-Glu-Pg for TAFIa-treated QSY-FDPs would be very similar to that of intact fibrin.

Model for Enhanced TAFIa Activity due to Plasminogen Binding

It was presumed that plasminogen would be a competitive inhibitor of C-terminal lysine cleavage by TAFIa; however, Glu- or Lys-Pg actually increased the catalytic
efficiency of TAFIa in cleaving lysines from FDPs. The model depicted in Figure 3-5 can be used to interpret the data and rationalize this phenomenon.

![Figure 3-5: Model of lysine cleavage by TAFIa.](image)

TAFIa may interact with FDPs directly and subsequently cleave lysines or the interaction between TAFIa and FDPs may be facilitated by plasminogen.

In the absence of plasminogen, the rate of lysine cleavage may be written as

\[
v = k_1 [FT] + k_2 [TPF]
\]  

(17)

where F=FDPs, T=TAFIa and P=Glu- or Lys-Pg.

The total concentration of TAFIa can be expressed as a sum of free and bound TAFIa;

\[
[T]_o = [T] + [FT] + [PT] + [TPF],
\]  

(18)

which can be rewritten as

\[
T_o = T \left( 1 + \frac{[F]}{k_{FT}} + \frac{[P]}{k_{TP}} + \frac{[P][F]}{k_{TP}k_{TPF}} \right)  
\]  

(19)

Substitution of [FT] and [TPF] by \( \frac{[F][T]}{K_{FT}} \) and \( \frac{[P][F][T]}{K_{TP}k_{TPF}} \), respectively, in equation (17) yields

\[
v = \left( \frac{k_1}{k_{FT}} + \frac{k_2 [P]}{k_{TP}k_{TPF}} \right) [F][T]
\]  

(20)
and upon substituting free TAFIa with equation (19) the rate is given by

\[
v = \frac{\left( \frac{k_1}{k_{FT}} + \frac{k_2}{k_{TP}k_{TPF}} \right) [F][T_0]}{1 + \left( \frac{[P]}{k_{TP}} \right) + [F]\left( \frac{1}{k_{FT}} + \frac{[P]}{k_{TP}k_{TPF}} \right)}.
\]

(21)

Since \( k_{FT} \cdot k_{FTP} = k_{TP} \cdot k_{TPF} \)

(22)

and

\[
\frac{k_{FT}}{k_{TP} \cdot k_{TPF}} = \frac{1}{k_{FTP}}
\]

(23)

the rate, after some algebraic manipulation, can be expressed as

\[
\frac{v}{[T]_0} = \frac{(k_1 k_{FTP} + k_2 [P])[F]}{k_{FTP} \left( k_{FTP} + \frac{k_{FTP}}{k_{TP}} [P] \right) + [F] \left( k_{FTP} + [P] \right)}
\]

(24)

Finally, the rate may be expressed in the form of the Michalis-Menten model of enzyme kinetics by dividing the numerator and denominator by \( (K_{TFP} + [P]) \). The result is:

\[
\frac{v}{[T]_0} = \frac{k_{cat(app)} [F]}{K_{m(app)} + [F]}
\]

(25)

Where

\[
k_{cat(app)} = \frac{k_1 k_{FTP} + k_2 [P]}{k_{FTP} + [P]}
\]

(26)

and

\[
K_{m(app)} = \frac{k_{FTP} \left( k_{FTP} + \frac{k_{FTP}}{k_{TP}} [P] \right)}{k_{FTP} + [P]}
\]

(27)
If we consider the kinetics of TAFIa in the absence of plasminogen

\[ k_{\text{cat(app)}} = k_1 \]  \hspace{1cm} (28)

And, using the relationship shown in equation (22)

\[ K_{m(app)} = k_{FT} \]  \hspace{1cm} (29)

This model shows that the kinetics of lysine cleavage from FDPs by TAFIa changes as the concentration of P changes.

As \([P] \rightarrow \infty\), \hspace{1cm} (30)

\[ k_{\text{cat(app)}} = k_2 \]

Again, using the relationship shown in equation (22)

\[ K_{m(app)} = k_{TPF} \]  \hspace{1cm} (31)

We show that the presence of plasminogen has very little effect on the \(k_{\text{cat}}\); however, the \(K_m\) is decreased in the presence of 5\(\mu\)M Pg which suggests that \(k_{FT} > k_{TPF}\).

We show that the \(K_d\) between TAFIa and Lys-Pg is approximately half that of TAFIa which is reflected in the \(k_{\text{cat}}\) and \(K_m\) of lysine cleavage. As \([P] \rightarrow \infty\), more Pg would be available to bind TAFIa which would cause the \(K_m(app)\) to be closer to \(k_{TPF}\) than \(k_{FT}\). This is reflective of a weaker interaction between FDP and TAFIa compared to the interaction between FDP and the TAFIa-plasminogen complex. Our model is consistent with the notion that plasminogen (Glu- or Lys-) binds TAFIa at a different site than it binds FDPs. If the tight interaction between Pg and FDPs (Figure 3-1) remains intact in the presence of TAFIa, plasminogen may act as a template that can be used to position TAFIa within
close proximity to its substrate, lysine residues on FDPs. Since our model predicts that $k_{FT} > k_{TPF}$, at high plasminogen concentrations, the binding energy between the plasminogen-TAFIa complex and FDPs is higher than the binding energy of TAFIa binding FDPs. This suggests that plasminogen dependent binding linkage, similar to the linkage described by Wyman et al. (72), occurs to lower the $K_m$ of plasminogen binding site removal by TAFIa.

The catalytic efficiency of TAFIa in removing plasminogen binding sites from FDPs is relatively high compared to other known substrates of TAFIa (28) and as a result very little TAFIa is required to effectively attenuate plasminogen activation and fibrinolysis. In the early stages of fibrinolysis, when very little TAFI has been activated, the plasminogen concentration greatly exceeds the TAFIa concentration and the $K_d$ (Figure 3-2) suggests that most TAFIa is bound to plasminogen. The interaction between TAFIa and plasminogen may play a pivotal role in lowering the $K_m$ of TAFIa-mediated lysine cleavage from FDPs.

Given the complex nature of plasminogen binding to FDPs, it is hard to speculate the location or number of lysines that make up a plasminogen binding site. This is further complicated by the different affinities of Glu- and Lys-Pg for internal lysines (62). The data shown here are consistent with the models shown and provides a reasonable physiological interpretation of the kinetics of TAFIa in removing lysines, whether involved in plasminogen binding or not, from fibrin degradation products.
Acknowledgements

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Chapter 4

Soluble thrombomodulin partially corrects the premature lysis defect in FVIII-deficient plasma by stimulating the activation of thrombin activatable fibrinolysis inhibitor
Abstract

Background: Previous work by others has shown that premature clot lysis occurs in plasmas deficient in components of the contact pathway, due to a failure to activate thrombin-activatable fibrinolysis inhibitor (TAFI). This suggests the hypothesis that bleeding in hemophilia is due not only to defective coagulation but also enhanced fibrinolysis. These studies were carried out to quantify the extent of TAFI activation over time in normal plasma (NP) and factor VIII deficient plasma (FVIII-DP) and to determine whether soluble thrombomodulin (sTM) can correct the lysis defect in FVIII-DP. Methods: The time courses of TAFI activation in both NP and FVIII-DP were monitored after clotting with thrombin, PCPS and Ca²⁺, ± sTM. Clotting and lysis were measured turbidometrically and TAFIa using a functional assay. Results: Premature lysis that occurs in FVIII-DP is corrected by mixing deficient plasma with 10% NP. However, this does not fully correct the defect in TAFI activation. FVIII-DP must be mixed with up to 50% NP to attain the same TAFIa potential as NP. In FVIII-DP, sTM can correct the defect in TAFIa-dependent prolongation of lysis at low tPA concentrations and partially correct this defect at high tPA concentrations. Conclusions: TAFI activation increases as the concentration of FVIII increases. FVIII at a level of 10% fully corrects the lysis defect in spite of the extent of TAFI activation being only one half that obtained with 100% FVIII. In addition, sTM increases TAFI activation sufficiently to correct the premature lysis defect in FVIII-DP.
Introduction

Upon injury, a minor fraction of available prothrombin is activated to thrombin via extrinsic tenase activation of factor (F) X (73), fibrinogen is converted to fibrin and a clot is formed. Following clot formation, the intrinsic pathway is triggered (74) and a positive feedback loop is established through activation of FVIII and FV by thrombin (75), which, when activated (FVIIIa, FVa) are cofactors for intrinsic tenase and prothrombinase, respectively. The events leading up to clot formation are collectively referred to as the initiation phase of coagulation, whereas those following clot formation are referred to as the propagation phase.

Plasmas lacking FVIII or FIX, such as those of individuals with hemophilia A or B, function nearly normally with respect to the initiation phase of coagulation. They, however, fail to show the robust thrombin generation that occurs in normal plasma in the propagation phase (76). Because the initiation phase and the extrinsic pathway are functional in hemophiliacs, the severe bleeding tendencies in these diseases appear to be associated with the failure of coagulation to enter the propagation phase.

Whereas the blood clotting system functions to form a clot, the fibrinolytic system functions to remove it. These opposing effects presumably are highly regulated so that hemostasis occurs when and where it is needed and blood fluidity is otherwise maintained throughout the vasculature. Work in the past decade indicates that the TAFI activation pathway might play a role in regulating hemostasis because through it thrombin formation leads to suppression of fibrinolysis (22,38,39,77).
TAFI is efficiently activated by the thrombin–thrombomodulin complex (22) and relatively inefficiently by thrombin alone. During the propagation phase in plasma, sufficient TAFI is activated to suppress tissue plasminogen activator (tPA) initiated fibrinolysis. Broze and Hiquich i (38) showed that clots formed in hemophiliac plasma lyse prematurely compared with clots formed in normal plasmas and this 'premature lysis' is due to reduced or absent TAFI activation. These results suggest that bleeding in hemophilia may also be a result of improperly regulated fibrinolysis.

The bleeding phenotype in hemophilia A is variable for patients sharing the same molecular defect. Cases have been reported showing that a given mutation can give rise to identical FVIII:C levels but different bleeding phenotypes (37). Many explanations for these phenomena have been offered. The presence of FVIII inhibitors (35,78,79), discrepancies in FVIII:C levels measured using the one-stage compared with the two-stage activity assay (80), deficiency in protein S and the presence of FV Leiden have been implicated in hemophiliacs with identical FVIII:C levels and differences in bleeding phenotypes. Recently, it has been shown in vitro that when a clot is eventually formed in FVIII-DP supplemented with tPA, fibrinolysis is not down-regulated and as a result clots lyse prematurely (38,39). Consequently, TAFIa should be considered when studying the bleeding tendencies of hemophiliacs with similar FVIII:C levels but different bleeding phenotypes.

The present studies were carried out to quantify the activated TAFI present during the clotting and lysis of normal and FVIII-deficient plasmas and determine whether the
premature lysis phenomena observed in FVIII-deficient plasma can be corrected by the addition of sTM or by reconstituting FVIII through the addition of normal plasma.

Materials and Methods

Thrombin and fibrinogen were prepared as previously described (63) with one exception: for the fibrinogen preparation, the solution was made to 1.2% PEG-8000 instead of 2% PEG-8000 by the addition of 40% (w/v) PEG-8000 in water, subsequent to β-alanine precipitation. This change in protocol allowed for a greater yield of fibrinogen. QSY-FDPs (fibrin degradation products that are covalently attached to the quencher, QSY9 C5-maleimide) and TAFIa standards used in the TAFIa assay were prepared as described (61,64) and recombinant human Pg (S741C) and the fluorescein derivative (5IAF-Pg) were prepared as described by Horrevoets et al. (62). S525C-prothrombin was purified and fluorescently labeled with 5-iodoamidofluorescein (5IAF) as previously described by Brufatto et al. (81). QSY9 C5-maleimide and 5-iodoamidofluorescein were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). Plasmin was purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA) and recombinant human soluble thrombomodulin (Solulin; sTM) was a generous gift from Dr Oliver Kops, Paion, GmbH (Aachen, Germany). Normal human pooled plasma (NP) was obtained from healthy donors at the blood bank in the Kingston General Hospital (KGH) in Kingston, Ontario, Canada, and FVIII-deficient plasma (FVIII-DP) was purchased from Affinity Biologica, Inc. (Hamilton, ON, Canada). TAFI-deficient plasma (TDP) was prepared by affinity chromatography of normal plasma on a column of immobilized anti-human TAFI monoclonal antibody, as described previously (30). The plasmin
inhibitor D-Val-Phe-Lys chloromethyl ketone (VFKck), the thrombin inhibitor D-Phe-Pro-Arg chloromethyl ketone (PPAck) and potato tuber carboxypeptidase inhibitor (PTCI) were purchased from Calbiochem (San Diego, CA, USA). Tissue-type plasminogen activator (Activase; tPA) was purchased from the pharmacy at KGH (Kingston, ON, Canada). All other reagents were of analytical quality.

Clot lysis assays and the preparation of samples to determine the extent of TAFI activation – FVIII-DP was mixed with NP so that the final percentage of NP was 0, 1, 6, 10, 50 or 100% (0–100% NP). Before mixing, each plasma was diluted to an optical density of 32 and added to an equal volume of a solution containing 1.5 nM tPA, 40 μM PCPS and 20 mM CaCl₂ in the presence or absence of 20 nM thrombin (final concentrations: 0.75 nM tPA, 20 μM PCPS, 10 mM CaCl₂, ±10 nM thrombin) and the samples were divided into multiple Eppendorf tubes and placed in a 37°C water bath. Clotting and lysis were stopped in these tubes at various time points by the addition of 10 μM PPAck and 10 μM VFKck to selectively inhibit thrombin and plasmin, respectively. The samples were mixed vigorously, then centrifuged for 30 s at 16 000 x g (room temperature) and immediately placed on ice to prevent thermal inactivation of TAFIa. The supernatant of each sample was serially diluted by 5-fold with TAFI-deficient plasma and TAFIa was measured using a functional assay described by Kim et al. (61). Identical experiments were conducted in a covered, 96-well plate and the turbidity was monitored at 400 nm over time using a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) to determine the timing of coagulation and
fibrinolysis. Similar experiments were conducted in the presence or absence of soluble thrombomodulin (0–100 nM) at 4 tPA concentrations (0.25, 0.75, 1.5 and 3 nM) to determine the effect of sTM on TAFI activation and lysis times. These experiments were also conducted in the presence of 5 μM PTCI to show the TAFIa dependent prolongation of lysis in normal and FVIII-deficient plasma.

**Determination of the time course of prothrombin activation in normal and FVIII-deficient plasma** – Normal and FVIII-deficient plasmas (0–100% NP) were supplemented with the prothrombin derivative (5IAF-II; 300 nM final) as well as 20 μM PCPS and 10 mM CaCl₂ in the presence of 10 nM thrombin to initiate clotting. These experiments were conducted in an opaque, plastic-covered 96-well plate. A SpectraMax GeminiXS (Molecular Devices, Sunnyvale, CA, USA) was used to monitor fluorescence intensities over time at 37°C with excitation and emission wavelengths of 495 nm and 535 nm, respectively, employing a 530-nm emission cut-off filter. Fluorescence was normalized to reflect the baseline and maximal fluorescence, which correlates with full prothrombin activation.

**TAFIa potential** – The area under the TAFIa plots was chosen as a parameter to quantify the effect of TAFIa over the course of the experiments. This parameter was designated the 'TAFIa potential' by analogy with the 'thrombin potential' defined by Hemker et al. (82). TAFIa potential, like thrombin potential, is proportional to the amount of substrate cleaved and is explained mathematically, as follows:
\[
\frac{dS}{dt} = -\frac{k_{cat}}{K_m} [TAFIa][S] 
\]

(1)

where \( \frac{dS}{dt} \) is the rate of substrate consumption and \( S \) is the substrate.

If \( S \) is constant (i.e. limited consumption of \( S \)),

\[
\frac{dS}{dt} = -S \frac{k_{cat}}{K_m} [TAFIa] 
\]

(2)

\[
dS = -S \frac{k_{cat}}{K_m} [TAFIa] \, dt 
\]

(3)

For some interval 0 to \( t \),

\[
\int_{S(0)}^{S(t)} dS = -S \frac{k_{cat}}{K_m} \int_{0}^{t} [TAFIa] \, dt 
\]

(4)

Realizing that the integral on the right in equation (4) is the area under the TAFIa plot,

\[
\Delta S(t) = -S \frac{k_{cat}}{K_m} \text{(area under curve)} 
\]

(5)

\[
\Delta S(t) = -S \frac{k_{cat}}{K_m} \text{(TAFIa Potential)} 
\]

(6)

Results

Lysis times in normal and FVIII-deficient plasmas – Clotting was initiated with 10 nM IIa, 10 mM CaCl\(_2\) and 20 \( \mu \)M PCPS vesicles to create a model where the clot structure is insensitive to the FVIII concentration. Because the clot structure is similar regardless of the FVIII concentration, the effect of FVIII on tPA-dependent (0.75 nM) clot lysis can be determined. Using this lysis model, lysis times increased as the percentage of normal plasma increased. Figure 4-1 shows the clot-lysis profiles for FVIII-DP with 0–100%
added normal plasma and the lysis times are summarized in Figure 4-1 (inset). In FVIII-DP the lysis time is 37 min and can be increased by approximately 50% by the addition of normal plasma. At 10% normal plasma the shortened lysis time associated with FVIII-DP has been corrected to that observed in normal plasma.

Figure 4-1. Clot-lysis profiles and lysis times of factor VIII deficient plasma (FVIII-DP), normal plasma (NP) and FVIII-DP mixed with NP. Clot lysis profiles are shown for 0 (—), 1 (······), 6 (— —), 10 (····), 50 (— —) and 100% (——) NP. From the clot-lysis profiles, the lysis time was determined by taking the time at which the clot has been degraded to one half of its highest optical density. In the inset, the lysis times are summarized, with the general trend being an increase in lysis time as the percentage of NP (and consequently amount of FVIII) is increased. The effect of adding NP on lysis time reaches a plateau at 10% NP.
TAFI and prothrombin activation in normal and FVIII-deficient plasmas – TAFI activation was measured in normal, FVIII-deficient and mixed plasmas to quantify the effect of FVIII on the time course of activation. A functional assay was used to measure TAFIa over the time course of clotting and lysis and the results are presented in Figure 4-2. When thrombin, calcium ion and PCPS were used to initiate clotting in FVIII-DP, approximately 30 pM TAFIa was measured after 5 min. As the percentage of normal plasma increased so too did the peak concentration of TAFIa. Although the lysis time was corrected by supplementing FVIII-DP with 10% normal plasma, this was not sufficient to fully correct TAFI activation. By calculating the area under the TAFIa time course plots (Figure 4-2A) it was determined that approximately the same TAFIa potential (Figure 4-2B) was achieved over the first 50 min in normal plasma and 50% normal plasma (16 800 pM-min and 14 100 pM-min, respectively) but FVIII-DP plasma mixed with 10% normal plasma had a TAFIa potential of only 50% of the TAFIa potential in normal plasma. In order to quantify the relationship between lysis time and TAFI activation over the range 0–100% FVIII, log lysis time vs. log TAFIa potential was plotted (Figure 4-2, panel, inset). As expected, the data show a strong positive correlation between lysis time and TAFIa potential in plasma containing 0–100% FVIII.
Figure 4-2. Thrombin activatable fibrinolysis inhibitor (TAFI) activation in plasma containing various percentages of FVIII

In FVIII-DP only 30 pM TAFIa was measured at its peak (●) compared with ~600 pM TAFIa in 50% NP (□) and 100% NP (Δ). These experiments were conducted in triplicate and the data represent the mean ± SE (panel A). The TAFIa potential (panel B), defined here as the area under the time course of activation plot (A) from the time of clot initiation to the last time point, increases as the percentage of NP increases to a plateau at 50% NP. The TAFIa potentials of 50% NP and 100% NP are similar (14 100 pM-min and 16 800 pM-min, respectively) despite the shape of their respective TAFI activation plots being quite different. The relationship between lysis time (Figure 4-1, inset) and TAFIa potential, as it relates to FVIII levels, is presented (Figure 4-2, inset) using a plot of log lysis time vs. log TAFIa potential.
The TAFI activation profile in Figure 4-2A can be rationalized by analyzing prothrombin activation in plasma (Figure 4-3) because thrombin is the activator of TAFI. The general trend is that as the percentage of normal plasma increased, the rate of prothrombin activation also increased (which can be determined by examining the slope of the curve in Figure 4-3). An exception occurs with normal plasma. In normal plasma the rate of prothrombin activation is lower than in FVIII-DP mixed with 50% normal plasma. While the rate is slower in normal plasma, prothrombin activation persists for about twice as long as in FVIII-DP mixed with 50% normal plasma. In every experiment, the timing of prothrombin activation corresponds well with TAFI activation.
Figure 4-3. Prothrombin activation in plasma containing various percentages of FVIII.

The time course of prothrombin activation is shown for FVIII-DP mixed with 0 (●), 1 (■), 6 (▲), 10 (○), 50 (□) and 100% NP (Δ). Generally, the rate of prothrombin activation increases as the percentage of NP increases. At 50% NP prothrombin activation occurs at a high rate (as determined by examining the slope of each plot) and appears to be over within 15 min, whereas 100% NP has a slower rate of prothrombin activation over a longer time period.

Normal plasma was also clotted using calcium ion and PCPS, without added thrombin. Calcium-induced coagulation does not occur immediately; it takes approximately 15 min for the clot to form in normal plasma. At this time, prothrombin activation enters the propagation phase (75,81) and as a result, TAFI is activated. The extent and timing of TAFI activation with respect to clot formation is the same whether clotting is initiated in the presence or absence of added thrombin, which suggests that TAFI activation is a result of thrombin generated in situ and not of thrombin added to induce clotting. In the
presence of thrombin there was a TAFIa potential of 16 800 pM-min compared with 14 150 pM-min in the absence of thrombin.

*TAFI activation in normal and FVIII-deficient plasmas in the presence of soluble thrombomodulin* – In normal plasma, peak TAFIa levels and TAFIa potential increased from 600 pM and 16 800 pM-min, respectively, in the absence of sTM to approximately 6000 pM and 150 000 pM-min, respectively, in the presence of 10 nM sTM. This increase in TAFI activation resulted in a 70% increase in the lysis time. The effect of 10 nM sTM on the relative prolongation of lysis in FVIII-DP was similar to normal plasma in that lysis was prolonged by 65% when FVIII-DP was clotted and lysed in the presence of sTM. In the presence of 10 nM sTM, 750 pM TAFIa was present at peak TAFIa concentration compared with 30 pM in the absence of sTM. In the time from clot initiation to the clot lysis time the TAFIa potential was measured to be 12 800 pM-min in the presence of 10 nM sTM compared with 600 pM-min in the absence of sTM.

*The concentration dependence of tPA and sTM on lysis times in normal and FVIII-deficient plasmas* – The effect of TAFI activation on lysis time was analyzed over a range of tPA and sTM concentrations to determine if the lysis defect in FVIII-DP could be corrected by stimulating TAFI activation. The lysis times summarized in Figure 4-4 are relative to lysis times from similar experiments containing PTCI, which is an inhibitor of TAFIa. In the presence of PTCI, there is no functional TAFIa so the relative lysis times presented in Figure 4-4 are representative of TAFIa-dependent prolongation of lysis. At the lowest concentration of tPA (0.25 nM), the maximal TAFIa-dependent prolongation
of lysis (2-fold) was observed when 1 nM sTM was added to normal plasma. Supplementing FVIII-DP with sTM caused a dose-dependent prolongation of the lysis time (Figure 4-4).

Figure 4-4. Soluble TM prolongs fibrinolysis in a TAFIa-dependent manner.
The effect of sTM on thrombin activatable fibrinolysis inhibitor (TAFI) activation in normal plasma (NP) and factor VIII deficient plasma (FVIII-DP) at various concentrations of both sTM (0–100 nM) and tPA (0.25–3 nM). The TAFIa-dependent defect in prolonging lysis in FVIII-DP is corrected by the addition of 100 nM sTM to plasma containing 0.25 nM tPA. As the concentration of tPA is increased only partial correction of the lysis defect is observed in FVIII-DP in the presence of 100 nM sTM. In these experiments, potato tuber carboxypeptidase inhibitor (PTCI) was used to create a condition in which there is no functional TAFIa. Therefore, any increase in lysis, as presented by the ratio lysis time/lysis time + PTCI is TAFIa dependent.
When 100 nM sTM was added to FVIII-DP the lysis time was fully corrected to that seen in normal plasma. As the tPA concentration increased, a higher concentration of sTM was required to get maximal TAFIa-dependent prolongation of lysis. For example, when 1.5 nM tPA (Figure 4-4) is present, 25 nM sTM is required to maximize the TAFIa-dependent prolongation of lysis in normal plasma and 100 nM sTM is required in FVIII-DP. Also, as tPA is increased in these clot lysis experiments TAFIa appears to have a much greater effect on lysis time (up to 5.2-fold at 1.5 nM tPA compared with 2.3-fold at 0.25 nM tPA). It appears that as the tPA concentration is increased, the concentration of sTM required to get any TAFIa-dependent prolongation of lysis also increases. At 0.25 nM tPA, no sTM was required to get prolongation of lysis in normal plasma whereas 25 nM sTM was required to get prolongation of lysis when 3 nM tPA (Figure 4-4) was added to normal plasma. In order to show how the actual lysis times are affected by tPA and sTM the lysis times in TAFIa inhibited normal and FVIII-deficient plasma are presented in Table 4-1.
Table 4-1. Summary of the data used to construct Figure 4-4, including the absolute lysis time in the presence of PTCI to enable determination of the lysis time under each condition.

<table>
<thead>
<tr>
<th>NHP</th>
<th>[sTM] (nM)</th>
<th>Absolute LT +PTCI¹ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[tPA] (nM)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0.25</td>
<td>1.70</td>
<td>2.16</td>
</tr>
<tr>
<td>0.75</td>
<td>0.91</td>
<td>2.52</td>
</tr>
<tr>
<td>1.5</td>
<td>1.00</td>
<td>1.08</td>
</tr>
<tr>
<td>3.0</td>
<td>0.94</td>
<td>0.97</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>F8DP</th>
<th>[sTM] (nM)</th>
<th>Absolute LT +PTCI¹ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[tPA] (nM)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0.25</td>
<td>1.07</td>
<td>1.24</td>
</tr>
<tr>
<td>0.75</td>
<td>1.06</td>
<td>1.14</td>
</tr>
<tr>
<td>1.5</td>
<td>0.99</td>
<td>1.03</td>
</tr>
<tr>
<td>3.0</td>
<td>1.00</td>
<td>1.02</td>
</tr>
</tbody>
</table>

¹In all cases, the lysis time is expressed relative to that obtained in the presence of the TAFIa inhibitor, PTCI. TAFI, thrombin activatable fibrinolysis inhibitor; PTCI, potato tuber carboxypeptidase inhibitor.
Discussion

At 10% FVIII, lysis time is normal. The TAFIa potential, however, as measured by the area under the TAFIa concentration vs. time profile, is only about one-half of normal. Thus, the potential achieved at 10% FVIII, although less than that achieved in normal plasma, is adequate to completely correct the premature lysis defect. This, along with partial correction of the clotting defect, presumably is adequate to prolong clot lysis and minimize bleeding in vivo.

In some experiments, soluble thrombomodulin was added to normal and FVIII-deficient plasmas to determine its effect on TAFI activation and lysis time. As expected, the addition of sTM resulted in increased TAFI activation and prolonged clot lysis. However, we did not observe a decrease in lysis time at high thrombomodulin concentrations as reported by Mosnier et al. (83). In our experiments, when sTM was increased to 100 nM the effect was antifibrinolytic in both normal and FVIII-deficient plasmas, whereas rabbit lung TM (rITM) at concentrations greater than 15 nM promoted fibrinolysis (83). This can be explained by the higher $K_d$ of the thrombin–sTM interaction (22) compared with the $K_d$ of the thrombin–rITM interaction (84) and it is expected that at higher concentrations of sTM (greater than 100 nM) the profibrinolytic effect of activated protein C would cause the clot to lyse prematurely. The concept of using a cofactor for protein C activation as a way of correcting premature lysis in hemophiliac plasma is paradoxical; therefore, F376A–sTM may be a better candidate for prolonging lysis, especially in hemophiliac plasma, because this mutant has greatly reduced cofactor
activity for protein C activation but normal cofactor activity for the activation of TAFI (85).

In the presence of 0.25 nM tPA the defect in TAFIa-dependent prolongation of lysis that is a characteristic of FVIII-deficient plasma (38) was corrected by the addition of 100 nM sTM. At higher concentrations of tPA the TAFIa-dependent prolongation of lysis defect is only partially corrected by 100 nM of added sTM. It is expected that as the tPA concentration is increased, plasminogen activation happens before sufficient TAFI can be activated to down-regulate fibrinolysis. As a result, much more sTM is required to get any TAFIa-dependent prolongation of lysis even in normal plasma. Because it is hard to predict how much plasminogen activator is available \textit{in vivo} it will be interesting to determine at what sTM concentration protein C activation becomes preferred over TAFI activation by the thrombin–sTM complex.

The matter of variable bleeding phenotypes among mild, moderate or severe hemophiliacs is a complex one that should take TAFI activation into consideration. Quantification of TAFIa in clotted normal, FVIII-deficient or mixed plasmas shows that TAFI activation and as a result, lysis times, are sensitive to FVIII levels. Also, stimulation of TAFI activation by sTM can prolong lysis times in FVIII-deficient plasma. These studies, along with those of Wang \textit{et al.} (85), suggest that selective activation of TAFI may facilitate therapy in hemophilia. Further studies should be directed at determining if sTM is an effective tool for correcting TAFI activation and lysis time in hemophiliac whole blood and other models of hemophilia.
Acknowledgements

We thank P. Y. S. Kim for purifying 5IAF-S525C-prothrombin for use in the prothrombin activation experiments. This work was supported by The National Institute of Health [HL46703], USA, and The Heart and Stroke Foundation of Ontario (T5575) and a R. Samuel McLaughlin fellowship (JHF).
Chapter 5
Thrombin-activatable fibrinolysis inhibitor activation and bleeding in hemophilia A
Abstract

Individuals with hemophilia A exhibit bleeding tendencies that are not always predicted by their factor (f)VIII level. It has been suggested that bleeding in hemophilia is due not only to defective prothrombin activation but also unregulated fibrinolysis. Thrombin activatable fibrinolysis inhibitor (TAFI) activation was measured in tissue factor initiated blood coagulation in blood samples of 28 hemophiliacs and 5 controls and correlated with bleeding phenotype. Reactions were quenched serially over 20–30 minutes with FPRck and citrate and assayed for TAFIa and thrombin-antithrombin (TAT). The TAFIa potential (TP) and the maximum rate were extracted from the time course of TAFI activation. The time course of TAFI activation closely follows thrombin generation regardless of the severity of hemophilia A. The magnitude of TP was similar among the control and mild subjects and reduced in severe subjects and the rate of TAFI activation was reduced in severe subjects. Both the TP and the rate were directly correlated with the rate of thrombin generation and fVIII levels. When considering all hemophilia subjects with bleeding score phenotype information, the TP was inversely and significantly correlated with hemarthrosis (-0.46, p=0.022), fVIII replacement (-0.52, p=0.017) and total bleeding score (-0.55, p=0.006). This study shows in whole blood that TAFI activation closely follows TAT formation and it quantifies the extent to which TAFI activation is attenuated in hemophilia A. The correlation between TP and the clinical bleeding phenotype is consistent with the hypothesis that differential TAFI activation contributes to variable bleeding in hemophilia.
**Introduction**

Hemophilia A is an extensively studied bleeding disorder in which mutations of the factor (f)VIII gene result in impaired fVIII activity and a heterogeneous phenotype (86,87). Individuals with hemophilia A are clinically divided into three categories of severity based on their coagulant fVIII activity (fVIII:C) (36). These categories are: mild (5-40% fVIII:C), moderate (1-5% fVIII:C) or severe (≤1% fVIII:C) fVIII deficiency. Activated fVIII profoundly increases the rate of fX activation (88) but fVIII:C does not adequately predict the bleeding phenotype in hemophilia, especially when fVIII:C is <1% (89). Previous studies have shown that fVIII inhibitors (35), discrepancies in fVIII:C using the one-stage compared to two-stage assay (80) or the presence of fV Leiden (90) can help explain some of the discrepancy between fVIII activity and the bleeding phenotype.

The molecular hallmark of hemophilia A is dampened fX activation (91) which has a negative downstream effect on thrombin generation, and therefore, clot formation (92). While most of the literature on hemophilia A focuses on diminished thrombin-dependent procoagulant activity, relatively little work has been conducted on the anti-fibrinolytic potential that thrombin affords through the activation of fXIII or thrombin activatable fibrinolysis inhibitor (TAFI), also known as procarboxypeptidase U, plasma procarboxypeptidase B or procarboxypeptidase R (27,48). At the site of vascular injury, thrombin initiates clot formation and influences clot structure directly by cleaving fibrinogen to form insoluble fibrin (93) and by activating platelets (94), and indirectly by stabilizing fibrin through the activation of TAFI and fXIII (95). Activated TAFI (TAFIa)
is a carboxypeptidase whose primary known physiological substrate is plasmin-modified fibrin (21,27). TAFIa removes C-terminal lysine and arginine residues created by plasmin, thus attenuating further plasminogen activation, which results in temporary inhibition of fibrinolysis (27). Activated fXIII (fXIIIa) is a transglutaminase that forms isopeptide bonds between fibrin monomers that increase the strength of the clot (96). Additionally, fXIIIa cross-links α2-antiplasmin to fibrin which delays clot lysis (97) by inhibiting plasmin, the enzyme responsible for digesting fibrin. It has been suggested that bleeding in hemophilia A is due in part to enhanced fibrinolysis (38,39,98) and consequently, enzymes that inhibit or prolong fibrinolysis, such as TAFIa, might influence bleeding in a positive way in hemophilia A.

A previous study by Antovic et al. (99) evaluated hemophilia A plasma for TAFI zymogen, TAFIa(i) (active and inactive TAFIa) and total TAFI antigen levels. Their results showed that TAFI zymogen levels are generally lower (~180nM) and TAFIa(i) antigen (~30pM) are increased in hemophiliac plasma compared to healthy controls (~300nM and ~15pM, respectively). This was suggested to be a result of the plasmin generated during enhanced fibrinolysis in hemophilia. Since only a small portion of the TAFI in plasma needs to be activated (<1%) to half-maximally prolong fibrinolysis (22), taking a snap-shot of total TAFI antigen levels in plasma is not very informative because of inherent variability between patients; however, an increase in TAFI zymogen would likely influence the thrombin-thrombomodulin-dependent rate of TAFI activation due to the relatively high $K_m$ of TAFI (22) compared to its plasma concentration.
TAFIa-dependent prolongation of the clot lysis time has been qualitatively demonstrated in hemophilia plasma by showing that a TAFIa inhibitor produces a small but significant reduction in the clot lysis time (38,39,100-102). The total TAFI antigen (77,103) and TAFI zymogen (77) antigen present in hemophilia A and healthy control plasmas have been measured by ELISA at a single time point. Conflicting results have been reported regarding the level of TAFI antigen in hemophilia A versus normal plasma. One study showed that both total TAFI antigen and TAFI zymogen antigen were decreased (77), whereas Guo et al. showed that there was no significant difference in total TAFI antigen in hemophilia versus normal plasma (103). Only one study exists where TAFIa was quantified at intervals over the course of clotting and fibrinolysis (98). This study, while informative, was conducted in plasma immuno-depleted of fVIII. In this current study, phlebotomy blood, maintained in the presence of corn trypsin inhibitor (CTI) was obtained from hemophiliacs and healthy individuals and induced to clot with relipidated tissue factor (TF). In this study, TAFI activation was quantified in the whole blood of subjects with hemophilia A over time and a new measure of fibrinolysis was extracted from the time course of TAFI activation to describe the potential cumulative effect of TAFIa on the clot over the course of the experiment. This new measure, termed TAFIa potential (98), gives a more informative assessment of TAFIa-dependent prolongation of fibrinolysis in hemophilia A than a single measurement of plasma zymogen or enzyme levels.
Subjects, Materials and Methods

Subjects

Individuals with hemophilia A and healthy controls were recruited and advised according to a protocol approved by the Institutional Review Board at the University of Vermont Human Studies Committee and the Centre Hospitalier Universitaire Sainte-Justine (Montreal, Canada). Informed written consent was obtained from 26 subjects with hemophilia A and 5 healthy controls. All hemophiliacs that required prophylaxis used fVIII replacement therapy and did not have any known inhibitors. All individuals that were included in the study were told not to withhold replacement therapy and did not need to self infuse with fVIII from 6 hours to 4 days prior to the blood draw. Subjects within the severe population were on different prophylaxis programs. For the purposes of this study, subjects were grouped according to their endogenous fVIII level at the time of the blood draw and also by severity at the time of diagnosis (mild, moderate or severe). The fVIII level at the time of blood collection as well as the severity at the time of diagnosis can be seen in Table 5-1. Fibrinogen, platelets, fVII, fVIIa, fX, fIX, prothrombin, fV, antithrombin, fXI, fXII, protein C and the aPTT and PT were in the normal range for all subjects.

Bleeding phenotype – Various degrees of clinical bleeding phenotypes were present in our hemophilia population (104). Briefly, the bleeding score used here takes hemarthrosis, soft tissue hematoma and annual fVIII unit kg$^{-1}$ usage into consideration. Bleeding score points were allocated as follows: hemarthrosis, 1–3/y 3, 4–6/y 6, 7–12/y...
9, > 12/y 12; soft tissue hematoma, 1–3/y 2, 4–6/y 4, 7–12/y 6, > 8/y 8; annual fVIII unit/kg usage, 0/y 0, < 1000/y 3, 1000–3000/y 6, > 3000/y 12. Surgery, dental extractions and major accidents were excluded for calculation of annual fVIII unit usage. The range of scoring was 0–32 (Table 5-1). Scores are reported as means of annual scores for a 5-year observation period for all hemophilia A subjects with available bleeding score phenotype.
Table 5-1. Clinical bleeding phenotype and fVIII level at the time of blood collection.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Original Classification</th>
<th>fVIII (%)</th>
<th>Hemarth. Mean (SD)</th>
<th>Soft Tissue Mean (SD)</th>
<th>FVIII Repl. Mean (SD)</th>
<th>Total Mean (SD)</th>
</tr>
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<tbody>
<tr>
<td>Mild 1</td>
<td>Mild</td>
<td>21</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Mild 2</td>
<td>Mild</td>
<td>16</td>
<td>0</td>
<td>2</td>
<td>1.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Mild 3</td>
<td>Mild</td>
<td>51</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mild 4</td>
<td>Mild</td>
<td>35</td>
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<td>0.3 (0.3)</td>
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<td>0.8</td>
</tr>
<tr>
<td>Mild 5</td>
<td>Mild</td>
<td>6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mild 6</td>
<td>Mild</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mod 1</td>
<td>Moderate</td>
<td>4</td>
<td>0.4 (0.4)</td>
<td>0.3 (0.3)</td>
<td>0.9 (0.6)</td>
<td>1.6</td>
</tr>
<tr>
<td>S1</td>
<td>Severe</td>
<td>16</td>
<td>12.0</td>
<td>6.4 (1)</td>
<td>12.0</td>
<td>30.4 (1)</td>
</tr>
<tr>
<td>S2</td>
<td>Severe</td>
<td>10</td>
<td>3.0</td>
<td>8.0</td>
<td>5.4 (0.6)</td>
<td>16.4 (0.6)</td>
</tr>
<tr>
<td>S3</td>
<td>Severe</td>
<td>22</td>
<td>2.5 (0.9)</td>
<td>0</td>
<td>9 (1.3)</td>
<td>11.5 (0.9)</td>
</tr>
<tr>
<td>S4</td>
<td>Severe</td>
<td>10</td>
<td>9.6 (1.1)</td>
<td>4.8 (0.8)</td>
<td>6</td>
<td>20.4 (1.3)</td>
</tr>
<tr>
<td>S5</td>
<td>Severe</td>
<td>11</td>
<td>6 (1.8)</td>
<td>3.3 (1.5)</td>
<td>6.0</td>
<td>15.3 (3)</td>
</tr>
<tr>
<td>S6</td>
<td>Severe</td>
<td>2.4</td>
<td>1.2 (1.2)</td>
<td>2.8 (0.5)</td>
<td>3.0</td>
<td>7 (1.5)</td>
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<tr>
<td>S7</td>
<td>Severe</td>
<td>1.65</td>
<td>6 (3)</td>
<td>3.3 (2.4)</td>
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<td>15.3 (5.4)</td>
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<tr>
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<td>Severe</td>
<td>1.2</td>
<td>8 (1)</td>
<td>8.0</td>
<td>6.0</td>
<td>22 (1)</td>
</tr>
<tr>
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<td>Severe</td>
<td>1</td>
<td>5.4 (0.6)</td>
<td>2.4 (0.4)</td>
<td>6</td>
<td>13.8 (0.8)</td>
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<tr>
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<td>Severe</td>
<td>4</td>
<td>2 (0.6)</td>
<td>1.7 (0.6)</td>
<td>6.0</td>
<td>9.7 (0.8)</td>
</tr>
<tr>
<td>S11</td>
<td>Severe</td>
<td>1</td>
<td>10.8 (0.7)</td>
<td>2.8 (0.8)</td>
<td>6</td>
<td>19.6 (1.3)</td>
</tr>
<tr>
<td>S12</td>
<td>Severe</td>
<td>3</td>
<td>7 (1.3)</td>
<td>2.7 (1)</td>
<td>4.5 (0.7)</td>
<td>14.2 (2.4)</td>
</tr>
<tr>
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<td>Severe</td>
<td>0.42</td>
<td>7.8 (1.2)</td>
<td>3.2 (0.8)</td>
<td>6.0</td>
<td>17 (0.6)</td>
</tr>
<tr>
<td>S14</td>
<td>Severe</td>
<td>0.42</td>
<td>0.6 (0.6)</td>
<td>1.6 (0.7)</td>
<td>6.0</td>
<td>8.2 (1.3)</td>
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<td>0.24</td>
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<td>3.2 (1)</td>
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<td>15.8 (1.3)</td>
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<tr>
<td>S16</td>
<td>Severe</td>
<td>0.16</td>
<td>3 (1.7)</td>
<td>1.3 (1.3)</td>
<td>12.0</td>
<td>16.3 (2.2)</td>
</tr>
<tr>
<td>S17</td>
<td>Severe</td>
<td>0.18</td>
<td>8.4 (1.1)</td>
<td>5.2 (1)</td>
<td>12.0</td>
<td>25.6 (2.1)</td>
</tr>
<tr>
<td>S18</td>
<td>Severe</td>
<td>0.42</td>
<td>2 (1)</td>
<td>3.3 (1.3)</td>
<td>6.0</td>
<td>11.3 (0.3)</td>
</tr>
<tr>
<td>S19</td>
<td>Severe</td>
<td>0.07</td>
<td>1.5 (0.9)</td>
<td>0.0</td>
<td>7.5 (1.5)</td>
<td>9 (2.1)</td>
</tr>
<tr>
<td>S20</td>
<td>Severe</td>
<td>0.1</td>
<td>3.6 (0.6)</td>
<td>4 (0.6)</td>
<td>9.6 (1.5)</td>
<td>17.2 (1.6)</td>
</tr>
<tr>
<td>S21</td>
<td>Severe</td>
<td>0.16</td>
<td>4.8 (1.4)</td>
<td>7.2 (0.9)</td>
<td>5.4 (0.4)</td>
<td>17.4 (0.8)</td>
</tr>
</tbody>
</table>
Materials

*Whole blood assay* – HEPES, Tris-HCl, citrate, TFA, 1-palmitoyl-2-oleoyl-phosphatidylserine (PS), 1-palmitoyl-2-oleoyl-phosphatidyl choline (PC) were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant TF was provided by Drs. Lundblad and Liu (Hyland division, Baxter Healthcare Corp, Duarte, CA) and was relipidated in PCPS (75% PC:25% PS) vesicles by a previously described protocol (105). CTI was prepared as described (106). D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (FPRck) was provided by Dr. Richard Jenny (Haematologic Technologies, Essex Junction, VT) or purchased from Calbiochem (San Diego, CA, USA).

*TAFI assay* – Thrombin and fibrinogen were prepared as previously described (63) with one exception: for the fibrinogen preparation, the solution was made to 1.2% PEG-8000 instead of 2% PEG-8000 by the addition of 40% (w/v) PEG-8000 in water, subsequent to β-alanine precipitation. Fibrin degradation products labeled with the quencher QSY9 C₅-maleimide (QSY-FDP), TAFIa standards, TAFI-deficient plasma (TDP), recombinant human Pg (S741C) and the fluorescein derivative (5IAF-Pg) were prepared as described (61,62,64). QSY9 C₅-maleimide and 5-iodoamidofluorescein were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). Plasmin was purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA). Solulin was a generous gift from Dr. Achim Schuttler, Paion, GmbH (Aachen, Germany). The plasmin inhibitor D-Val-Phe-Lys chloromethyl ketone was purchased from Calbiochem (San Diego, CA, USA). All other reagents were of analytical quality.
Methods

Whole blood coagulation assay – Experiments were performed as previously described (3,107) in a temperature controlled chamber (37°C) on a rocking platform. Venous blood obtained by antecubital phlebotomy from 28 hemophiliacs and 5 controls was added (1mL) to tubes preloaded with CTI (100μg/mL) and relipidated TF (5pM, PCPS 1:2000 protein:lipid). A time course was set up over the time period from 0-20 (18 subjects) or 30 (13 subjects) minutes; dependent on the amount of whole blood obtained from each individual. The control tube contained CTI and no TF and did not clot within 30 minutes. At appropriate times, samples were quenched with FPRck (50μM) and citrate (9 parts blood mixed with 1 part sodium citrate, 3.2%; final concentration 10.8mM). The zero time point contained the inhibitors prior to the addition of blood. Each sample was centrifuged at 1200 x g for 15 minutes to remove cells and fibrin and promptly placed on ice to prevent thermal inactivation of TAFIa. Samples were stored at -80°C until assayed for TAFIa. A second set of samples from contemporaneous experiments were quenched (EDTA:25mM final, benzamidine-HCl:10mM and FPRck:50μM, pH 7.4) and identically prepared and stored and subsequently assayed using an ELISA for the TAT complex (Behring, Westwood, MA, USA) as previously described (108).

TAFIa assay – Samples were thawed by moving the samples between 4°C and ambient temperature to prevent thermal inactivation of TAFIa while ensuring solubility of all plasma proteins. All samples were then diluted serially by 5-fold using TAFI deficient plasma and assayed for TAFIa as previously described (61). To measure TAFI zymogen,
each sample was diluted 1000-fold and incubated with 25nM thrombin, 100nM solulin in the presence of 5mM CaCl₂ for 15 minutes at room temperature to quantitatively activate TAFI. The samples were then assayed for TAFIa.

Determination of the rate of TAFI activation and the TAFIa potential – TAFIa concentration was measured over time using a functional assay for TAFIa (61). These data were plotted and the rate of TAFI activation was determined using the maximal slope as determined by linear regression of the data where possible and TAFIa potential (TP) was determined by calculating the area under the curve during the time course of the experiment for each set of data.

Statistics – Between groups comparisons were conducted using the Student’s unpaired \( t \) test for normally distributed data. Data were presented as mean ± standard error of the mean. To correlate the rate of TAFI activation and TP to TAT, fVIII or clinical bleeding score Spearman rank correlation analysis was used. For all statistical analysis \( p<0.05 \) was considered statistically significant. The Grubbs’ outlier test was used to detect outliers where appropriate.

Results

Time courses of TAFI activation in control and hemophilia A blood – TAFI activation was monitored over time in phlebotomy blood of 5 control subjects and 28 hemophiliacs and the time courses of TAFI activation are presented. Figure 5-1 shows that TAFI
activation commenced after a lag period ranging from 5-15 minutes in the control subjects. The TAFIa concentration then propagated between 5-20 minutes and the concentration reached a plateau thereafter. The lag period ranged from 5 to 15 minutes in hemophilia subjects that showed measurable TAFI activation (Figures 5-2 and 5-3) and the propagation of TAFI occurred in the 15 minutes following the lag period. After propagation, the concentration subsequently reached a plateau.

Figure 5-1. TAFI activation in control subjects.
These data demonstrate that TAFI activation is highly variable in controls. The rate of TAFI activation and TP were extracted from these data.
Figure 5-2. TAFI activation in mild and moderate Hemophilia A.
These data demonstrate that TAFI activation is somewhat variable mild and moderate subjects with hemophilia A. Both the rate of TAFI activation and TP were extracted from these data.
Figure 5-3. TAFIa activation in severe Hemophilia A.

Severe subjects were grouped according to their fVIII level at the time of blood collection. Panel A shows subjects with >5% fVIII at the time of blood collection, while subjects with 1-5% fVIII or <1% fVIII at the time of blood collection are shown in panels B and C, respectively. These data demonstrate that TAFI activation is variable regardless of endogenous fVIII activity. Both the rate of TAFI activation and TP were extracted from these data.

Within the hemophilia subjects, the patterns of TAFIa formation were similar but changes in the lag period, propagation and plateau appear to be influenced by the fVIII concentration. When the clinically severe hemophilia subjects were grouped according to their fVIII level at the time of blood collection (Figure 5-3) the lag period increased and TAFIa propagation decreased as the fVIII level decreased. Within the severe subjects (n=16) with less than 5% fVIII at the time of blood collection (Figure 5-3, panels B and C), most subjects exhibited both a reduced TAFIa potential (Figure 4) and a reduced rate of TAFI activation (Figure 5-5) compared to subjects with > 5% fVIII. In Figures 5-4 and
5-5, subjects have been grouped by both original diagnosis (panel A) and fVIII level at the time of blood collection (panel B). Both the rate and the TP decreases as the fVIII:C level decreases regardless of how the subjects are grouped which is expected since many of the subjects had fVIII levels at the time of blood collection that were consistent with their original diagnosis. Some caution should be taken when comparing the average TAFI activation profiles since not all subjects could be analyzed over the full 30 minute time course.

In all experiments that ran for 30 minutes, rates were substantially greater in the 10 to 20 minute interval than in the 20-30 minute interval, and in the majority of cases, the TAFIa level reached a plateau and began to decline in the latter interval. This indicates that the majority of TAFI activation occurred in the first 20 minutes. This is consistent with the time courses of the TAT formation (see below).

*Rate of TAFI activation and TAFIa potential in hemophilia A* – The time courses of TAFI activation (Figures 5-1, 5-2 and 5-3) were used to extract the rates of TAFI activation and TP as described in *Methods*. When subjects were grouped by their original classification, the average TP of the controls (15930±4030pM-min) and mild subjects (17300±3025pM-min) were indistinguishable. In severe subjects the TP was reduced by 63 percent (5850±1320pM-min, p=0.02) (Figure 5-4A). The TP in the severe group was significantly reduced compared to the control and mild groups. When subjects were grouped by their fVIII level at the time of blood collection, the average TP in subjects
with >5% fVIII was reduced by 14%. The TP was reduced by 52% and 87% in subjects with 1-5% fVIII or <1% fVIII, respectively (Figure 5-4B). The TP in the group with <1% fVIII was significantly reduced compared to all other groups.

The average rate of TAFI activation was reduced by 41 – 49% in the mild, moderate and severe groups compared to the controls (233±36pM-min) (Figure 5-5A). When subjects were grouped by their fVIII level at the time of blood collection, the average rate of TAFI activation in subjects with >5% fVIII was reduced by 35%. The TP was reduced by 33% and 75% in subjects with 1-5% fVIII or <1% fVIII, respectively (Figure 5-5B). Like TP, the rate of TAFI activation in the group with <1% fVIII was significantly reduced compared to all other groups.
Figure 5-4. Average TAFIa potential in hemophilia A and control subjects. Subjects were grouped by the original classification (panel A) or their fVIII level at the time of blood collection (panel B). Between group comparisons were conducted using a t test. *p<0.05
Figure 5-5. Average rate of TAFI activation in hemophilia A and control subjects. Subjects were grouped by their original classification (panel A) or their fVIII level at the time of blood collection (panel B). Between group comparisons were conducted using a t test. *p<0.05
The temporal relationship between thrombin formation and TAFI activation in control and hemophilia subjects – Upon analyzing the time course of thrombin formation, presented as TAT, and TAFI activation it became evident that there is a temporal relationship between thrombin generation and TAFI activation. Figure 5-6 shows the time course of thrombin generation and TAFI activation in the control (panel A) and hemophilia subjects (panels B-D). TAFI activation closely mirrors the thrombin generation profile, with TAFI activation lagging behind thrombin generation by approximately 5 minutes.
Figure 5-6. The temporal relationship of thrombin generation and TAFI activation in subjects grouped by their fVIII level at the time of blood collection. Thrombin-antithrombin (●) and TAFIa (○) were assayed over time in whole blood from controls (panel A), and hemophilic subjects with > 5% fVIII (B), 1-5% fVIII (C) and < 1% fVIII (D). The maximal concentration of thrombin-antithrombin and the rate of TAT formation decreased as the fVIII concentration decreased. Similarly, both the rate and maximal concentration of TAFIa decreased as the fVIII concentration decreased. In all groups, TAFI activation was slightly delayed compared to thrombin generation.
When the hemophilia subjects are separated by fVIII level at the time of blood collection, the extent of thrombin generation is similar in all groups, with the maximum level of thrombin generated being approximately 400nM. In contrast, the rate of TAT formation decreases as the fVIII concentration decreases; control (38.2nM/min), >5% fVIII (22.8nM/min), 1-5% fVIII (18.7nM/min) and <1% fVIII (11.6nM/min). When thrombin generation is compared to TAFI activation, a similar trend with respect to the rate is evident. The maximal TAFIa concentration is more sensitive to fVIII than TAT since TAFIa levels are decreased in the group with >5% fVIII but TAT levels are not noticeably decreased in groups with >1% fVIII.

_TAFI zymogen levels_ – Hemophilia A subjects had significantly reduced TAFI levels (127.0±7.7nM) compared to controls (174.9±10.4nM) (p<0.05). While these data differed significantly, both groups fell within the normal range (73-250nM) (109). The difference between controls and hemophiliacs is consistent with previous observations (99).

_Correlations between TAFI activation, thrombin generation and bleeding phenotype in hemophilia A_ – In hemophilia subjects, the rate of TAFI activation was significantly correlated with the rate of TAT formation (0.42), the TAT level at 20 minutes (0.43) and fVIII (0.51) while the TP was significantly correlated with the rate of TAT formation (0.66), the TAT level at 20 minutes (0.67) and fVIII level at the time of blood collection (0.74). As shown in Table 5-2, TP was also significantly correlated with the clinical
bleeding phenotype (score) and these correlations were always stronger than the corresponding values for TAT level at 20 minutes.

<table>
<thead>
<tr>
<th>Table 5-2. Spearman correlation of the clinical phenotype to TAFIa potential and TAT.</th>
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</thead>
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<td>Hemarthrosis</td>
</tr>
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<td>TP</td>
</tr>
<tr>
<td>TAT</td>
</tr>
<tr>
<td>Soft Tissue</td>
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<td>TAT</td>
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<tr>
<td>Total Score</td>
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<td>TP</td>
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</table>

**Discussion**

The hypothesis that bleeding in hemophilia is partially due to enhanced fibrinolysis was first suggested by Broze and Higuchi in 1996 (38). Recently others have demonstrated that the dampened thrombin generation associated with hemophilia A has a negative effect on TAFI activation and clot stabilization (39,99).

Only a relatively small percentage (<1%) of available TAFI is activated in plasma when clotting is initiated with low levels of thrombin (68). As recently reported, the percentage of fVIII in plasma greatly influences TAFI activation over the interval between clot formation and tPA induced clot lysis (98) especially in the range of 0-10% fVIII. We expanded upon this previous study to determine how TAFI activation
correlated with other factor levels in hemophilia A plasma and to gain insight into the rate and extent of TAFI activation in the whole blood of hemophilia A subjects. Our results confirm that fVIII aids TAFI activation since fVIII was significantly correlated with the rate of TAFI activation (0.51) and TP (0.74). The correction of TP in subjects with > 5% fVIII to control levels suggests that as little as 5-10% fVIII:C is required to normalize TAFI activation in whole blood, whereas up to 50% fVIII is required to correct TAFI activation in plasma (98).

Data presented in Figure 5-6 shows that thrombin generation has essentially ceased after 20 minutes in controls and in most hemophiliacs, suggesting that the propagation phase has ended. This is also reflected by the lower rate of TAFI activation after 20 minutes compared to the first 20 minutes. Since most TAFI activation occurs during the propagation phase, 20 minutes is a reasonable interval for measuring the rate of TAFI activation and TP. In the interval after thrombin generation has ended and, therefore, TAFI activation has ceased, TAFIa levels can be predicted using the rate constant for spontaneous decay of TAFIa (110).

TAFI levels were previously determined to be decreased in hemophilia A patients compared to healthy controls (99). Our findings are consistent with these results; however, the TAFI zymogen concentrations in hemophilia still fall within the reported normal range (73-250nM) (109). TAFI plasma levels can vary considerably but this may depend on the assay used (109). A reduction of TAFI concentration in hemophilia A may be significant in vivo since the $K_m$ of thrombin-thrombomodulin-dependent TAFI activation is relatively high (22) compared to the concentration of TAFI in plasma (109).
and, therefore, the rate of TAFI activation would be proportional to the TAFI concentration. In addition, Mosnier et al. have shown a direct correlation between the TAFI concentration and the time to lyse a clot in plasma (111).

Recently, it was demonstrated that the dampened thrombin generation in severe hemophilia A led to reduced fXIII activation, which coincided with increased fibrinopeptide A (fpA) release prior to the time of clot formation (92). Consequently, hemophiliac blood clots are more porous than normal clots. Presumably, the decreased fXIII activation observed in hemophiliac blood would also negatively influence the cross-linking of anti-plasmin to fibrin which, combined with the decrease in TAFI activation described here, would result in a clot that is predisposed to enhanced fibrinolysis.

It has been demonstrated, using our whole blood assay (104), that thrombin generation is decreased in hemophilia A. Thus, decreased TAFI activation in hemophilia A is expected. TAFI activation, however, should be considered as more than a corollary to thrombin generation. TAFI activation and activity can vary due to the presence of soluble thrombomodulin (22), the plasma TAFI concentration (22) or the TAFI genotype (30). Thus, the impact of a fixed thrombin concentration on TAFI activation and activity can result in differing clot stability and clot lysis times in different individuals. The data presented here are consistent with the hypothesis that variable TAFI activation contributes to variable bleeding in hemophilia. In the subjects analyzed here, we confirm that TP is strongly correlated with thrombin generation and also more strongly correlated with the clinical bleeding phenotype than TAT levels at 20 minutes. Fibrinolysis is an often overlooked component of hemostasis and the TP may prove useful in giving a more
comprehensive, global assessment of hemostasis in pathological bleeding since it accurately reflects thrombin generation but also is a measure of the clot’s resistance to fibrinolysis.

Empirical research using whole blood from subjects with severe hemophilia is complicated by fVIII replacement therapy which makes it difficult to assign a mechanism to variable bleeding at a given fVIII level. The issue of whether to classify hemophiliacs based on their fVIII level at the time of blood draw or their original fVIII level is complex since biochemical data derived from whole blood clotting assays are a reflection of the fVIII at the time of the blood draw but historical data such as the clinical bleeding phenotype (bleeding score) is based more upon the original diagnosis. To clarify this issue, we have grouped subjects by both fVIII levels at the time of blood collection and by original diagnosis.

Currently, patients with hemophilia A are treated as required or by prophylaxis with factors that correct or bypass the defective tenase complex (35). It is unclear if these treatment strategies also fully correct secondary thrombin-dependent events such as fXIII and TAFI activation. Enhancing the activation of these zymogens would potentially protect hemophilia A clots from premature lysis by decreasing plasminogen activation and increasing plasmin inhibition. Future studies will examine what percentage of fVIII is required to fully correct both TAFI and fXIII activation. We have determined that both the rate of TAFI activation and the TP are significantly reduced in the blood of severe hemophiliacs and the methods described here could be used to determine which tenase correcting or bypassing agent is most effective in correcting TAFI activation.
Acknowledgements

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Chapter 6
Soluble thrombomodulin improves the hemostatic balance in whole blood from canines with hemophilia A
**Abstract**

Hemophilia A is a debilitating disease that affects approximately 1 in 5,000 males. Severe hemophiliacs are prone to spontaneous bleeding which increases the morbidity and mortality of the disease and many affected individuals develop inhibitors to fVIII which makes fVIII replacement therapy ineffective. Soluble thrombomodulin (sTM), an indirect stabilizer of blood clots, may stabilize the clot to an extent where less fVIII may be needed to control bleeding in hemophilia. Clot-lysis experiments were conducted in normal and hemophilic (±fVIII neutralizing inhibitors) dog plasma and whole blood and clotting and fibrinolysis were monitored by turbidity and thrombelastography. Canine TAFI exists in plasma at a concentration of 100nM and the half-life of canine TAFIa is 23 minutes. In hemophilic plasma, the clot-lysis time and the TAFIa potential increased linearly with the sTM concentration. In hemophilic whole blood, the area under the clot-lysis (elasticity) curve (AUEC) can be used as a measure of clot firmness and 100nM sTM increased this parameter by approximately 5-fold. The clot lysis time in whole blood was increased from 20 minutes (no sTM) to >1.5 hours in the presence of 100 or 250nM sTM. Finally, in hemophilic whole blood with fVIII-neutralizing inhibitors (>150BU), the AUEC was normalized with 390nM sTM and the clot-lysis time increased from 18 to 46 minutes. STM increases both the clot lysis time and clot firmness in hemophilic plasma and whole blood. This improvement in the hemostatic balance suggests that sTM may be used to suppress fibrinolysis and stabilize clots in hemophilia.
Introduction

Patients with hemophilia A have a bleeding diathesis that is usually predicted by their factor (f)VIII activity level (fVIII:C) (35,112). The primary form of treatment for severe hemophilia A is replacement therapy which involves administration of recombinant or plasma derived fVIII. FVIII can be given either on demand or by prophylaxis (113) and the amount given can vary drastically depending on the treatment schedule and the type and severity of the bleed in the case of on demand treatment (114). While these forms of treatment are reasonably effective it causes 25% of severe hemophilic patients to develop neutralizing fVIII antibodies (115,116), termed fVIII inhibitors, which can render fVIII replacement therapy ineffective.

When fVIII is no longer useful in treating the bleeding diathesis of hemophilia A, other products such as recombinant fVIIa or (activated) prothrombin complex concentrates (a)PCC may be used. These forms of treatment have typically been given on demand (117,118), however, prophylactic treatment is becoming more common (119,120).

In addition to the development of fVIII inhibitors, the major drawback of fVIII (and fVIIa and (a)PCC) is the high cost. Recently, a cost analysis showed that the cost to treat a severe hemophilic patient on demand with fVIII during the years 1978-1998 was almost $60,000/patient/year (121) and the cost of prophylactic treatment has been estimated to be in excess of $300,000/patient/year (122). It is the cost of fVIII replacement therapy, whether on demand or by prophylaxis, that prevents most people in undeveloped countries from getting the required treatment (123). Because of the high
cost of fVIII replacement therapy and the risk of developing fVIII inhibitors, other forms of treatment are being explored.

Many new and adjunctive therapeutic options have been explored including platelet infusion (124), tranexamic acid (125), ε-amino caproic acid (126) a fucoidan called AV513 (127) and a combination of phospholipid and fXa (128). Recently, we demonstrated that soluble thrombomodulin (sTM) may be used to partially correct the premature lysis defect in fVIII-deficient plasma through a TAFIa-dependent mechanism (98) which supports the hypothesis that bleeding in hemophilia may be due to unregulated fibrinolysis (38) in addition to the well documented clotting defect (87). This hypothesis is also supported by a preliminary clinical study showing that ε-ACA, an anti-fibrinolytic lysine analogue, may be used adjunctively with FEIBA or aPCC to control bleeding (126). Full length thrombomodulin (TM) has been shown to tightly bind to thrombin (84) which prevents cleavage of (129) fibrinogen and therefore, fibrin formation. Furthermore, the cofactor activity of TM for thrombin mediated protein C activation diminishes thrombin generation by proteolytically inactivating coagulation cofactors fVa and fVIIIa (130). It is for these reasons that TM was thought an unlikely candidate for the treatment of bleeding in hemophilia. An important difference between sTM and full length TM is its reduced affinity for thrombin which greatly reduces its anti-coagulant function but still adequately promotes TAFI activation (22).

In the present study, we show that sTM may be used to attenuate fibrinolysis in whole blood from hemophilic dogs from the Queen’s University canine hemophilia A colony. These dogs are an ideal model for human hemophilia A since they share many
characteristics of the human pathology. Some of the dogs develop fVIII neutralizing antibodies, while others do not (131,132) and in all of the dogs, the reduced fVIII:C is a result of an intron 22 inversion which is strikingly similar to the mutation that causes severe hemophilia A in 45% of afflicted humans (133). Finally, the Queen’s University hemophilic dog colony has previously been used for preclinical studies on the development of fVIII (134,135), fXa/PCPS (128) and AV513 (127). Here we present in vitro data which support the hypothesis that sTM may be used to stabilize blood clots in hemophilia A. These preliminary data provide information that will be useful during preclinical in vivo studies.

**Materials and Methods**

*Materials* – Thrombin was prepared as previously described (63). Recombinant human soluble thrombomodulin (Solulin; sTM) was a generous gift from Dr Achim Schuttler (Paion, GmbH (Aachen, Germany). The thrombin inhibitor D-Phe-Pro-Arg-chloromethylketone (PPAck) and potato tuber carboxypeptidase inhibitor (PTCI) were purchased from Calbiochem (San Diego, CA, USA). Tissue-type plasminogen activator (Activase; tPA) was purchased from the pharmacy at KGH (Kingston, ON, Canada). All other reagents were of analytical quality and the buffer used in all experiments was HEPES buffered saline (20mM HEPES, 150mM NaCl pH 7.4).
Animals – The dogs with hemophilia A were bred and maintained at Queen’s University, Kingston, Ontario. All procedures were in compliance with the institutional animal care committee and the Canadian Council for Animal Care.

Decay of Canine TAFIa – To activate canine TAFIa, hemophilic canine plasma was diluted 1:100 and incubated with 25nM thrombin, 100nM sTM and 5μM CaCl₂ for 15 minutes at room temperature (as per (30)). The thrombin was subsequently inhibited using 1μM PPA-chloromethyl ketone and the sample was centrifuged (16,000 x g for 2 minutes) to remove any insoluble fibrin. The sample was then incubated at 37°C in a water bath. At regular intervals, a small aliquot was removed and placed on ice to prevent further thermal inactivation of TAFIa. After all samples were collected they were assayed for TAFIa activity using a previously described functional assay (61).

Clot-lysis assays – Canine hemophilic plasma (40μL) was added to wells of a clear bottom 96-well microtitre plate. A solution (60μL) containing sTM (0 – 500nM) and tPA (1nM) (final concentrations) was added to each well. These experiments were also conducted in the presence of PTCI (5μM final concentration) to show the TAFIa dependent prolongation of lysis in canine hemophilic plasma. Finally, coagulation was induced by the addition of a solution (20μL) containing 12nM thrombin and 60mM CaCl₂. Coagulation and fibrinolysis were monitored continuously (1 minute intervals) by turbidity (400nm) at 37°C using a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).
Thrombelastography – Canine hemophilic whole blood (±fVIII neutralizing antibodies) (320μL) was added to channels of a Haemoscope TEG® 5000 (Haemonetics Corp. Braintree, MA) containing a 40μL solution of 90nM thrombin, 9nM tPA and 0 – 3510nM sTM. After mixing thoroughly, the pin was seated and coagulation and fibrinolysis were monitored continuously. The Haemoscope TEG® 5000 allows for measurement of the clot time, clot kinetics, clot strength and clot stability (fibrinolysis) by measuring the torque on a wire which is connected to through the pin. As a clot forms the torque on the pin increases and is represented by an increase in the amplitude (output). Similarly, during fibrinolysis, degradation of the clot results in a decrease of the torque and a decrease in the amplitude.

Results

Canine TAFI – Using standard methods, TAFI was quantitatively activated in a 1:100 dilution of hemophilic canine plasma and the concentration of TAFIa assayed was 1037pM, which implies a plasma concentration of 104nM, a value similar to the concentration of TAFI in human plasma (109). By fitting the data to an exponential decay function the half-life of canine TAFIa was determined to be approximately 23 minutes and unlike human TAFIa, not all TAFIa activity decayed at 37°C (Figure 6-1). After a 3 hour incubation period at 37°C approximately 120pM of TAFIa-like activity remained.
Figure 6-1. Decay of canine TAFIa.
Canine TAFIa was incubated at 37°C and assayed over time for activity. An exponential decay function was used to determine that the half-life of canine TAFIa is 23 minutes and not all TAFIa-like activity decays. Since canine TAFIa was quantitatively activated from canine plasma (1/100 dilution) the TAFIa activity at t=0 implies a TAFI plasma concentration of ~100nM.

TAFI activation in hemophilic canine plasma in the presence of sTM – In hemophilic canine plasma, peak TAFIa levels and TAFIa potential increased from 2000pM and 10,000pM-min, respectively, in the absence of sTM to approximately 8100pM and 250,000pM-min, respectively, in the presence of 100nM sTM (Figure 6-2, panels A and D). Soluble TM linearly increased the TAFIa potential (Figure 6-2, panel E) which resulted in a 4-fold prolongation of fibrinolysis (Figure 6-2, panels A-D). Clot lysis times were also determined in the presence or absence of the TAFIa inhibitor, PTCI, and a ratio of the times (lysis time, no PTCI divided by the lysis time with PTCI) were determined to
show the TAFIa-dependent prolongation of fibrinolysis as shown in Figure 6-3. In the absence of sTM, the lysis time is the same in the presence or absence of PTCI resulting in a ratio of approximately 1. As the sTM concentration increases the TAFIa-dependent prolongation of fibrinolysis increases. At 25nM sTM, the lysis time is doubled and this trend continues up to 200nM sTM where fibrinolysis is attenuated by 8-fold. This 8-fold prolongation of fibrinolysis is constant at sTM concentrations ranging from 200 – 500nM.
Figure 6-2. Soluble TM dependent prolongation of clot-lysis.
In hemophilic plasma, the clot-lysis time (panels A-D, —) and the extent of TAFI activation (•) increased with the sTM concentration (Panel A: 0nM; B: 25nM; C: 50nM; D: 100nM). As the concentration of sTM increased the lysis time and the extent of TAFI activation increased. Panel E shows the TAFIa potential (area under the time courses of TAFI activation in panels A-D).
TAFIa-dependent Fold Prolongation of Lysis Time (LT, No PTC/LT + PTCI)

$[sTM]$ (nM)

Figure 6-3. Soluble TM increases the TAFIa-dependent prolongation of fibrinolysis in plasma. Fibrinolysis is prolonged by approximately 2-fold in the presence of 25nM sTM. A plateau in the fold-prolongation of lysis is reached at 200nM sTM.

sTM improves clot firmness and attenuates fibrinolysis in canine hemophilic whole blood in the presence or absence of fVIII neutralizing antibodies – Thrombelastography experiments were conducted in hemophilic whole blood and the data are presented in Figure 6-4A. In hemophilic whole blood, the clot lysis time was increased from 20 minutes in the absence of sTM to >1.5 hours in the presence of 100 or 250nM sTM (Figure 6-4, panel A). When using thrombelastography, the area under the clot-lysis (elasticity) curve (AUEC) can be used as a measure of clot firmness. Figure 6-4, panel B
shows that 100nM or 250nM sTM increased the AUEC by approximately 5-fold. When the sTM was increased to 390nM both the clot lysis time and the AUEC decreased substantially.
A

Time (minutes)

Amplitude (mm)

-150
-100
-50
0
50
100
150

No sTM
100nM sTM
250nM sTM
390nM sTM

B

Lysis Time (minutes)

200
180
160
140
120
100
80
60
40
20
0

[sTM] (nM)

0 100 200 300 400

100
80
60
40
20
0
Figure 6-4. Thrombelastographs showing the sTM-dependent prolongation of lysis in whole blood from a dog with hemophilia A (panel A). The clot lysis time is the time after clotting when the amplitude is <2mm. The clot lysis time (estimate for 100 and 250nM sTM) in whole blood was increased from 20 minutes in the absence of sTM to >3 hours in the presence of 100 or 250nM sTM (panel B). The area under the clot-lysis curve (AUEC) can be used as a measure of clot firmness and 100nM sTM increased this parameter by approximately 5-fold (panel C).

Similar thrombelastography experiments were conducted in hemophilic whole blood with fVIII-neutralizing inhibitors (>150BU) and the data are presented in Figure 6-5A. In canine whole blood with fVIII inhibitors, the clot-lysis time was increased from 18 to 46 minutes and the AUEC was normalized with 390nM sTM (Figure 6-5, panel B).
Figure 6-5. Thrombelastographs showing the sTM-dependent prolongation of lysis in whole blood from a canine with hemophilia A and fVIII neutralizing antibodies. The clot lysis time is the time after clotting when the amplitude is <2mm. As shown in panel B, the AUEC was normalized with 390nM sTM and the clot-lysis time was increased from 18 to 46 minutes.
Discussion

Thrombomodulin (TM) has long been recognized as an effective anti-coagulant due to its cofactor activity for protein C activation (130) and its ability to tightly bind thrombin which consequently prevents cleavage of fibrinogen (129) and fibrin formation. In fact, the soluble thrombomodulin variant used in these studies is currently being explored for the treatment of stroke. During phase I clinical trials, sTM was determined to be safe and demonstrated a clear proof of principle, that being effective inhibition of thrombin (136). The concentrations of sTM used in our studies are far lower than those that may be effective in treating stroke and since sTM has a relatively high $K_d$ for thrombin (22) compared to full length thrombomodulin (84) it minimally impacts fibrinogen cleavage at low concentrations. Mosnier et al. (83) demonstrated that less than 15nM full length rabbit lung (rl) TM may be used to prolong fibrinolysis but at higher concentrations the balance shifts toward the anticoagulant effect of activated protein C and inhibition of fibrinogen cleavage.

In hemophilic canine plasma we showed that sTM can be used to prolong fibrinolysis at concentrations ranging from 25 – 500nM. In hemophilic canine whole blood the anti-fibrinolytic effect of sTM is demonstrated at sTM concentrations up to 390nM. The apparent shift from an anti-fibrinolytic to an anti-coagulant appears to occur between 250 and 390nM sTM. At 250nM sTM, the maximal anti-fibrinolytic effect is achieved (no lysis in approximately 90 minutes), whereas, at 390nM sTM the lysis time is shortened significantly (40 minutes). Since the maximum amplitude of the TEG tracing does not change between 250 and 390nM sTM, it is unlikely that fibrinogen cleavage is
inhibited; therefore, the shortening of the lysis time can be attributed to an increase in protein C activation which results in decreased thrombin generation and consequently, decreased TAFI activation. Since sTM does have anti-coagulant properties, pre-clinical studies will be very important in determining what dose adequately stabilizes the clot without causing unwanted bleeding. There are 2 sTM mutants that may be useful in increasing the safety of the potential drug. Both oxidized M388-sTM and F376A-sTM retain most of their cofactor activity for TAFI activation but only a small fraction of their cofactor activity for protein C activation. Using these constructs may increase the therapeutic window of sTM by making it safer at higher doses.

It has been our experience that canine blood and plasma differ significantly with respect to parameters of both coagulation and fibrinolysis. One such example is the extent of TAFI activation in canine plasma compared to human plasma. Here, we have reported that in the absence of sTM, hemophilic canine plasma (<1% fVIII:C) when clotted with 2nM thrombin and 10mM CaCl₂ yields a TAFIa potential of 10,000pM-min. This is in contrast to the reported TAFIa potential of 2000pM-min in human plasma containing 1% fVIII:C (98). The increased TAFI activation in canines when combined with the 3-fold longer half-life of canine TAFIa (23 minutes compared to 8 minutes for human TAFIa) gives rise to clots that are more resistant to fibrinolysis.

Surprisingly, when measuring the decay of canine TAFIa we observed that not all TAFIa activity decayed at 37°C suggesting the presence of a stable isoform of TAFIa or another enzyme displaying TAFIa activity. In the original paper describing our TAFIa assay it was noted that there was a residual rate in TAFI-deficient plasma suggesting
some TAFIa-like activity from another enzyme. Carboxypeptidase N (CPN) or cleaved CPN are considered the most likely candidates. It is expected that the residual TAFIa-like activity measured here is from cleaved-CPN which has been shown to inhibit fibrinolysis (137) and as a result should also cleave the fibrin degradation products that act as the TAFIa substrate in our TAFIa assay.

When comparing the sTM-dependent prolongation of lysis in the hemophilic dog to the hemophilic dog with inhibitors it is evident that the effect of sTM is much more pronounced in the absence of inhibitors. Since the inhibitor titre was so high in the dog with inhibitors (>150 BU), fVIII:C was completely neutralized and as a result, these data suggests that very little fVIII (<1%) is sufficient in enhancing the anti-fibrinolytic effect of sTM in hemophilic canine blood. While it is promising that very little fVIII is required to enhance the anti-fibrinolytic properties of sTM, more research is required before sTM may be considered as an adjuvant for fVIII.

As previously mentioned hemophilic dogs, especially those of the Queen’s University colony are a suitable model of human hemophilia because of the similarity of their condition to that of afflicted humans. The preclinical data presented here suggests that these dogs may also prove effective in the preclinical development of sTM for the treatment of hemophilia A; however, given the differences between the extent of TAFI activation and the half-life of TAFIa in canines compared to humans, any prolongation of fibrinolysis in the canine model may be exaggerated compared to the potential effect in human hemophilic whole blood.
Acknowledgements

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Chapter 7

TAFI activation is increased in protein C deficiency but is decreased in individuals anti-coagulated with Warfarin or with no history of thrombosis.
Introduction

The processes of coagulation and fibrinolysis are inextricably linked through the activation of TAFI by thrombin (22). A blood clot is formed after thrombin cleaves fibrinogen to yield fibrin monomers that spontaneously assemble to form a mature blood clot. Thrombin also contributes to the down-regulation of fibrinolysis through the activation of TAFI. Activated TAFI (TAFIa, also known as carboxypeptidase U) attenuates fibrinolysis by removing lysine residues from plasmin-modified fibrin that act as cofactors for plasminogen activation (27). While unregulated (138-140) or excessive (141) thrombin formation is the primary cause of pathological hemostasis, the indirect role of thrombin as an attenuator of fibrinolysis (through the activation of TAFI) may also contribute to hemostasis. While TAFI activation is generally predictable based on the amount of thrombin generated, other factors contribute to the extent of TAFI activation and the associated anti-fibrinolytic potential. TAFI activation and activity can vary due to the presence of soluble thrombomodulin (22), the plasma TAFI concentration (22) or the TAFI genotype (30). Recently, 325-Ile-TAFI, which is present in 20% of the population and has a 2-fold longer half-life than 325Thr-TAFIa (16 minute half-life compared to 8 minutes) (30) was shown to be associated with the incidence of stroke and the age at onset of first stroke in patients of the LURIC cohort (142). This suggests that 325Ile-TAFI may be an independent or secondary risk factor for thrombosis.

Protein C (PC) deficiency has been shown to be an independent risk factor for venous thrombosis (138). Since many afflicted individuals have no history of thrombosis, it has been suggested that another factor may, together with a deficiency of PC, be
responsible for thrombosis (143). Recently, cell adhesion molecule 1 was identified as a probable thrombophilia gene that increases the risk of venous thrombosis in PC deficiency (144). Previously, it was suggested that fibrinolytic activity may contribute to the variable pattern of thrombosis among PC deficient individuals (138). In the current study, the time course of TAFI activation was measured in the whole blood clotting experiments (107) using blood from healthy individuals or individuals with heterozygous PC deficiency (138). All subjects were recruited and advised according to a protocol approved by the Institutional Review Board at the University of Vermont Human Studies Committee and gave informed written consent prior to the study.

Results and Discussion

Here, we used TAFIa potential to describe the potential cumulative effect of TAFIa over time. TAFIa potential (area under the TAFIa curve) is a single measurement that incorporates the timing, rate and extent of TAFI activation. In Figure 7-1A we show that the timing, rate and extent of TAFI activation is somewhat variable resulting in a TAFIa potential (0-20 minutes) ranging from 4700pM-min to 27400pM-min (12300±3900pM-min, n=6).
Figure 7-1. The time course of TAFI activation for healthy controls (panel A) and individuals with PC deficiency (panel B).

The TAFIa potential (the area under the TAFI activation curve) was determined for each individual and is summarized in panel C. TAFIa potential is marginally increased in PC-def 1 compared to the mean TAFIa potential for the healthy controls. PC-def 2 and PC-def 3 (on Warfarin) and PC-def 4 (no history of thrombosis) had drastically reduced TAFIa potentials when compared to the healthy controls or PC-def 1.

Due to the low prevalence of PC deficiency (~0.3%, (145)) and difficulty recruiting patients, to date only 4 individuals with PC deficiency have been enrolled in our study. The relevant patient information and clinical histories are presented in Table 7-1. PC levels represent levels prior to anti-coagulation with Warfarin.
Table 7-1. Summary of the clinical histories for PC deficient subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age</th>
<th>Thrombosis* (age)</th>
<th>PC (%)</th>
<th>Warfarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-def 1</td>
<td>Female</td>
<td>35</td>
<td>DVT† (22)</td>
<td>50</td>
<td>No</td>
</tr>
<tr>
<td>PC-def 2</td>
<td>Female</td>
<td>60</td>
<td>DVT‡, PE (18)</td>
<td>55</td>
<td>Yes</td>
</tr>
<tr>
<td>PC-def 3</td>
<td>Male</td>
<td>54</td>
<td>DVT‡ (20)</td>
<td>&lt;10%**</td>
<td>Yes</td>
</tr>
<tr>
<td>PC-def 4</td>
<td>Female</td>
<td>75</td>
<td>No</td>
<td>34%</td>
<td>No</td>
</tr>
</tbody>
</table>

* All DVTs were confirmed by leg ultrasound
† DVT occurred while taking oral contraceptives
‡ with reoccurrences
** PC measurement not available before on Warfarin

As shown in Figure 7-1B, the PC deficient subject with a history of thrombosis but not on Warfarin (PC-def 1) at the time of blood draw had a much higher rate and maximal level of TAFI activation compared to those on Warfarin (PC-def 2 and PC-def 3) or with no history of thrombosis (PC-def 4).

Blood drawn from PC-def 1 showed a 54% increase in the TAFIa potential (18900pM-min) compared to mean TAFIa potential of the control group (12300±3900pM-min, n=6); however, the TAFIa potential was within the range reported for controls. This individual had only one DVT with no reoccurrences and it might have been predicted by a secondary risk factor such as oral contraceptives (OC). Previously, both 2nd and 3rd generation OC have been shown to increase the plasma TAFI concentration (146). Since the K_m for TAFI activation by thrombin is relatively high compared to the normal plasma TAFI concentration this elevation of TAFI plasma concentration would likely elevate the TAFIa potential while on OC which may have
further contributed to the DVT. Unfortunately, we do not have samples from the time of DVT so this cannot be confirmed but these data are consistent with decreased fibrinolysis (caused by increasing TAFIa above a certain critical threshold (52,53)) being a secondary risk factor for venous thrombosis.

Individuals anti-coagulated with Warfarin to prevent the reoccurrence of thrombosis (PC-def 2 and PC-def 3) showed a marked decrease in TAFIa potential (16700pM-min and 960pM-min, respectively) when compared to the healthy controls or PC-def 1. This suggests that in addition to the well established decrease in prothrombin activation by Warfarin (147) a secondary benefit of this type of anti-coagulation is a decrease in TAFI activation. A lower TAFIa potential would have the added benefit of allowing for efficient fibrinolysis in individuals with a thrombotic diathesis such as those with a PC deficiency. If “diminished fibrinolysis” (138) can be confirmed as a secondary risk factor for thrombosis then it would be interesting to determine what effect, if any, TAFIa inhibitors have on thrombosis. By increasing fibrinolysis, individuals predisposed to, or having recurrent thrombosis, may have fewer or less severe clots. In addition, a TAFIa inhibitor could potentially be given with a reduced dose of Warfarin in order to achieve the same anti-coagulant effect which might decrease necrosis associated with Warfarin treatment (148).

Finally, we show that an individual with heterozygous PC deficiency (34% PC) and no history of thrombosis (PC-def 4) has a TAFIa potential of 6000pM-min which is 68% less than that observed for PC-def 1 and 50% of mean TAFI potential reported for the healthy controls. This is consistent with “diminished fibrinolysis” being a secondary
risk factor for thrombosis among the PC deficient kindred. Individuals with a higher fibrinolytic potential (i.e. lower TAFIa potential) may be less likely to develop pathological hemostasis than those with a lower fibrinolytic potential.

Figure 7-1C shows the TAFIa potential for the healthy controls, PC deficient subjects on Warfarin anti-coagulation and a PC deficient individual with no history of thrombosis. The results from this small study are consistent with “diminished fibrinolysis” being a secondary risk factor for thrombosis in heterozygous PC deficiency. Our priority is to increase the number of subjects in this study in order to further evaluate the role of fibrinolysis in pathological hemostasis and determine if TAFI activation is associated with thrombosis in these PC deficient kindred with a heterogeneous thrombotic phenotype.

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Chapter 8
General Discussion

The current studies were undertaken to investigate the biochemistry and enzymology of TAFI and TAFIa and to determine the time course of TAFI activation in hemophilia and protein C deficiency. Based on the TAFI activation deficit in hemophilia described here, we sought to increase the stability of clots formed in hemophilic plasma and whole blood using sTM, a cofactor for TAFI activation, in hopes that sTM would prevent the premature lysis defect which is evident in hemophilia (38,126).

As previously described here and elsewhere, coagulation and fibrinolysis are linked via the thrombin-thrombomodulin complex (22). This complex efficiently activates both protein C and TAFI which, when activated, attenuate coagulation and fibrinolysis, respectively. Here we have shown using a whole blood clotting assay (3,107) that both the rate of TAFI activation and the TAFIa potential are significantly depressed in hemophilic whole blood. In chapter 5 we showed that, when subjects are grouped by fVIII level, the rate of TAFI activation and TAFIa potential are 233±36pM/min and 15930±4030pM-min, respectively, in healthy controls and these measures of TAFI activation are reduced significantly in severe hemophilia. While correlation does not prove causation it is interesting to note that the TAFIa potential was significantly and negatively correlated with the bleeding phenotype of the hemophilic individuals recruited to the study. FVIII levels are generally but not always associated with TAFI activation
(data not shown) and differences in the TAFIa potential may provide a plausible explanation of why some individuals with <1% fVIII (classified as severe hemophilia) have a severe clinical bleeding phenotype while others do not (89). In chapter 3 we have shown that the kinetics of TAFIa in removing Glu- or Lys-Pg binding sites are such that only a very small percentage of plasma TAFIa must be activated to get substantial attenuation of fibrinolysis. Bajzar et al. showed previously that the lysis time is prolonged half-maximally at 1nM TAFIa which is approximately 1% of plasma TAFI (22). The high catalytic efficiency of plasminogen binding site removal by TAFIa (16.41 μM⁻¹s⁻¹) compared to other substrates of TAFIa, including inflammatory mediators such as bradykinin, C3a and C5a (28) suggests that fibrin degradation products are the primary substrate of TAFIa during periods of active fibrinolysis and that TAFIa very potently attenuates fibrinolysis by removing plasminogen binding sites. Since the Kₘ of plasminogen binding site removal by TAFIa is quite low (143nM) TAFIa is able to slow plasminogen activation from the very early stages of fibrinolysis (i.e. after limited fibrin cleavage by plasmin).

TAFI zymogen has been shown to have carboxypeptidase activity toward small substrates (30) and small peptides (28) including those from fibrin (55). When considering the carboxypeptidase activity of TAFI zymogen and the concentration of TAFI zymogen (~100nM) (109) compared to the amount of TAFIa required to prolong lysis (<1nM) it is interesting to consider the possibility that TAFI zymogen, not TAFIa, is responsible for the attenuation of fibrinolysis. In chapter 2, we showed that TAFI is activated when clotting is initiated with low concentrations of thrombin and that the
higher the concentration of TAFI present in plasma the more TAFI is activated which is consistent with a \( K_m \) that is relatively high compared to the plasma concentration of TAFI (22). We also show that PTCI may be used to inhibit the carboxypeptidase activity of TAFIa but not TAFI zymogen. Since PTCI inhibits TAFIa but not TAFI zymogen any prolongation of lysis in plasma in the presence of PTCI is due to TAFI zymogen not TAFIa. In our experiments no prolongation of lysis was observed in the presence of PTCI. Finally, we showed using QSY-FDPs, our macromolecular substrate for TAFIa, that it cannot be cleaved by TAFI zymogen. This is consistent with the known structure of TAFI. TAFI zymogen has a formed active site that is largely covered by the activation peptide (31). Small substrates and even small peptides may bypass the activation peptide and gain access to the active site giving TAFI zymogen its intrinsic carboxypeptidase activity toward small substrates. We show that no carboxypeptidase activity is observed even at 500nM TAFI zymogen when the substrate is QSY-FDP.

Since TAFI activation is significantly decreased in hemophilia and the catalytic efficiency of TAFIa is quite high, a small increase in its activation could have a substantial effect on prolonging the clot lysis time in hemophilia. In chapter 4 we show that the clot lysis time in fVIII-deficient plasma is corrected with 10% fVIII; however, 25% fVIII gives only an 80% correction in TAFI activation. Individuals with 10% fVIII are classified as having mild hemophilia and the relatively low bleeding tendency of this group of patients (37) is consistent with the correction of the clot lysis time reported here. The potency of TAFIa described by the kinetics of plasminogen binding site removal (chapter 3) implies that full correction of TAFI activation is not necessary to get full
correction of the clot lysis time. To investigate this further, we supplemented plasma with sTM, a cofactor for TAFI activation to determine the effect of increasing TAFI activation on clot lysis time. In our studies, we showed that 100nM sTM fully corrects the clot lysis time in a TAFIa-dependent manner in fVIII-deficient plasma at a low tPA concentration (0.25nM). As the tPA concentration is increased, sTM can be used to partially correct the premature lysis defect in fVIII-deficient plasma.

Our results showing that sTM prolongs fibrinolysis in fVIII-deficient plasma are very promising. To further evaluate the potential of sTM to stabilize clots and prevent premature clot lysis in hemophilia we used the hemophilic dog colony at Queen’s University as a model for hemophilia A as described in chapter 6. Similar experiments were conducted in hemophilic dog plasma as those presented in fVIII-deficient plasma (chapter 4) with similar results. Soluble TM not only increased the clot lysis time in hemophilic dog blood at plasma concentrations ranging from 25 - 390nM but it also unexpectedly increased the clot strength as measured by the maximum amplitude. At the highest concentration of sTM the clot lysis time was shorter than at a lower concentration of sTM suggesting that protein C activation was increasing. This is a very important point that must be considered before in vivo studies are conducted. At high concentrations of sTM (higher than those used here), an anticoagulant effect will likely occur (as demonstrated in (83)) and this would be very detrimental to the bleeding diathesis in hemophilia. Finally, we showed that sTM effectively increased the clot lysis time in hemophilic dog blood with greater than 150 Bethesda units of fVIII neutralizing antibodies (fVIII inhibitors), albeit not to the extent seen in hemophilic dogs lacking
fVIII inhibitors. Since these dogs have no functional fVIII and the severe dogs have <1% fVIII it can be concluded that a very small amount of fVIII improves the efficacy of sTM in stabilizing clots. This is of great importance for the treatment of hemophilia since fVIII replacement therapy is very expensive. It is plausible that sTM may be given with a reduced dose of fVIII in order to achieve hemostasis at a much lower cost. To verify this hypothesis, further in vivo pre-clinical studies are required.

Most of the work in this dissertation is related to the quantification of TAFI activation in samples obtained from individuals with hemophilia; however, we are able to briefly comment on TAFI activation in pathological thrombosis. As shown in chapter 5, healthy individuals have a TAFIa potential (over the interval 0 - 20 minutes) of 12260±3880pM-min (n=6). In chapter 7, we measured the time course of TAFI activation in individuals with a heterozygous protein C deficiency (138) and showed that TAFIa potential was increased 54% in an individual with protein C deficiency (18930pM-min) with a history of DVT. Protein C deficient subjects who were actively being treated for thrombosis with Warfarin had a much lower TAFIa potential compared to either the healthy controls or the protein C deficient individual not on Warfarin. This is consistent with the decreased thrombin generation seen in patients receiving Warfarin anticoagulant therapy (147). Elevated TAFI activation may play a role in preventing adequate fibrinolysis which may contribute to the tendency for pathological thrombosis. In the original article describing this protein C deficient kindred, the authors suggested that “diminished fibrinolytic activity [may] contribute to the intra-familial heterogeneity in the expression of clinical manifestations” (138). In all cases reported here, the TAFIa
potential is consistent with the individual’s history of thrombosis suggesting a potential link between “fibrinolytic activity” and thrombosis. To verify this connection between TAFI activation and thrombosis in the protein C deficient kindred more extensive studies are required.

Initially, we sought to measure TAFI activation in normal pooled plasma and fVIII-deficient plasma in order to relate the extent of TAFI activation to the clot lysis time (chapter 4). In addition to this primary goal, we were able to develop a novel functional assay for TAFIa (61) (data not shown); (1) show that TAFI zymogen is not a physiologically relevant carboxypeptidase (68); (2) determine the kinetics of TAFIa in removing plasminogen binding sites and lysine residues from FDPs; (3) quantify TAFI activation in normal and fVIII-deficient plasma and identify sTM as a potential TAFIa-dependent clot stabilizer (149); and (4) quantify TAFI activation in normal, hemophilic and (5) protein C deficient whole blood. The data presented here suggests that fibrin degradation products are the primary physiological substrate for TAFIa during active fibrinolysis and that TAFI activation is involved in the maintenance of hemostasis in pathological bleeding. Finally, the data presented here provides that rationale and proof of principle necessary to further explore the potential for using sTM as a therapy for bleeding in hemophilia. Beginning in July 2010, in vivo pre-clinical studies using the hemophilic dog colony at Queen’s University will begin to test sTM as a potential therapy for the treatment of bleeding in hemophilia.
Future Studies

The data provided here are promising and may be used to generate hypotheses for future studies. We have shown that plasminogen binds to TAFIa and it is known that plasminogen binds TAFI (47). Further studies may include examining if plasminogen recruits TAFI to the thrombomodulin containing cell surface via and endothelial plasminogen receptors, such as annexin-II (150) or α-enolase (151) thereby acting as a cofactor for TAFI activation on endothelial cells. This proposed mechanism is similar to the mechanism of protein C recruitment to the endothelium by the endothelial protein C receptor. It will also be interesting to determine the structural requirements of the interaction between TAFI or TAFIa and plasminogen and determine if TAFI competes with plasminogen for binding to C-terminal lysine residues. Using our fluorescent-plasminogen and fibrin degradation model to determine the kinetics of TAFIa we may also determine if other carboxypeptidases remove plasminogen binding sites. A particularly interesting carboxypeptidase is cleaved-carboxypeptidase N which has been shown to attenuate fibrinolysis (137). Finally, we have shown in vitro that sTM may be used to attenuate fibrinolysis in human and dog plasma and dog blood. The next step in this project is to determine the concentration of sTM that positively influences hemostasis in human whole blood and determine if a similar effect is observed in vivo in the hemophilic dog.
References