SECRETION AND ANTIFIBRINOLYTIC FUNCTION OF TAFI FROM HUMAN PLATELETS

by

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Abstract

Thrombin activatable fibrinolysis inhibitor (TAFI) is a human plasma-derived zymogen that is activated through proteolytic cleavage by thrombin, thrombin in complex with thrombomodulin, or plasmin. Active TAFI attenuates fibrinolysis by removing carboxyl-terminal lysine residues from partially degraded fibrin, thereby inhibiting a potent positive feedback loop in the fibrinolytic cascade. In addition to the plasma pool of TAFI arising from expression in the liver, a distinct pool of TAFI has been reported to be present in platelets. While the antifibrinolytic effect of plasma-derived TAFI has been well-documented by *in vitro* and *in vivo* clot lysis assays, characterization of the platelet-derived form has been limited. Here, we not only confirm the presence of TAFI in the medium of washed, thrombin-stimulated platelets, but also that platelet-derived TAFI is capable of attenuating platelet-rich thrombus lysis *in vitro* independently of plasma TAFI using a novel thrombus lysis assay. Fluorescent thrombi were generated by suspending washed human platelets in plasma immunodepleted of TAFI containing fluorescently-labeled human fibrinogen such that the only TAFI present in the system was of platelet origin. Following platelet activation and clot retraction induced by thrombin, t-PA-dependent platelet-rich thrombus lysis was observed by removal of timed aliquots from the medium of retracted thrombi followed by measurement of fluorescence. When supplementary thrombomodulin was added to the thrombus medium, a 2.3-fold reduction in lysis rate was observed, indicating platelet-derived TAFI could attenuate the fibrinolytic cascade *in vitro*. Furthermore, when supplementary recombinant TAFI (rTAFI) was included in the medium, platelet-derived TAFI and rTAFI were observed to combine for greater inhibition of fibrinolysis. Taken together, these observations indicate that the secretion of platelet-derived TAFI can augment concentrations of TAFI already present in plasma to enhance attenuation of the fibrinolytic cascade. This could be significant at sites of vascular damage or regions of

pathological thrombosis, where activated platelets are known to accumulate and secrete the contents of their granules. Finally, we have purified platelet-derived TAFI from platelet releasates for future characterization studies and mass spectrometry.

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List of Abbreviations

ε-ACA	ε-aminocaproic acid
AA	Arachidonic acid
ADP	Adenosine diphosphate
APC	Activated protein C
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CFTB	Calcium-Free Tyrode's buffer
COX-1	Cyclooxygenase-1
CPN	Carboxypeptidase N
DAG	Diacylglycerol
DTS	Dense tubular system
FDP	Fibrin degradation product
Fluo-Pg	Fluorescently-labelled plasminogen
Gla	γ-carboxyglutamic acid
GEMSA	2-guanidinoethylmercaptosuccinic acid
GP	Glycoprotein
GPCR	G protein-coupled receptor
HBS	HEPES-buffered saline
HBS/Tween	HBS containing 0.01% (v/v) Tween 80
Hip-Arg	Hippuryl-arginine
IP_3	Inositol triphosphate

HTB	HEPES-Tyrode's buffer
IIa	Thrombin
JAM	Junctional adhesion molecule
K_m	Michaelis constant
MK	Megakaryocyte
MLCK	Myosin light-chain kinase
OCS	Open canalicular system
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease activated receptor
PC	Phosphatidyl choline
PCPS	75% phosphatidyl choline: 25% phosphatidyl serine
PE	Phosphatidyl ethanolamine
PECAM-1	Platelet/endothelial cell adhesion molecule-1
PF4	Platelet factor 4
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PKC	Protein Kinase C
PLC	Phospholipase C
PMSF	Phenylmethanesulphonylfluoride
PPAck	D-phenylalanylprolylarginyl chloromethylketone
PS	Phosphatidyl serine
PSF	Penicillin/streptomyocin/fungizone
pTAFI	Plasma-derived TAFI

PTCI	Potato tuber carboxypeptidase inhibitor
QSY-FDPs	QSY-conjugated fibrin degradation products
ROCK	Rho kinase
rTAFI	Recombinant TAFI
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Serpin	Serine protease inhibitor
t-PA	Tissue plasminogen activator
TAFI	Thrombin activatable fibrinolysis inhibitor
TAFIa	Active TAFI
TDP	TAFI-deficient plasma
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
TP	Thromboxane-prostanoid receptor
TXA_2	Thromboxane A_2
u-PA	Urokinase
VFKck	Valylphenylalanyllysyl chloromethylketone
VWF	Von Willebrand factor

Chapter 1
Introduction

1.1 Introduction to Haemostasis

Humans have evolved a delicately balanced system of blood coagulation to arrest bleeding following vascular injury. Physical damage to blood vessels rapidly induces a mechanism that seals the injured site to limit critical hemorrhage: a process referred to as *haemostasis* (*1*, *2*). The haemostatic response is tightly regulated, utilizing a highly localized process of clot formation and removal, so that the fluid state of blood is maintained elsewhere in the vasculature. Two opposing pathways regulate this phenomenon: the coagulation cascade, whose end product is the insoluble fibrin clot, and the fibrinolytic cascade, which serves to degrade the clot into soluble products. Both pathways are constantly active, yet specific stimuli can promote either cascade, thereby initiating the deposition or dissolution of a fibrin clot. The result is an intricately orchestrated balancing act which, when functioning properly, results in a haemostatic response strictly at the site of injury. On the other hand, erroneous regulation of enzymatic activity and feedback communication within these cascades can result in either the propensity to thrombose, whereby inappropriate blood clot formation occurs, or the inability to form a stable fibrin clot, leading to vascular blockage or excessive bleeding respectively.

Platelets are a critical component of the haemostatic response (*3*). Under normal circumstances, platelets are quiescent and flow through the vasculature as discoid-shaped cell fragments. Following damage to the endothelium, platelets become activated to form a semi-stable mass that serves to plug the leak in the vessel wall. Formation of the platelet plug represents one of the preliminary events of the haemostatic response, where platelets aggregate and adhere to components of an exposed subendothelial matrix. Through a diverse set of structural changes and secretion events mediated by adhesion to the injured site and subsequent

intracellular signalling, platelets amplify and regulate a localized coagulant response, thereby preventing systemic activation of the coagulation cascade. Local procoagulant activity facilitates assembly of an integrated fibrin network into and around the initial platelet plug, forming the principal impediment to hemorrhagic bleeding: an adhesive mass of blood elements known as the *thrombus* (*4*). Subsequent activation of the fibrinolysis cascade promotes clearance of the thrombus in conjunction with wound healing. Platelets also have significant implications in fibrinolysis, as they secrete factors directly involved in the cascade and are a key component of the thrombus itself (*5*). A brief overview of the coagulation and fibrinolysis cascades is provided below, with particular emphasis on the interactions of platelets with these two pathways.

1.1.1 Platelets and Coagulation

When the endothelial cell wall lining blood vessels is compromised by physical damage, proteins and cell components normally sequestered from flowing blood become exposed and initiate the haemostatic response (*2*, *3*). Subendothelial collagen and von Willebrand factor (VWF) are integral in this regard to facilitate platelet adhesion and activation. These and other critical platelet interactions as well as platelet plug formation are discussed in detail in Section 1.3. The focus here will be on initiation of the coagulation cascade, which typically begins with the expression or exposure of the glycoprotein tissue factor (TF) to flowing blood (*6*).

TF is an integral membrane protein of subendothelial cells like fibroblasts, but is also expressed by monocytes, endothelial cells, and possibly platelets following injury-induced stimulation (Figure 1-1) (*3*, *6*). In the preliminary stages of the coagulant response, trace amounts of activated factor VII (factor VIIa) present in plasma interact directly with membrane-bound TF. Binding with TF enhances the enzymatic activity of factor VIIa, a serine protease, thereby initiating autoactivation of the plasma pool of factor VII. The TF-VIIa complex or *extrinsic tenase* also catalyzes the activation of two other plasma zymogens, factor X and factor IX, to their

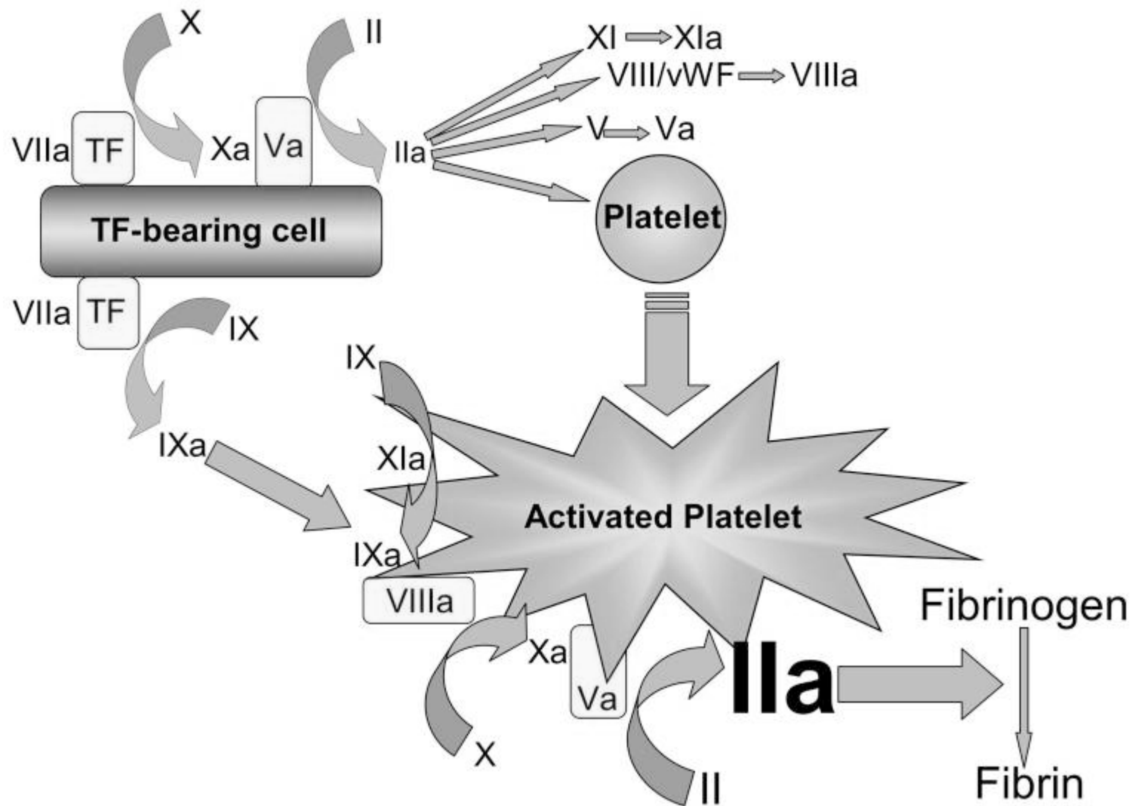

Figure 1-1. Interaction of cells and platelets with the coagulation cascade.

The extrinsic pathway is initiated upon formation of the TF-VIIa complex (extrinsic tenase) on the surface of a TF-bearing cell. Factor Xa formed by extrinsic tenase then binds with its membrane-bound cofactor, factor Va, to form the prothrombinase complex. Thrombin (IIa) generated by the complex activates platelets as well as factors V, VIII, and XI. Extrinsic tenase and thrombin can also activate factor IX to factor IXa, which goes on bind with its membrane-bound cofactor, factor VIIIa, to form intrinsic tenase. Intrinsic tenase activates factor X with 50-fold greater efficiency than extrinsic tenase, which results in increased prothrombinase complex generation on the platelet surface. While the small quantities of thrombin produced by extrinsic tenase are sufficient to give rise to fibrin formation and initiate the intrinsic pathway, the large quantities of IIa produced by the intrinsic pathway are required to propagate the coagulation cascade and facilitate formation of a stable fibrin clot. (Figure adapted from Wolberg AS and Campbell RA (*7*)).

corresponding serine protease forms, factor Xa and factor IXa. Factor Xa then interacts with its membrane-bound cofactor, factor Va, to form the prothrombinase complex, which activates prothrombin to thrombin. Thrombin is the central procoagulant factor that, among a wide array of regulatory activities, is a potent platelet activator or *agonist* and also catalyzes assembly of the insoluble fibrin network from soluble circulating fibrinogen (*7*). This mode of thrombin generation is historically referred to as the *extrinsic* pathway of coagulation due to the involvement of extravascular components.

Only limited amounts of factor Xa, and thus thrombin, are generated by the extrinsic pathway due to rapid inhibition by circulating tissue factor pathway inhibitor (TFPI) (*8*). TFPI is capable of inhibiting factor Xa directly, but the TFPI-Xa complex goes on to inhibit the TF-VIIa complex as well. Consequently, the low levels of factor Xa generated by the extrinsic pathway in the presence of TFPI are insufficient to sustain a robust haemostatic response (*9*). Still, factor X activated by extrinsic tenase catalyzes the formation of enough thrombin to stimulate platelet activation, give rise to fibrin formation, and initiate the *intrinsic* pathway of coagulation.

The intrinsic pathway is the driving force behind propagation and consolidation of the coagulant response, which is facilitated by massive prothrombin activation (*7*). Like the extrinsic pathway, thrombin generated by the intrinsic pathway is also dependent upon the activation of factor X, albeit through an entirely different mechanism. In this case, factor IXa, activated either by thrombin or extrinsic tenase, forms a complex with the activated form of its membrane-bound cofactor, factor VIIIa (Figure 1-1). The factor IXa-VIIIa complex is known as *intrinsic tenase* as it also activates factor X, but with 50-fold greater efficiency than extrinsic tenase (*10*). As a result, factor Xa concentration elevated by the intrinsic pathway amplifies the coagulant response, again through formation of the prothrombinase complex (factor Xa-Va). The subsequent burst of thrombin promotes thrombus formation and propagates coagulation through positive feedback.

Among these key feedback interactions is the thrombin-mediated cleavage of factor XI to factor XIa, which goes on to activate additional factor IX and upregulate the intrinsic pathway.

As shown in Figures 1-1 and 1-2, activated platelets serve to localize, amplify, and sustain the coagulant response at the site of injury (*11*). Regulation of the coagulation cascade is mediated in part by noncovalent interactions between activated platelet membranes and specific factors in the pathway. When platelets are activated, a redistribution of plasma membrane components results in the exposure of negatively charged phospholipids on the platelet surface that enable interactions with key elements of the coagulation cascade. The vitamin K-dependent coagulation proteins, factors VII, IX, X, and prothrombin all contain a γ-carboxyglutamic (Gla) domain that allows for high affinity interaction with calcium and anionic membrane surfaces. Additionally, platelet activation exposes surface receptors for the membrane-binding coagulation cofactors Va and VIIIa. Hence, both intrinsic tenase and the prothrombinase complex can be assembled on the surface of activated platelets, which serves to further localize the procoagulant response at the site of injury. Moreover, membrane binding of these complexes greatly enhances their catalytic efficiency and protects them from inhibition or inactivation, thereby enabling eventual thrombus formation through the accumulation of large amounts of thrombin in close proximity with the damaged region. Apart from the implications of their membranes, activated platelets sustain the coagulant response through secretion of platelet-derived coagulation factors like fibrinogen, factor XI, and a unique factor V molecule.

Activation of prothrombin to thrombin by the extrinsic and intrinsic pathways facilitates the stable assembly of an interconnected network of fibrin strands, an integral component of the developing thrombus (*7*). The precursor of fibrin is fibrinogen, a dimer of three pairs of disulfide-bonded polypeptides designated the α, β, and γ chains. The chains fold to form two globular ‘D’ domains at either end of the molecule separated by a smaller globular ‘E’ domain in the centre.

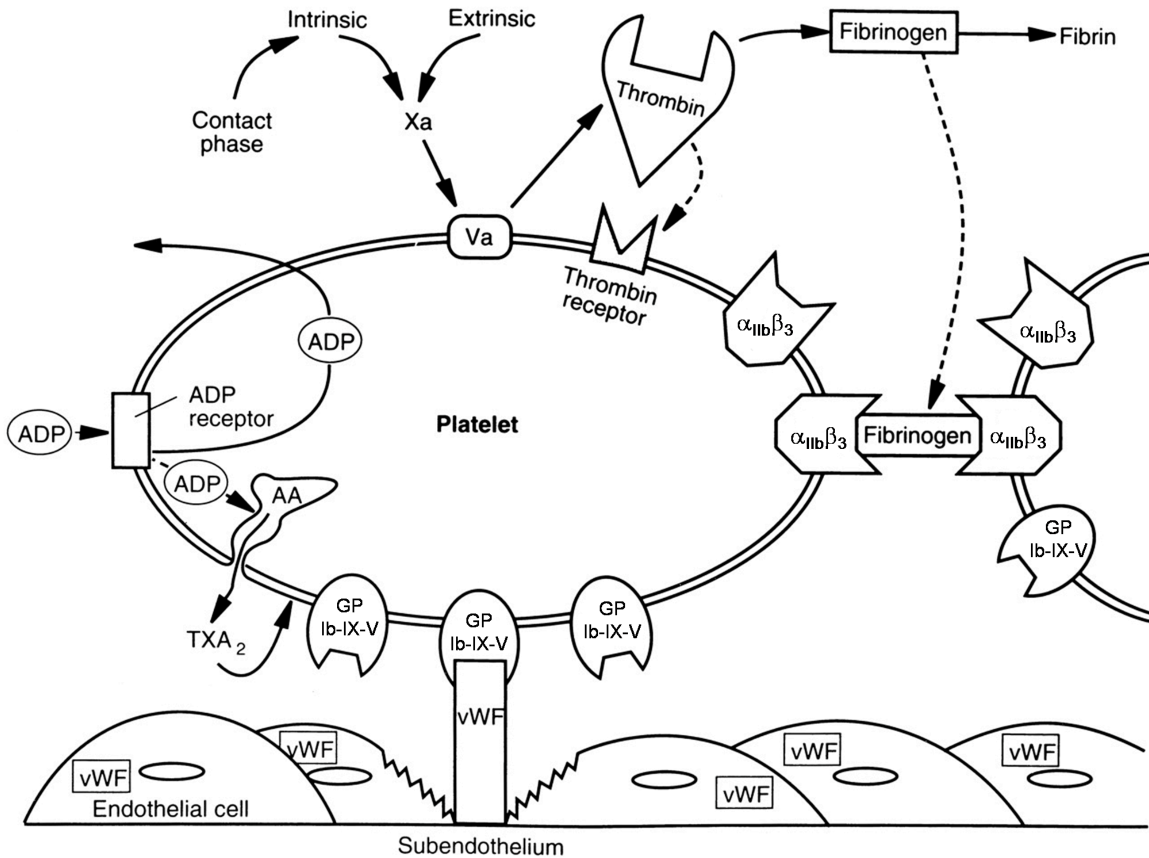

Figure 1-2. Platelet tethering, activation, and aggregation.

When the endothelial cell wall is damaged, exposed collagen in complex with von Willebrand factor (VWF) mediates initial platelet tethering through GP Ib-IX-V on the platelet surface. Subsequent intracellular signaling results in secretion of soluble agonists like ADP, which can activate other circulating platelets through ADP receptors on the plasma membrane. Thromboxane (TXA_2), another platelet agonist, is secreted upon platelet activation following enzymatic modification of arachidonic acid (AA) in the platelet cytoplasm. Thrombin generated by the prothrombinase complex on the platelet surface can also activate circulating platelets. Platelet activation results in the conversion of integrin $\alpha_{IIb}\beta_3$ to a high affinity state that interacts with multivalent fibrinogen and VWF to form a cohesive aggregate of activated platelets at the site of vascular injury. (Figure adapted from Camacho A and Dimsdale JE (*12*)).

Conversion of this soluble fibrinogen into insoluble fibrin occurs through a multi-step polymerization process. First, thrombin-mediated cleavage of two peptides (fibrinopeptides A and B) from the central E-domain results in the formation of the fibrin monomer. This modification by thrombin initiates a non-covalent assembly process in which the D-domain of one fibrin monomer interacts with newly exposed binding sites on the E-domain of an adjacent monomer. The resulting fibrin dimer has a half-staggered overlap structure that allows for rapid bimolecular polymerization of additional monomers to form two-stranded *protofibrils*. These protofibrils then undergo a lateral association process to form the thicker fibrin fibers that extend and branch to form the characteristic fibrin mesh around the initial platelet plug.

In addition to promoting the assembly of coagulation factor complexes, platelet activation also influences the process of clot formation (*3*, *11*). Another of the multifunctional roles of thrombin generated on activated platelet surfaces is described above, in which fibrin polymerization is initiated by the protease activity of thrombin. Additionally, platelets express glycoproteins that interact directly with components of the fibrin mesh like fibronectin, thrombospondin, and fibrin itself. Binding of these adhesive proteins with platelets contributes to consolidation of thrombus constituents and attachment to the damaged vessel wall. Furthermore, platelets influence a clot stabilization process through secretion of factor XIII, the zymogen form of a transglutaminase. Factor XIII is activated to factor XIIIa by thrombin, which goes on to catalyze covalent isopeptide bond formation between the γ-chains of fibrin monomers throughout protofibril and thick fiber assembly (*7*). This crosslinking reaction improves the stability of fibrin polymers and increases their resistance to proteolysis. Although factor XIII is expressed endogenously in plasma, studies have shown that platelet- and monocyte-derived factor XIII fully crosslinks fibrin polymers in the absence of the plasma-derived counterpart (*13*).

1.1.2 Platelets and Fibrinolysis

As introduced above, the pathway directly opposing coagulation is fibrinolysis. In response to thrombus assembly, initiation of the fibrinolysis cascade promotes dissolution of the fibrin network in conjunction with wound healing and regrowth of endothelial cells (angiogenesis) (*2*, *14*). Fibrinolytic degradation of aggregated coagulation elements in the vasculature is necessary for eventual recanalization of the previously damaged vessel and maintenance of vascular patency. Like the coagulant response, fibrinolysis occurs by a sequential mechanism of zymogen activation steps and feedback interactions that regulate its activity.

As shown in Figure 1-3, the initiating factors of the fibrinolysis cascade are tissue plasminogen activator (t-PA) and urokinase (u-PA) (*15*). t-PA is released from endothelial cells in response to thrombin, while u-PA is synthesized following endothelial perturbation. Both t-PA and u-PA catalyze the cleavage of the abundant plasma zymogen plasminogen to its corresponding serine protease, plasmin. Plasmin is the central fibrinolytic factor that catalyzes proteolysis of the fibrin network into solubilized fibrin degradation products (FDPs). Plasminogen activation is enhanced in the presence of fibrin, which serves as a scaffold for the formation of a ternary complex consisting of t-PA, plasminogen, and fibrin. Consequently, the fibrin component of the thrombus is rapidly degraded to FDPs by plasmin following initiation of the fibrinolytic cascade.

Fibrinolysis is tightly regulated in order to localize the response and prevent inappropriate or premature dissolution of developing thrombi (*15*). For example, α_2-antiplasmin is a serine protease inhibitor (serpin) that rapidly inhibits free circulating plasmin (Figure 1-3). Alternatively, factor XIIIa from plasma or platelets can crosslink α_2-antiplasmin directly to the fibrin mesh, thereby increasing resistance of the thrombus to degradation by plasmin. Likewise, t-PA and u-PA are inhibited by another plasma- and platelet-derived serpin, plasminogen activator

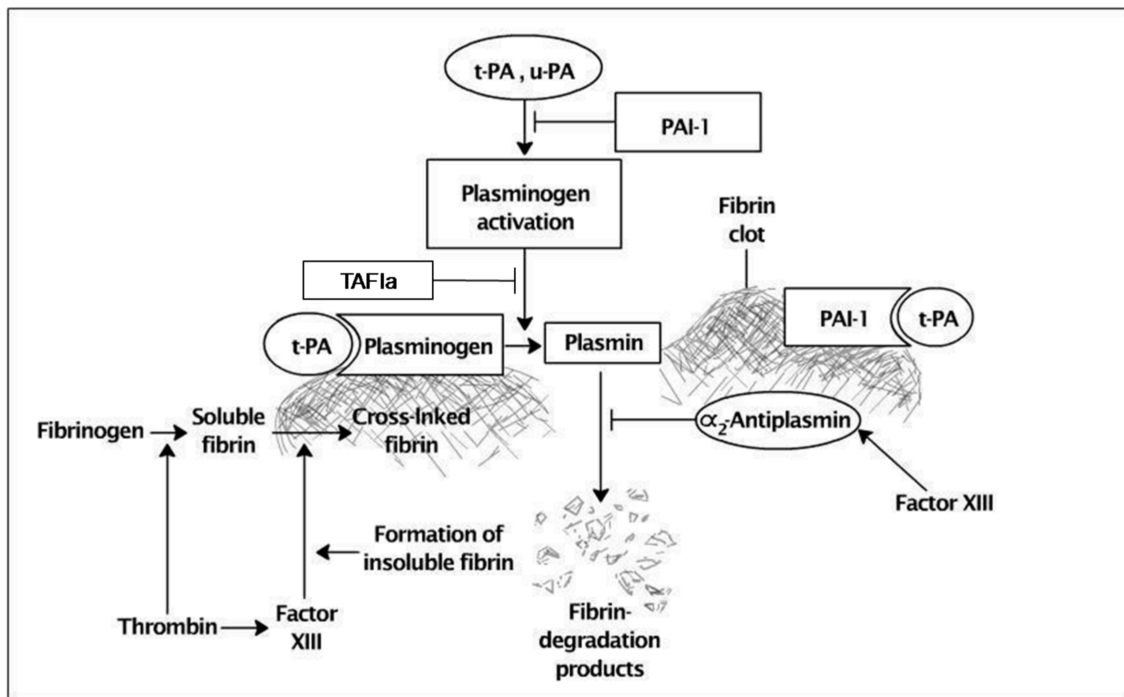

Figure 1-3. Initiation and regulation of the fibrinolytic cascade.

Fibrinolysis is initiated by the activating cleavage of plasminogen to plasmin by t-PA or u-PA. t-PA and u-PA are inhibited by plasminogen activator inhibitor-1 (PAI-1). Plasmin proteolyzes fibrin, resulting in dissolution of the fibrin clot to form fibrin degradation products. Plasminogen activation by t-PA is enhanced in the presence of fibrin, which results in the localization of plasminogen activation to the vicinity of the clot. Activated TAFI (TAFIa) modifies the fibrin substrate and interferes with this positive feedback mechanism in plasminogen activation. Thrombin cleaves circulating fibrinogen to soluble fibrin monomers and also activates factor XIII. Activated factor XIII catalyzes formation of isopeptide bonds between fibrin polymers to form fibrinolysis-resistant cross-linked fibrin and also crosslinks α_2-antiplasmin to the fibrin clot. α_2-Antiplasmin in serum or bound to the fibrin clot inhibits the activity of plasmin. (Figure adapted from Kohler HP and Grant PJ (*16*)).

inhibitor-1 (PAI-1). Fibrinolysis can also be attenuated by a zymogen present in plasma and, possibly, in platelets, thrombin-activatable fibrinolysis inhibitor (TAFI). TAFI operates through an indirect mechanism, in contrast to the covalent, stable, 1:1 stoichiometric complexes formed by serpins and their target proteins. In this case, activated TAFI (TAFIa) modifies the fibrin substrate and interferes with a potent positive feedback mechanism in plasminogen activation. A thorough discussion of TAFI and the feedback interactions mediating the fibrinolysis cascade is provided in Section 1.4.

Platelets influence fibrinolysis through secretion of fibrinolytic factors and direct interaction with elements of the cascade (*5*). Like coagulation complex formation, the platelet surface also supports assembly of proteins in the plasminogen activation system by providing binding sites for t-PA, plasminogen, and plasmin (*17*). Indeed, it has been shown that plasminogen activation is enhanced in the presence of platelets. Binding of t-PA and plasmin to the platelet surface also confers protection from inhibition by PAI-1 and α_2-antiplasmin respectively (*17*). Moreover, activated platelets secrete plasminogen directly and augment the local concentration of the plasmin precursor.

As platelets are a principal constituent of the thrombus, their influence on clot architecture has implications in fibrinolysis. For example, platelet-rich thrombi are more resistant to fibrinolysis than clots composed of fibrin alone (*18*). This differential lysis rate is likely the result of structural differences between thrombi dictated by the number and distribution of platelets, but also due to platelet secretion of antifibrinolytic factors including α_2-antiplasmin, PAI-1, and, possibly, TAFI (*18*). Taken together, the observations outlined above indicate that platelets have a regulatory role in fibrinolysis in addition to their well-established effects on the coagulation cascade.

1.2 Platelet Formation, Structure, and Function

Platelets originate from megakaryocytes (MKs). The current model of platelet formation *in vivo* involves the assembly of proplatelets, in which long cytoplasmic extensions from MKs in bone marrow fragment into individual platelets (*19*). MKs are localized on the abluminal side of endothelial cells, where these pseudopod-like proplatelets can readily extend into the vasculature (*20*, *21*). Each MK produces thousands of platelets, which circulate as anuclear discoid shaped cell fragments measuring 2.0-5.0 µm in diameter with a lifespan of 7-10 days. This discoid shape causes platelets to flow at the apical surface of the vessel wall, where they rapidly detect vascular injury and function in a highly regulated haemostatic response.

The structure of resting platelets facilitates an initial interaction with regions of vascular damage. Receptors on the platelet surface mediate tethering and adhesion with components of an exposed subendothelial matrix like collagen and von Willebrand factor (VWF) (Figure 1-2). Adhesion is followed quickly by platelet activation, in which platelets undergo a drastic structural rearrangement that allows them to spread over the site of injury and form a physical barrier to excessive blood loss. The activation response also results in secretion of soluble platelet agonists and activation of integrins in the plasma membrane, promoting recruitment, activation, and aggregation of additional platelets at the injured site. Furthermore, as discussed in Section 1.1.1, the activated platelet membrane serves as a cofactor for reactions in the coagulation cascade and provides a structural base for the subsequent accumulation of fibrin. An overview of mechanisms and signal transduction involved in platelet adhesion, activation, and aggregation is provided below.

Reorganization of the actin cytoskeleton is responsible for many of the characteristic platelet activation responses. Initially, receptor ligation on the platelet surface during adhesion or stimulation by soluble agonists activates phospholipase C (PLC). Activated PLC then hydrolyzes

polyphosphoinositides (specifically $PI(4,5)P_2$) on the cytosolic side of the plasma membrane to form the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG) (*22*). IP_3 subsequently interacts with receptors on the dense tubular system (DTS), a region in the cytoplasm responsible for calcium sequestration in the resting platelet, thereby inducing the release of stored calcium (*23*). The resultant increase in the cytosolic free calcium concentration promotes the activation of gelsolin, an F-actin severing protein whose activity exposes free barbed ends in actin filaments (*24*). The actin nucleation and remodeling that follows is regulated by activation of the Arp2/3 complex, which is in turn mediated by additional downstream signaling from surface receptors, polyphosphoinositides in the plasma membrane and a host of other proteins including those in the WASp and Rho GTPase families. The consequences of this cytoskeletal rearrangement are an initial rounding of the platelet followed by membrane ruffling, formation of lamellipodia, and extension of filopodia. These responses function in the spreading and recruitment of platelets at the injured site as well as secretion of platelet proteins.

Multiple platelet-derived proteins, ions, and small molecules are secreted into the vasculature following platelet activation. Three types of secretory organelles exist in platelets: α-granules, dense granules and lysosomes, all of which are exocytosed via fusion with the plasma membrane during platelet shape change (*25*). Each platelet contains approximately 80 α-granules that house proteins involved in the haemostatic response as well as growth factors, chemokines, and adhesion molecules. Additionally, α-granules contain membrane proteins like integrin $\alpha_{IIb}\beta_3$, CD36, and P-selectin that are exposed on the platelet surface following fusion of granules with the platelet membrane. The less abundant dense granules contain other haemostatically active substances including platelet agonists like ADP and serotonin as well as calcium, a critical cofactor in the coagulation cascade. Platelets also contain lysosomes, although the release of acid hydrolases stored therein is slow and incomplete relative to granular secretion. The mechanism by

which granules are secreted is poorly understood, but increases in intracellular calcium concentration following platelet stimulation and the associated activation of protein kinase C (PKC) by DAG are critical to generate a robust secretion response (*26*). The shape change initiated by contraction of actin and myosin is thought to mediate an initial centralization of granules in activated platelets prior to their secretion (*27*). Subsequent fusion of granules with the open canalicular system (OCS) is likely analogous to exocytosis in neurons, which occurs by a SNARE protein-dependent mechanism (*28*).

Granular proteins are either endogenously expressed by MKs or endocytosed from plasma prior to processing and storage. Platelet factor 4 (PF4) and β-thromboglobulin are platelet-*specific* proteins, as they are expressed exclusively in MKs and unique to platelets. On the other hand, VWF and fibrinogen are considered platelet-*selective* as they are synthesized by MKs and endocytosed respectively, but also exist in plasma and other cell types. Uptake of plasma proteins like fibrinogen into MKs occurs through receptor-mediated endocytosis facilitated by glycoproteins on the platelet surface (*29*). In humans, factor V is also endocytosed from plasma but undergoes partial cleavage prior to storage in α-granules (*30*). These posttranslational modifications of factor V alter its cofactor activity and interaction with haemostatic factors like activated protein C (APC), making platelet-derived factor V distinct from its plasma-derived counterpart (*31*).

In addition to secreted proteins directly involved in the haemostatic response, platelets exert a regulatory influence on the coagulation cascade by altering the distribution of phospholipids in their plasma membranes. Phospholipid scramblase-1, a calcium-activated enzyme in platelets facilitates the redistribution of anionic phospholipids like phosphatidylserine to the outer leaflet of the plasma membrane (*32*). The negatively charged extracellular surface of the platelet promotes the assembly of all physiologically relevant procoagulant enzyme

complexes, with the exception of the extrinsic tenase complex (TF-VIIa). As discussed in Section 1.1.1, thrombin is a potent platelet agonist and its accumulation in the vicinity of activated platelet surfaces is critical for platelet recruitment and fibrin assembly leading up to formation of the thrombus.

Thrombin induces platelet activation, degranulation, and aggregation through protease-activated receptors (PARs) on the platelet surface. PARs are activated by proteolytic cleavage of an extracellular loop that exposes a new N-terminus that serves as a tethered ligand for the receptor after refolding (*33*). Human platelets express PAR1 and PAR4, both of which are members of the GPCR family coupled to G_q- and $G_{12/13}$-mediated pathways. Activation of PARs by thrombin results in the platelet activation response outlined above, characterized by activation of PLC, increase in cytosolic calcium levels, and cytoskeletal reorganization. Additionally, GP Ibα on the platelet surface has a high affinity thrombin-binding site that has been observed to accelerate hydrolysis of PARs, thereby promoting platelet activation (*34*).

In addition to secreted ADP and locally generated thrombin, thromboxane A_2 (TXA_2) is also an important platelet agonist (Figure 1-2). Stimulated platelets activate phospholipase A_2, which hydrolyzes phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in the plasma membrane to arachidonic acid (AA) (*35*). Cyclooxygenase-1 (COX-1) subsequently catalyzes the metabolism of arachidonic acid to prostaglandin G_2 (PGG_2) and then to prostaglandin H_2 (PGH_2). Thromboxane synthase then converts PGH_2 to TXA_2, which crosses the platelet membrane and promotes activation and aggregation from the external milieu, through interaction with thromboxane-prostanoid (TP) receptors on the platelet surface. This pathway explains the inhibition of platelet function by aspirin, as aspirin-mediated inhibition of COX-1 limits TXA_2 generation and thus platelet activation (*36*). Like thrombin, both TXA_2 and ADP activate receptors of the GPCR superfamily that go on to activate PLC.

1.3 Platelet Plug Formation

Platelet plug formation requires platelets to activate and adhere with an injured vessel bed. The platelet activation response can be subdivided into three overlapping phases: initiation, extension, and perpetuation. In the initiation phase, adhesion of platelets with the vessel wall is mediated by glycoproteins (GPs) on the platelet surface and components of the subendothelial matrix. As shown in Figure 1-2, when the multimeric adhesive glycoprotein VWF interacts with exposed subendothelial collagen, its affinity for interaction with the GP Ib-IX-V complex on the platelet surface increases. In the context of arterial injury, shear stress induces a conformational change in collagen-bound VWF and the GP Ib-IX-V complex that further promotes their association (*37*). This interaction causes platelets to tether and translocate at the injured site with reduced velocity, facilitating other receptor-matrix interactions that induce platelet adhesion, secretion, and aggregation (*38*). For example, downstream signaling by another surface receptor, GP VI, initiates the activation response outlined above through interaction with collagen, which in turn results in activation of multiple cell surface integrins (*39*). In particular, activation of integrin $\alpha_2\beta_1$ allows it to bind collagen and immobilize platelets at the damaged site (*40*). The ensuing monolayer of platelets formed on the vessel surface during the initiation phase is critical for platelet plug formation, but recruitment of additional platelets during the extension phase is required to consolidate the thrombus and prevent blood loss.

In the extension phase, activation of integrin $\alpha_{IIb}\beta_3$ induces platelet aggregation through its interaction with fibrinogen or VWF (Figure 1-2) (*41*). As fibrinogen and VWF are divalent and multivalent respectively, their binding with $\alpha_{IIb}\beta_3$ on adjacent platelets facilitates cohesion and aggregation. Additional recruitment of circulating platelets is promoted by the accumulation of soluble agonists like ADP, TXA_2, and thrombin in the injury microenvironment. ADP and TXA_2 are directly released from platelets upon activation while thrombin is rapidly generated on

the extracellular surface of the activated membrane. These and other agonists act synergistically to stimulate platelets through GPCRs that in turn induce PLC-mediated calcium mobilization and reorganization of the actin network. Hence, a positive feedback loop is formed in which flowing platelets activate, aggregate, and stimulate additional platelets in the absence of direct contact with the subendothelial matrix.

Finally, in the perpetuation phase, the thrombus retracts and is further stabilized by platelet-platelet interactions. Though not fully characterized, these effects are thought to be mediated by outside-in signaling through platelet surface integrins (*42*). In the case of $\alpha_{IIb}\beta_3$, binding of VWF or fibrinogen facilitates interaction of cytoskeletal components with the integrin cytoplasmic domain. Subsequent downstream signaling from activated integrins mediates phosphorylation of the myosin light-chain through MLCK or ROCK. In association with activated integrins, actin/myosin complexes can generate a contractile force that narrows the gaps between adjacent platelet membranes, which in turn stabilizes the platelet plug in a number of ways. Firstly, additional interactions occur on platelet surfaces in *trans* through cell and junctional adhesion molecules (CAMs and JAMs) like PECAM-1 (*43*), JAM-A, and JAM-C (*44*). Likewise, other platelet receptors can interact with membrane-bound ligands in *trans* as has been observed in the case of Eph kinases and ephrin ligands, eliciting additional downstream signals (*45*). Accumulation of agonists or other platelet-derived molecules in the protected spaces between platelets may also contribute to a sustained activation signal and stabilization of large aggregates (*46*).

The incorporation of activated integrin $\alpha_{IIb}\beta_3$ into actin/myosin complexes also facilitates the phenomenon of clot retraction, in which a fibrin clot pulls in on itself and shrinks to a reduced volume. In addition to gradually narrowing the gaps between platelets, clot retraction also serves to protect the clot from premature dissolution by the fibrinolytic cascade (*47*). The resistance of

retracted platelet-rich clots to lysis is likely based on a reduced number of accessible t-PA binding sites as well as a reduced lysis front velocity. It has been suggested that the increase in fibrin density following clot retraction restricts the access of t-PA binding sites in the fibrin network, thereby attenuating fibrinolysis. Indeed, manipulation of platelet-rich clot structure *in vitro* has been observed to alter lysis rates (*47*). For example, lysis front velocity in platelet-rich areas was significantly reduced relative to platelet-poor regions, resulting in a heterogeneous lysis pattern that left platelet-rich regions largely unlysed. Furthermore, treatment of platelets with $\alpha_{IIb}\beta_3$ antagonists restored lysis rates to levels similar to that observed in platelet-poor clots (*47*, *48*). In addition to these effects, the extrusion of plasma and reduction in gap space following clot retraction may limit the access or accumulation of plasmin in retracted thrombi. Taken together, these results suggest the process of clot retraction is a powerful mediator of fibrinolysis *in vivo*.

1.4 Thrombin-Activatable Fibrinolysis Inhibitor

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a single-chain glycoprotein zymogen that circulates in human plasma at approximately 50-250 nM (*49-51*). As a result of its characterization by multiple groups in independent studies, TAFI is also designated procarboxypeptidase U (*52*), plasma carboxypeptidase B (*53*), and carboxypeptidase R (*54*), though all four titles refer to the same protein. The biochemical characteristics, structure, and function of TAFI are discussed in detail below, with particular emphasis on its role in the haemostatic response.

Human plasma TAFI is expressed in the liver, where cleavage of a 22 amino acid signal peptide precedes secretion into the vasculature (*49*, *55*). Mature, circulating TAFI consists of 401 amino acids with an apparent molecular mass of 60 kDa on SDS-PAGE (*49*). This contrasts from its predicted peptide mass of 45 999 Da as a result of *N*-linked glycosylation at four sites on the amino-terminal activation peptide (Asn22, Asn51, Asn63, and Asn86) (*56*, *57*). Proteolytic

cleavage of this 92-amino acid N-terminal fragment gives rise to activated TAFI (TAFIa), a 35-kDa zinc ion-dependent carboxypeptidase B-like enzyme (*53*, *58*, *59*). Once exposed, the active site of the enzyme moiety catalyzes the removal of carboxyl-terminal lysine and arginine residues from protein and peptide substrates.

Proteolysis of the Arg92-Ala93 bond that results in dissociation of the activation peptide and accessibility of the active site is catalyzed by thrombin and plasmin (Figures 1-4 and 1-5) (*49*, *60*). Whereas both these proteases are relatively inefficient activators of the TAFI zymogen in isolation, when thrombin is in complex with the endothelial cell surface cofactor thrombomodulin (TM), its efficiency of TAFI activation is enhanced 1250-fold (*61*). This substantial increase has led to hypotheses that the thrombin-thrombomodulin complex is the physiological activator TAFI. It should be noted, however, that activation of the TAFI pathway *in vivo* is not necessarily thrombomodulin-dependent. Since the half-maximal effect of TAFIa on prolongation of clot lysis time occurs at about 1 nM *in vitro*, activation of only a fraction (<2%) of the total plasma TAFI pool could result in significant inhibition of the fibrinolytic cascade (*61*). Consequently, it has been postulated that explosive prothrombin activation elicited by positive feedback in the intrinsic pathway of coagulation could generate thrombin at levels sufficient to induce TAFIa-mediated inhibition of fibrinolysis. This notion is supported by *in vitro* clot lysis assays, in which TAFIa-dependent prolongation of lysis time can be observed in the absence of thrombomodulin provided the factor XI-dependent (intrinsic) pathway remains intact (*61*). Furthermore, *in vivo* thrombosis models have demonstrated that impairment of the intrinsic pathway via inclusion of a factor XI antibody enhances thrombolysis due to diminished activation of the TAFI pathway by the thrombin produced by the intrinsic pathway (*62*).

Plasmin can also catalyze activation of TAFI but, like free thrombin, it is relatively inefficient in doing so. In the presence of glycosaminoglycans like heparin, however, plasmin-

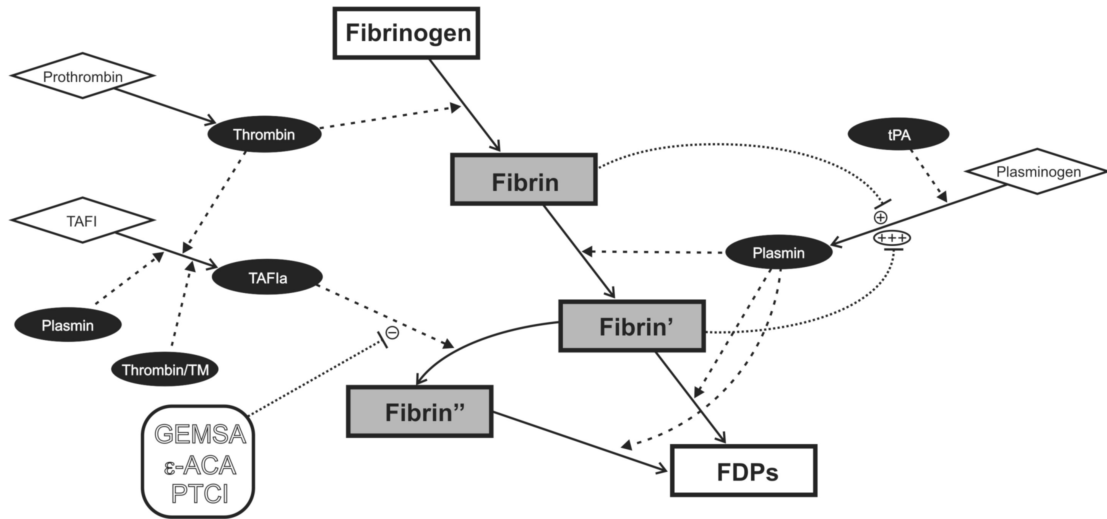

Figure 1-4. TAFI activation and its role in haemostasis.

The end product of the coagulation cascade is the insoluble fibrin clot formed by cleavage of circulating fibrinogen by thrombin. Fibrin is a potent positive cofactor in the activation of plasminogen by tissue plasminogen activator (tPA). The plasmin generated thereafter cleaves fibrin to soluble fibrin degradation products (FDPs) in a multi-step process. Initial partial cleavage results in plasmin-modified fibrin (Fibrin') with exposed carboxyl-terminal lysine residues. These lysines serve as binding sites for t-PA and plasminogen which improves the cofactor activity of fibrin three-fold in plasminogen activation. The increased generation of plasmin through this positive feedback loop results in the propagation of fibrinolysis and rapid dissolution of the clot. Activated TAFI (TAFIa), a basic carboxypeptidase, interferes with this positive feedback loop through cleavage of the carboxyl-terminal lysines from Fibrin'. Due to the decrease in available binding sites for tPA and plasminogen, the cofactor activity of TAFIa-modified fibrin (Fibrin'') is reduced approximately 100-fold. The TAFI zymogen can be activated to TAFIa by plasmin, thrombin, and most efficiently by the thrombin-thrombomodulin (TM) complex. The activity of TAFIa can be inhibited by GEMSA, ε-ACA, and PTCI. (Figure adapted from Boffa MB, Koschinsky ML, and Nesheim ME (*63*)).

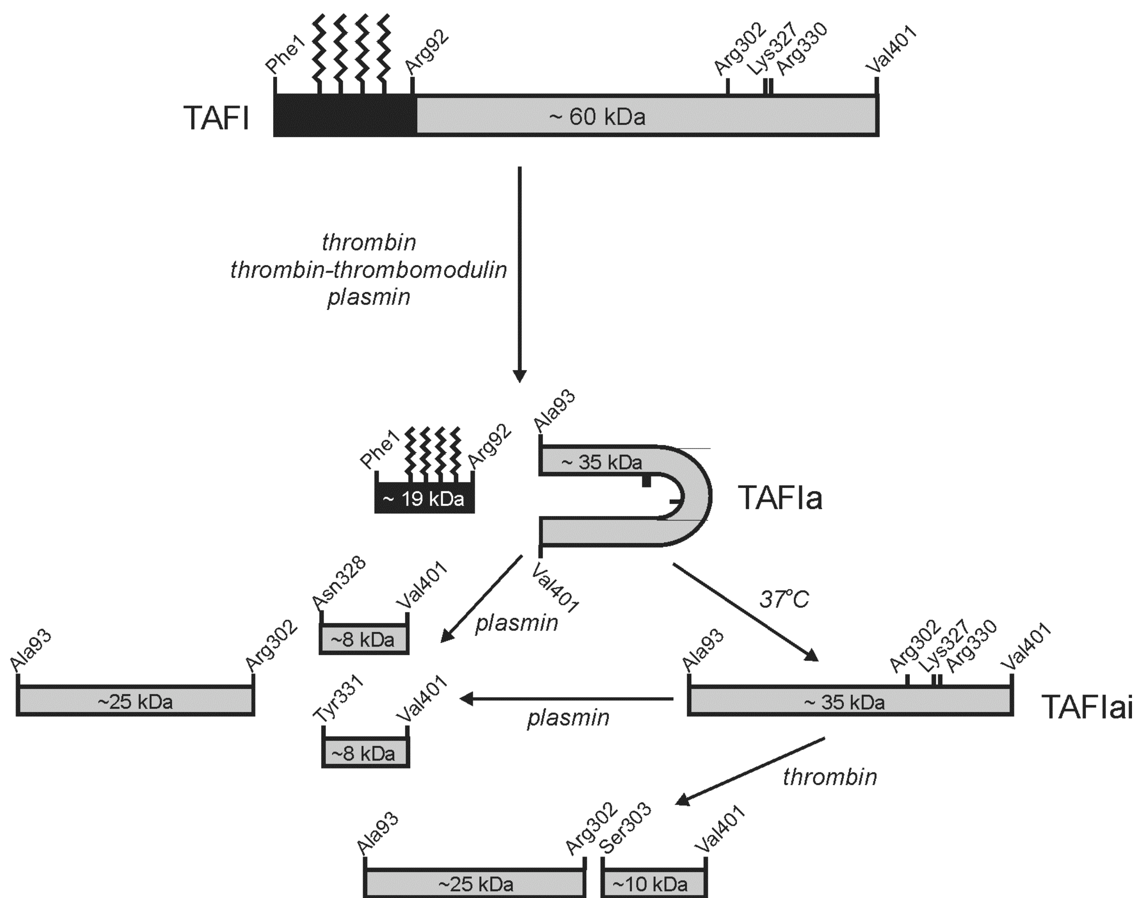

Figure 1-5. Activation of TAFI and inactivation of TAFI and TAFIa.

TAFI consists of an activation domain (black), containing four N-linked glycans, and an enzyme domain (grey). Cleavage of TAFI at Arg92 results in the release of the activation domain and formation of TAFIa. TAFIa is unstable at body temperature, and undergoes a conformational change that is associated with the disappearance of enzyme activity (TAFIai). This conformational change also exposes Arg302 for cleavage by thrombin and accelerates cleavage by plasmin at Arg302, Lys327 and Arg330. Not shown are the small fragments (Ser303 to Lys327; Ser 303 to Arg330; Asn328 to Arg330) that would also be expected to be formed following plasmin cleavage of TAFIa or TAFIai. (Figure adapted from Boffa MB, Koschinsky ML, and Nesheim ME (*63*)).

mediated TAFI activation is enhanced up to 20-fold (*64*). This could be significant in the context of vascular damage, as substantial amounts of glycosaminoglycans may be accessible from an exposed subendothelial matrix. Still, the catalytic efficiency of the thrombin-thrombomodulin complex remains 10-fold higher than that of plasmin in the presence of heparin (*64*).

Activation of the TAFI pathway *in vivo* could occur by any of the respective mechanisms outlined above. The relative contribution of each pathway is unknown, but likely dependent upon the spatiotemporal context of the haemostatic response. That is, extent of activation of the coagulation and fibrinolytic cascades as well as structure and integrity of the endothelium all can be expected to have significant influence on the degree of activation of the TAFI pathway.

Unlike many of the proteases in the coagulation and fibrinolysis cascades, no endogenous inhibitor of TAFIa activity has been described. However, TAFIa activity can be inhibited by addition of exogenous molecules like the lysine analogue ε-aminocaproic acid (ε-ACA), the arginine analogue 2-guanidinoethylmercaptosuccinic acid (GEMSA), and the globular peptide potato tuber carboxypeptidase inhibitor (PTCI) (Figure 1-4) (*50*, *53*, *65*). TAFIa is likely regulated *in vivo* by its intrinsic instability, whereby TAFIa spontaneously decays with a half-life of about 10 minutes at body temperature (*56*, *66*). This instability is temperature dependent, with the half-life of TAFIa increasing to about 2 hours at room temperature; at 0˚C TAFIa is stable indefinitely (*66*).

Recent crystallographic analyses have provided a structural basis for the characteristic instability of TAFIa, which appears to be the result of a dynamic flap region (residues 296-350) in the enzyme moiety (*67*, *68*). In the intact zymogen, this highly mobile flap is stabilized by hydrophobic interactions between residues Val35 and Leu39 in the activation peptide and Tyr341 in the dynamic flap (*67*). Following cleavage of the activation peptide and loss of the associated stabilizing interactions, increased mobility of the dynamic flap compromises the structural

integrity of TAFIa. The irreversible unfolding that follows is accompanied by a loss of TAFIa activity due to displacement of critical active site residues contained in the dynamic flap. Unfolding of this region also exposes the cryptic thrombin cleavage site at Arg302, which is subject to proteolysis only following inactivation of TAFIa (*66*, *69*).

The dynamic flap region also contains the naturally occurring polymorphism at residue 325, which exists as either threonine or isoleucine (*70*, *71*). Notably, the presence of Ile at this position results in a two-fold increase in TAFIa half-life and a 60% increase in antifibrinolytic effect (*71*). This too can be explained by the TAFI structure, as Ile325 is better suited to form hydrophobic interactions with Arg384, Glu385, and Ala388, thereby reducing plasticity of the dynamic flap (*67*). This increased stability translates to an extended half-life for the Ile325 variant, which also corresponds with increased antifibrinolytic potential.

A discussion of the process by which TAFI suppresses fibrinolysis must be preceded by a brief overview of the relevant portion of the fibrinolytic cascade. As introduced in Section 1.1.2, plasmin, the central fibrinolytic protease, catalyzes digestion of fibrin into soluble FDPs (Figures 1-3 and 1-4). Intact fibrin enhances the catalytic efficiency of t-PA-mediated plasminogen activation over 500-fold by providing binding sites for t-PA and plasminogen, thereby serving to restrict relevant activation of plasminogen to the vicinity of the fibrin clot (*72*, *73*). The resultant plasmin then cleaves fibrin after specific lysine and arginine residues in the α-, β-, and γ-chains of fibrin protomers in a multi-step degradation process. As shown in Figure 1-4, the early stages of plasmic cleavage yield plasmin-modified fibrin with exposed carboxyl-terminal lysine residues, to which t-PA and plasminogen can bind through lysine binding sites in their kringle domains (*74*). The basic carboxypeptidase activity of TAFIa enables it to cleave these lysine residues, resulting in downregulation of fibrinolysis by three distinct mechanisms discussed in detail below.

In the first mechanism, TAFIa interferes with a potent positive feedback loop in plasminogen activation. Exposure of the carboxyl-terminal lysines enhances the cofactor activity of fibrin on plasminogen activation approximately three-fold (*74*, *75*). Additionally, the same lysine residues serve as a cofactor in the plasmin-mediated cleavage of native Glu-plasminogen to truncated Lys-plasminogen. Being a 20-fold improved substrate for t-PA, Lys-plasminogen generation corresponds with increased plasminogen activation, which in turn accelerates fibrinolysis (*72*, *73*). By cleaving these carboxyl-terminal lysine residues, TAFIa reduces the cofactor activity of plasmin-modified fibrin on plasminogen activation approximately 100-fold due to a decrease in available binding sites for t-PA and plasminogen (*75*). Likewise, removal of the carboxyl-terminal lysines also downregulates production of Lys-plasminogen (*75*). The net effect of this mechanism is a marked reduction in overall plasminogen activation, thereby attenuating the fibrinolytic cascade.

The second mechanism by which TAFIa suppresses fibrinolysis is related to a protective effect on plasmin provided by partially degraded fibrin. In serum, free plasmin is rapidly and irreversibly inhibited by α_2-antiplasmin (*76*). When plasmin interacts with newly exposed carboxyl-terminal lysine residues on partially degraded fibrin, however, this bound plasmin is protected from inhibition by α_2-antiplasmin (*77*). Accordingly, it has been shown that TAFIa-modified fibrin is unable to facilitate this lysine-dependent shielding of plasmin from its endogenous inhibitor (*78*, *79*). Thus, the carboxypeptidase activity of TAFIa on partially degraded fibrin corresponds with a reduced local plasmin concentration, in turn prolonging the process of clot lysis.

Finally, TAFIa inhibits fibrinolysis by disrupting a cooperative mechanism in plasmin-mediated dissolution of the fibrin clot. Newly exposed carboxyl-terminal lysine residues create a cooperative cleavage response by orchestrating a highly localized recruitment of plasmin to

specific sites within fibrin (*80*, *81*). It has been shown that cleavage of all α-, β-, and γ-chains in fibrin fibers is not necessary for complete solubilization of the clot. Still, for the D and E domains in the fibrin protomers to be separated, six individual plasmin-mediated cleavages are required. Because these cleavage events are in close proximity, the ensuing exposure of carboxyl-terminal lysine residues promotes localized accumulation of plasmin at a specific site and cleavage of the remaining chains. In effect, a non-random, cooperative proteolysis of connections within the protomer occurs after initial partial degradation by plasmin. In this case, carboxyl-terminal lysine removal by TAFIa abolishes this plasmin cooperativity and increases the randomness with which these cleavages occur, necessitating more individual cleavage events for complete clot digestion. Consequently, the time required to lyse the clot is increased, representing another inhibitory pathway of TAFIa on the fibrinolytic cascade (*80*, *81*).

The TAFI pathway defines a unique mode of inhibition by only transiently attenuating the fibrinolytic cascade. *In vitro* clot lysis assays have determined that the antifibrinolytic effect of TAFIa obeys a threshold-dependent mechanism (*82*, *83*). That is, concentrations of TAFIa above a certain threshold value prevent fibrinolysis from entering a propagation phase associated with rapid clot dissolution by enhanced plasmin production. The intrinsic instability of TAFIa plays a mediating role in this regard, as its brief half-life limits the time period in which TAFIa activity can be maintained above the threshold. In fact, maximal prolongation of clot lysis by TAFIa is directly proportional to the half-life of the enzyme (*66*, *71*). As such, this phenomenon explains the increased antifibrinolytic potential of the more stable naturally occurring TAFI variant (Ile325) (*71*). Presumably, once TAFIa concentration falls below the threshold value, positive feedback in plasminogen activation increases and fibrinolysis proceeds.

Aside from its antifibrinolytic effect, an emerging role of the TAFI pathway is its potential for modulating vascular inflammation. It has been shown that the inflammatory peptides

bradykinin and anaphylatoxins C3a and C5a are inactivated by the carboxypeptidase activity of TAFIa (*54*, *59*, *84*). Similarly, cleavage by thrombin exposes a carboxyl-terminal arginine residue in the proinflammatory cytokine osteopontin, making it a substrate for TAFIa (*85*). TAFIa interferes with the ability of thrombin-modified osteopontin to interact with cell surface integrins, as a result downregulating the enhanced inflammatory response associated with proteolytic cleavage of osteopontin by thrombin (*85*). Of particular note is the observation that TAFIa cleaves these inflammatory peptides with greater catalytic efficiency than plasma-derived constitutively active carboxypeptidase N (CPN) (*85*). Although it is unclear whether this implies a physiological role for TAFIa in regulating inflammation, nevertheless these observations suggest activation of the TAFI pathway has implications beyond the attenuation of fibrinolysis.

1.4.1 Potential Physiologic Roles of the TAFI Pathway

A growing body of evidence indicates that the TAFI pathway can regulate thrombolysis *in vivo.* While a clear physiological function of TAFI in humans remains to be elucidated, roles for the TAFI pathway have been observed in animals given thrombotic and other challenges. For example, activation of the TAFI pathway has been confirmed in the context of arterial thrombosis in dogs, in turn suggesting subsequent TAFI activity could influence thrombolysis *in vivo* (*86*). Indeed, multiple studies have shown that inhibition of the TAFI pathway in both venous and arterial thrombolysis models can enhance the thrombolytic efficacy of t-PA (*87-90*, *62*).

While the data outlined above indicate a regulatory role for the TAFI pathway *in vivo*, TAFI knockout mice are viable with no overt bleeding tendencies (*91*), in contrast to what might be expected in animals lacking a fibrinolysis inhibitor. Even in the context of thrombosis models like acute thromboembolism and disseminated intravascular coagulation, TAFI deficiency did not alter survival rates relative to wild-type mice. Notably however, TAFI knockout mice did show increased endogenous fibrinolysis when challenged with batroxobin-induced pulmonary

embolism (*92*). Similarly, mice that were both TAFI deficient and partially plasminogen deficient were observed to have accelerated rates of fibrinolysis in pulmonary clot lysis models and increased leukocyte migration in peritoneal inflammation (*93*). Here, TAFI deficiency may have resulted in increased availability of plasminogen binding sites, in turn promoting positive feedback in the plasminogen activation system. Consequently, enhanced fibrin cleavage and matrix degradation by plasmin could account for the accelerated clot lysis and cell migration rates respectively.

The effects of the TAFI pathway on cell migration are also reflected in the observation that TAFI-deficient mice are impaired with respect to cutaneous wound healing (*94*). Like the situation described above, a lack of TAFI activity could result in increased plasminogen activation and enhanced degradation of extravascular fibrin, thereby abolishing the provisional matrix required to support cell migration into the wound. Taken together, observation of the TAFI knockout mouse suggests that while not essential for normal development, the TAFI pathway might be a significant mediator of the haemostatic response in the context of vascular disease or other pathophysiological states.

1.4.2 Platelet-Derived TAFI

In addition to its presence in plasma, TAFI has also been identified in platelets. In the only study published on the subject, Mosnier *et al.* detected TAFI in the secretory granules as well as the releasates of platelets stimulated by various agonists (*95*). Based on its electrophoretic mobility on SDS-PAGE, this platelet-derived TAFI was observed to be smaller in molecular weight than its plasma-derived counterpart, with an apparent molecular weight of 50 kDa. This variation was attributed to differing glycosylation patterns, as platelet- and plasma-derived TAFI showed similar mobility on SDS-PAGE following deglycosylation with PNGase F. Coupled with the detection of TAFI mRNA in human megakaryocytic cell lines, these results suggested that

platelet-derived TAFI originates from expression in MKs, rather than in the liver like its plasma-derived counterpart (*95*).

While a thorough analysis of differences in source, primary sequence, and function of these two TAFI isoforms has not been published, preliminary data suggest that the platelet-derived zymogen is at least activatable and functional in cleaving both small and large substrates *in vitro* like hippuryl-arginine (Hip-Arg) and minimally degraded fibrin (Desafib X), respectively (*95*). Whether activated platelet-derived TAFI is capable of attenuating the fibrinolytic cascade is unknown. Presumably, however, the release of TAFI from platelets augments overall TAFI abundance in the vascular milieu, particularly at sites of vascular damage or pathological thrombosis that induce platelet activation and secretion. Since the rate of TAFI activation *in vivo* is proportional to the initial plasma concentration, this auxiliary source of TAFI may be significant in modulating degree of activation of the TAFI pathway. Indeed, the apparent concentration of TAFI within platelets (~80 nM) is sufficient to support a presumptive antifibrinolytic role (*95*).

1.5 Overall Hypothesis and Specific Objectives

An emerging role of platelets is their influence on the plasminogen activation system. First, the presence of platelets in thrombi has significant effects on clot structure that reduces t-PA binding site accessibility, thereby attenuating fibrinolysis. Second, platelets secrete antifibrinolytic factors upon activation that limit plasminogen activation. In particular, PAI-1 secretion from activated platelets has been shown to reduce lysis rates of platelet-rich thrombi (*96*). The observation of TAFI in the granules and releasates of platelets suggests that the secretion of TAFI may provide another means for platelets to modulate plasminogen activation. Like platelet-derived PAI-1, local accumulation of platelet-derived TAFI at the injury site could

contribute to inhibition of fibrinolytic cascade. Since platelets are known to aggregate in the context of a developing thrombus and the intraplatelet TAFI concentration is approximately 80 nM, platelet-derived TAFI activated following platelet stimulation could affect fibrinolysis. In the present study, we hypothesize that upon formation of platelet-rich thrombi *in vitro*, thrombin stimulated platelets will secrete TAFI at levels sufficient to attenuate the fibrinolytic cascade.

The specific objectives of the current study are as follows:

1. Confirm the presence of activatable TAFI in the releasates of human platelets.
2. Assess the ability of the platelet pool of TAFI to inhibit thrombus lysis *in vitro.*
3. Purify platelet-derived TAFI from platelet releasates.

Chapter 2
Materials and Methods

2.1 Materials

Fresh frozen citrated human plasma and all platelets were obtained from the blood bank of Kingston General Hospital (Kingston, Canada). The plasmin inhibitor, D-valylphenylalanyllysyl chloromethylketone (VFKck), thrombin inhibitor, D-phenylalanylprolylarginyl chloromethylketone (PPAck), and potato tuber carboxypeptidase inhibitor (PTCI) were obtained from Calbiochem (San Diego, CA, USA). Amicon Ultra centrifugal filtration devices were purchased from Millipore (Etobicoke, ON, Canada). Dulbecco's modified Eagle's medium/nutrient mixture F-12, Opti-MEM, Trypsin-EDTA, penicillin/streptomyocin/fungizone (PSF) were obtained from Gibco/Invitrogen (Burlington, ON, Canada). Newborn calf serum was obtained from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). Recombinant t-PA (Alteplase) was obtained from Kingston General Hospital Pharmacy. Thrombin, rabbit lung thrombomodulin and plasmin were obtained from Haematologic Technologies (Essex Junction, VT, USA). Monoclonal antibody MA-T4E3 (a kind gift of Dr. Ann Gils, Leuven, Belgium) was coupled to CNBr Activated Sepharose 4B (GE Healthcare Life Sciences, Mississauga, ON, Canada) according to manufacturer's instructions (2-6 mg antibody/mL resin). The cysteine-specific fluorescent probe 5-iodoacetamidofluorescein, cysteine-specific quencher QSY® 9 C5-maleimide, and Alexa Fluor® 488 fibrinogen conjugate were obtained from Invitrogen (Burlington, ON, Canada). Sheep anti-human TAFI, sheep anti-human PF4, and sheep anti-human VWF antibodies were purchased from Affinity Biologicals Inc. (Ancaster, ON, Canada). Synthetic 75% phosphatidylcholine: 25% phosphatidylserine (PCPS) vesicles were prepared as described by Schneider *et al.* (*97*). Recombinant plasminogen

with a single mutation of the latent active site serine to a cysteine (S741C) was isolated and labeled with the fluorescent derivative 5-iodoacetamidofluorescein (Fluo-Pg) as described by Horrevoets *et al.* (*98*). QSY® 9 C_5-maleimide-conjugated high molecular mass fibrin degradation products (QSY-FDPs) and TAFIa standards used in the TAFIa assay were prepared as described by Kim *et al.* (*99*).

2.2 Methods

2.2.1 Expression and Purification of Recombinant TAFI

For recombinant TAFI production, stably expressing lines were cultured in triple flasks (500 cm^2, Nunc, Roskilde, Denmark) in Opti-MEM containing 1% (v/v) PSF and 40 μM $ZnCl_2$. Conditioned medium was harvested at 48 hour intervals and replaced with fresh medium. Harvested conditioned medium was centrifuged at 3000 × *g* for 20 minutes at 4˚C, supplemented with Tris-HCl, pH 8.0 (to 5 mM), reduced glutathione (to 0.5 mM) and phenylmethane-sulphonylfluoride (PMSF) (to 2 μM) and stored at -20°C.

To isolate recombinant TAFI, 2 liters of conditioned, supplemented medium was passed through a 0.22 μM filter and then over a 2-mL MA-T4E3-Sepharose column at 4°C. The column was then washed extensively with HEPES-buffered saline (HBS; 20 mM HEPES, 150 mM NaCl, pH 7.4). TAFI was eluted with 0.2 M glycine pH 3.0 and 2-mL fractions were collected into tubes containing 1-mL aliquots of 1 M Tris-HCl, pH 8.0. Protein containing fractions were pooled, concentrated, and exchanged into HBS containing 0.01% (v/v) Tween 80 (HBS/Tween) using Amicon centrifugal filtration devices. Purified recombinant TAFI was quantified by measurement of absorbance at 280 nm ($\varepsilon_{1\%, 280}$ = 26.4, M_r = 60 000), snap frozen, and stored at -70°C.

2.2.2 Clot Lysis Assay

TAFI-deficient plasma was diluted 1:3 in HBS/Tween and supplemented with PCPS vesicles (20 μM) in the presence or absence of TM (10 nM). The mixture was then added to wells of a microtitre plate containing small, separated aliquots of thrombin (5 nM final), $CaCl_2$ (10 mM final), and t-PA (2 nM final) to a final volume of 100 μL. Clot lysis was monitored by the change in turbidity at 405 nm at 37˚C using a SpectraMax M2e plate reader (Molecular Devices, Sunnyvale, CA).

2.2.3 TAFI-Deficient Plasma

To make TAFI-deficient plasma (TDP), 50 mL of fresh-frozen citrated human plasma was thawed at 37˚C and passed over a 2-mL MA-T4E3-Sepharose column at ambient temperature. The plasma was passed over the column repeatedly, and between each pass the column was washed with HBS. After each pass, TAFI was eluted from the column with 0.2 M glycine, pH 3. An aliquot of the plasma was then used for an *in vitro* clot lysis assay in the presence or absence of TM. When no difference in clot lysis times was observed in the presence or absence of TM, the plasma was considered to be TAFI deficient. Aliquots of TDP were then snap-frozen and stored at -70˚C.

2.2.4 Preparation and Activation of Washed Human Platelet Suspensions

Human platelets were obtained from either outdated platelet apheresis packs or pooled four-unit buffy coat donor packs obtained from the Kingston General Hospital. Platelets were pooled and centrifuged at 1200 x *g* for 25 min at ambient temperature. The supernatant was decanted and platelets were resuspended in a total of 1 litre of calcium-free Tyrode's buffer (CFTB; 137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 0.26 mM EGTA, 5 mM PIPES, pH 6.5) containing 0.35% (w/v) bovine serum albumin. Red blood

cells and platelets not readily resuspended were discarded. Suspended platelets in CFTB were centrifuged at 1200 x *g* for 25 minutes at ambient temperature. Platelets were washed a second time in fresh CFTB, centrifuged again at 1200 x *g* for 25 minutes at ambient temperature, and then resuspended in HEPES-Tyrode's buffer (HTB; 137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.4). Platelet counts were determined visually in a haemacytometer (Reichert Analytical Instruments, Buffalo, NY) and concentrations required for subsequent assays were adjusted with HTB. For platelet-derived protein detection experiments, the final suspension of isolated platelets was adjusted to a concentration of 1 x 10^9 platelets/mL in HTB and activated by the addition of 5 nM thrombin for 30 minutes at room temperature with occasional mixing. For the thrombin activation time course, 1 x 10^{10} platelets/mL were activated with 5 nM thrombin to reflect the platelet concentrations used in the thrombolysis assay.

2.2.5 TAFIa Assay

Eighty microlitres of a solution of 1.0 µM QSY-FDPs and 50 nM Fluo-Pg in HBS was added to wells of a white opaque microtitre plate and fluorescence intensity was monitored with excitation and emission wavelengths of 480 and 520 nm, respectively, with a 495 nm emission cutoff filter using a SpectraMax M2e fluorescence plate reader (Molecular Devices). After the fluorescence signal stabilized, TAFIa standards or samples (20 µL) were added to the 96-well plate between readings. Initial rates (dRFU/dt) were measured for each TAFIa standard, and a standard curve was generated. The TAFIa concentration in samples was determined by interpolation of initial rates on the standard curve.

2.2.6 TAFIa Quantification in Human Platelet Releasates

A suspension of platelets at a concentration of 2.7×10^9 platelets/mL was activated with 5 nM thrombin for 30 minutes at ambient temperature with gentle mixing. Aggregated platelets were then pelleted by centrifugation for 5 minutes at 3000 x g at ambient temperature. The supernatant was removed and supplemented with an additional 20 nM thrombin (25 nM final) and 100 nM thrombomodulin for 20 minutes at room temperature to completely activate any TAFI present. Thrombin was then inhibited by the addition of 5 μM PPAck to the supernatant. The concentration of TAFIa in the system was then determined using the TAFIa assay.

2.2.7 Thrombolysis Assay

Isolated human platelets in HTB were brought to a final concentration of 1×10^{10} platelets/mL in TAFI-deficient plasma (TDP; 1:3 dilution) containing 5 mM $CaCl_2$, 100 μg/mL Alexa Fluor® fibrinogen conjugate, and 0.05% (v/v) Tween 80. Thrombi (600 μL) were formed by the addition of 594 μL of the mixture to 6 μL of 500 nM thrombin (5 nM final). Thrombus formation and retraction was allowed to proceed for 30 minutes at ambient temperature.

Retracted fluorescent thrombi were lysed by the addition of various concentrations of t-PA (0-10 nM) to the fluid surrounding the thrombus and incubation at 37˚C for 2 hours. Samples of the fluid (10 μL) were removed every 15 minutes and diluted 1:15 in HBS/Tween in the wells of a white opaque microtitre plate. The fluorescence intensity in the supernatants was then measured with excitation and emission wavelengths of 496 and 520 nm, respectively, with a 515 nm emission cutoff filter using a SpectraMax M2e fluorescence plate reader (Molecular Devices). Inhibition of thrombolysis was accomplished by supplementation of the fluid surrounding the thrombus with TM (to 10 nM) with or without supplementary recombinant TAFI (20 nM) at the

time of t-PA addition. TAFI-dependent inhibition of thrombolysis was overcome by inclusion of 25 μg/mL PTCI during thrombus formation.

2.2.8 Isolation of Platelet-Derived TAFI

Platelets from outdated pooled 4-unit buffy coat donor packs were isolated from plasma proteins as described above. Final platelet suspensions in HTB were incubated at 37˚C for 15 minutes and then activated with 5 nM thrombin. Aggregation and degranulation was allowed to proceed for 25 minutes at ambient temperature with occasional mixing. The suspension was then supplemented with 5 μM PPAck and centrifuged for 30 minutes at 3000 x *g* at 4˚C. Platelet releasates were aliquoted, snap frozen and stored at -70˚C.

Releasates from ~50 donor packs were thawed rapidly at 37˚C and passed over a 2-mL MA-T4E3-Sepharose at ambient temperature. The column was then washed extensively with HBS. TAFI was eluted with 0.2 M glycine pH 3.0 and 2-mL fractions were collected into tubes containing 1-mL aliquots of 1 M Tris-HCl, pH 8.0. Protein containing fractions were pooled, concentrated, and exchanged into HBS containing 0.01% (v/v) Tween 80 (HBS/Tween) using Amicon centrifugal filtration devices. Purified platelet-derived TAFI was quantified by measurement of absorbance at 280 nm ($\varepsilon_{1\%,\ 280} = 26.4$, $M_r = 60{,}000$), snap frozen, and stored at -70°C.

Chapter 3
Results

3.1 TAFI Detection in Human Platelet Releasates

Previous studies by Mosnier and colleagues had established that TAFI is released from human platelets upon activation by agonists such as thrombin (*95*). As this report was the first and only report of the existence of TAFI in platelets, our first priority of the current work was to verify the existence of this pool of TAFI. Like the Mosnier group, western blot analysis of releasates obtained from washed, thrombin-stimulated platelets was performed. Preliminary experiments failed to reproduce the finding of Mosnier that platelet-derived TAFI was substantially greater in electrophoretic mobility than plasma-derived TAFI. As such, a primary concern was that the TAFI immunoreactive material that we were detecting in the releasates of platelets was in fact contaminating plasma-derived TAFI carrying over from the plasma in which the platelet concentrates were stored. Therefore, we designed a series of experiments to rule this possibility out.

The efficacy of the isolation protocol in eliminating contaminating plasma proteins from the platelet suspensions was assessed by western blot analysis using a polyclonal anti-TAFI antibody (Figure 3-1). The absence of detectable TAFI in the final platelet suspension indicates this sample was free of the TAFI initially present in the plasma used for platelet storage in the donor packs. Addition of 5 nM thrombin to the platelet suspension induced a characteristic aggregation response, indicative of platelet activation and degranulation. The subsequent detection of TAFI in platelet releasates following activation by thrombin is consistent with the localization of TAFI within the secretory granules of human platelets. The immunoreactive material detected in the platelet releasate was confirmed to be TAFI by the addition of 10 nM

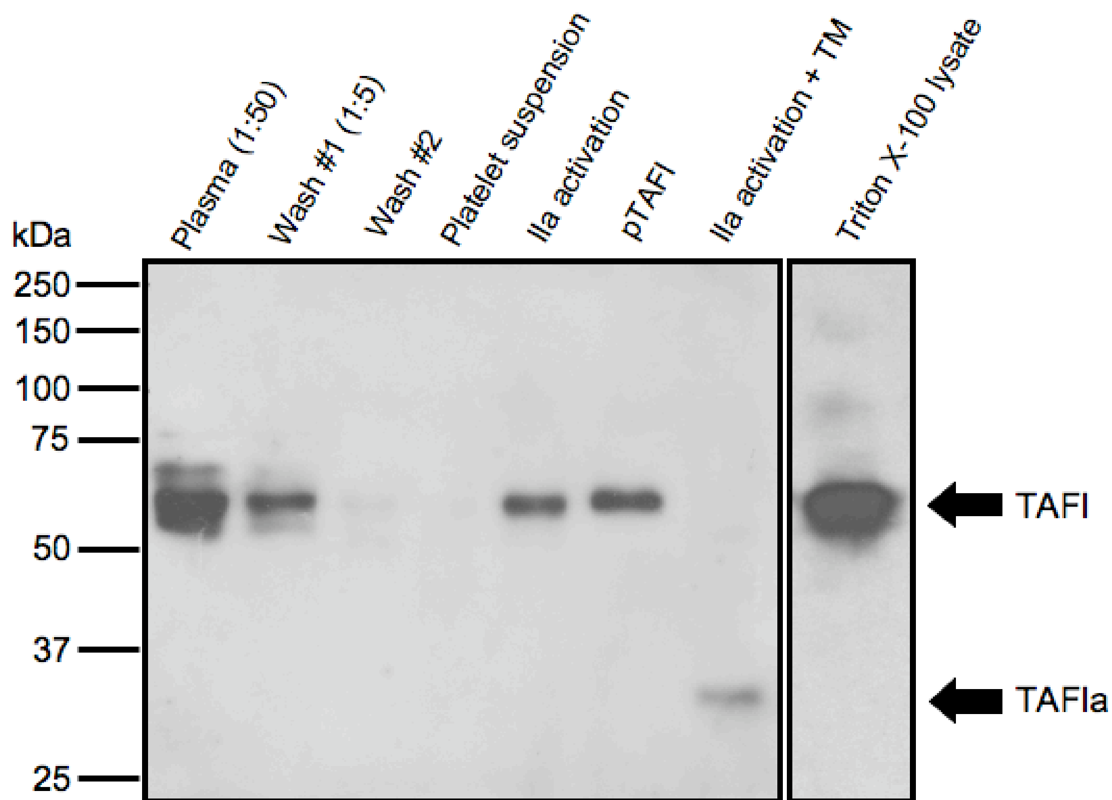

Figure 3-1. Washing of human platelets and secretion of activatable TAFI.

The presence of TAFI in the medium of human platelets throughout the isolation procedure and following activation was analyzed by western blot. Human platelets were centrifuged and the supernatant (Plasma) was decanted. Platelets were then resuspended and washed twice in CFTB (Wash #1 and #2). The washed platelets, presumed to be free of contaminating plasma proteins, were suspended in HTB (Platelet Suspension). Concentration of the platelet suspension was adjusted to 1 x 10^9 platelets/mL in HTB and activation was induced by addition of 5 nM thrombin for 30 minutes at room temperature (IIa activation). To activate platelet-derived TAFI, the platelet releasate was supplemented with 10 nM TM (IIa activation + TM) for 20 minutes at room temperature. A separate platelet suspension was lysed with 0.1% (v/v) Triton X-100. Samples of the platelet wash buffers, suspension, releasates, and lysates were subjected to 10% SDS-PAGE under reducing conditions followed by western blot analysis using a TAFI-specific polyclonal antibody raised in sheep. The western blot was subsequently incubated with rabbit anti-sheep IgG and immunoreactive bands were visualized by chemiluminescence. A sample of plasma-derived TAFI was included on the gel as a positive control (pTAFI). Positions of the molecular mass markers are shown to the *left* of the blot and the apparent migration of TAFI and TAFIa is shown on the *right.*

thrombomodulin, resulting in cleavage of the activation peptide and detection of the characteristic 35-kDa species representing the TAFIa enzyme moiety. Additionally, any TAFI zymogen contained within platelets was confirmed to be intact following lysis with 0.1% (v/v) Triton X-100. Notably, both the platelet-derived TAFI zymogen and a plasma-derived TAFI zymogen control migrated at approximately 60 kDa on SDS-PAGE, suggesting any difference in size between the two isoforms is not as significant as that reported by Mosnier and coworkers (*95*).

To further assess the activated platelet suspensions for platelet-derived protein content, western blot analyses using antibodies for VWF and PF4 were performed in parallel to the TAFI detection blots described above (Figure 3-2). Being a platelet-selective protein contained in plasma and also the α-granules of human platelets, detection of VWF throughout the isolation protocol was similar to the detection of TAFI in Figure 3-1. As expected, VWF was initially present in the plasma used for platelet storage in the donor pack, but was close to undetectable in the final platelet suspension. Following stimulation with 5 nM thrombin, a 270-kDa species representing monomeric VWF was detected in platelet releasates. This observation suggests the isolation protocol was successful in the clearance of contaminating plasma proteins and VWF was secreted directly from platelets into the surrounding medium following activation by thrombin.

The detection of a 7.8-kDa band representing monomeric PF4 was limited to the thrombin-stimulated platelet releasates and was otherwise absent from other platelet suspensions, wash steps, and plasma. This observation indicates that platelets were maintained in an unactivated, quiescent state throughout the wash procedure and activation was induced solely through cleavage of PARs on the platelet surface by thrombin and not by mechanical means. Taken together, these observations suggest the secretion of TAFI is concurrent with other proteins known to be stored within the α-granules of human platelets.

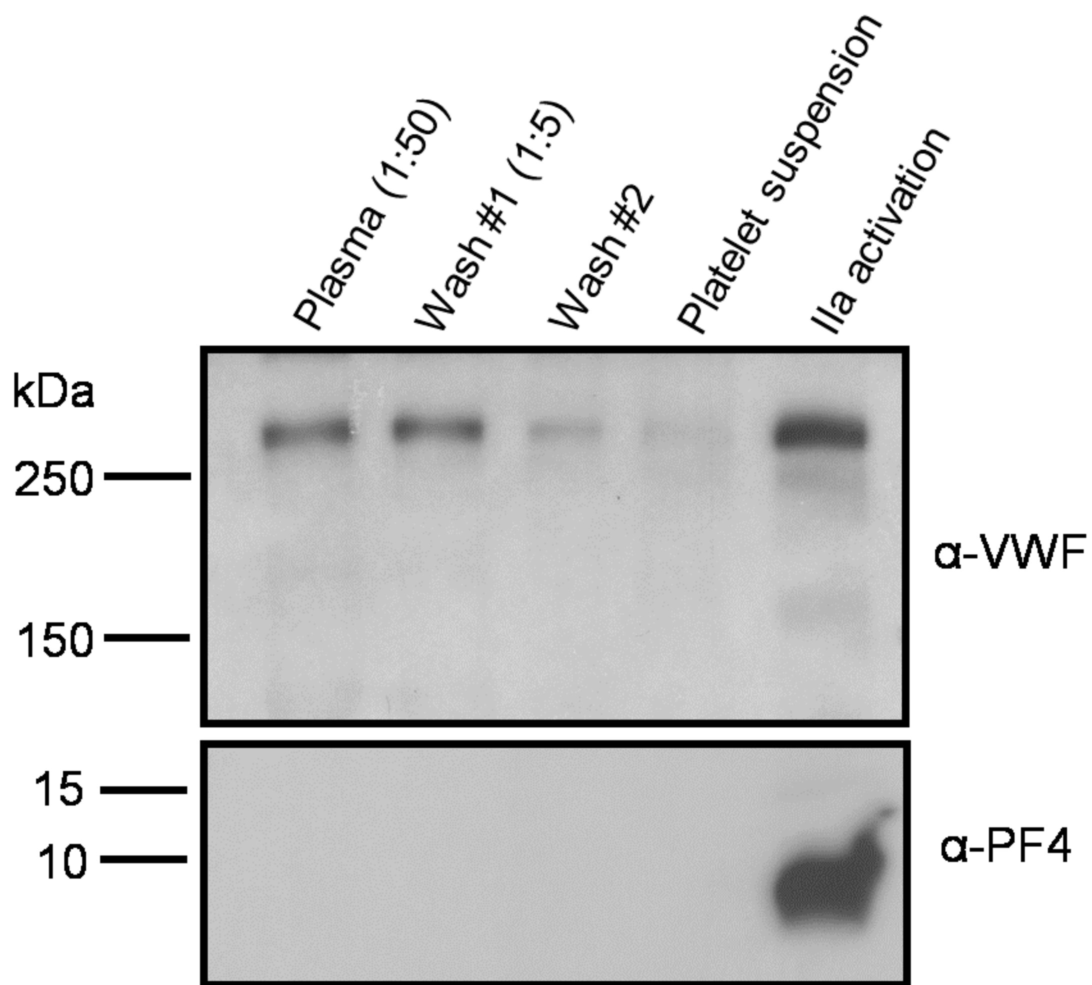

Figure 3-2. Washing of human platelets and secretion of VWF and PF4.

The presence of VWF and PF4 in the medium of human platelets throughout the isolation procedure and following activation was analyzed by western blot. Human platelets were washed and then activated by thrombin, and aliquots were taken from the respective platelet wash buffers, suspension, and releasates, as described in the Legend to Figure 3-1. Samples were subjected to 8% or 20% SDS-PAGE under reducing conditions followed by western blot analysis using VWF- and PF4-specific polyclonal antibodies, respectively. The western blots were subsequently incubated with rabbit anti-sheep IgG and immunoreactive bands were visualized by chemiluminescence. Positions of the molecular mass markers are shown to the *left* of the blots.

3.2 Time Course of Secretion of TAFI from Isolated Human Platelets

The temporal profile of secretion of TAFI from thrombin-stimulated platelets was analyzed by western blotting over a 30-minute period (Figure 3-3). A suspension of isolated human platelets at a concentration of 1 x 10^{10} platelets/mL in HTB was activated with 5 nM thrombin for 30 minutes at ambient temperature; at 5-minute intervals, platelet suspensions were centrifuged and the supernatants subjected to western blot analysis. Initially, no detectable TAFI was present in the medium of unactivated platelets (time = 0), suggesting all plasma-derived TAFI had been removed during the isolation procedure. Increasing amounts of TAFI were detected in the platelet suspensions at time points subsequent to the addition of 5 nM thrombin. The gradual appearance of TAFI in the medium of activated platelet suspensions is consistent with the localization of TAFI in the secretory granules of platelets, with its release into the surrounding medium being dependent on platelet activation and eventual degranulation.

3.3 Quantification of Activatable TAFI Secreted from Activated Human Platelets

An isolated suspension of platelets in HTB was activated with 5 nM thrombin to induce secretion of TAFI into the platelet medium. Platelet aggregates were subsequently removed by centrifugation and any TAFI present in the platelet medium was subsequently quantitatively converted to TAFIa by the addition of 20 nM thrombin and 100 nM thrombomodulin. The concentration of TAFIa in the medium of 2.7 x 10^9 thrombin-stimulated platelets was determined to be 1.053 ± 0.023 nM using the TAFIa assay described in Section 2.2.6. This TAFIa concentration corresponds to the secretion of 23.4 ± 0.3 ng of activatable TAFI per 10^9 thrombin-stimulated platelets, as compared to the value of 25 ± 5 ng per 10^9 thrombin-stimulated platelets reported by Mosnier and colleagues (*95*). Since the half-maximal antifibrinolytic effect of TAFIa is known to occur at about 1 nM *in vitro* (*61*), this observation further suggests that in the context

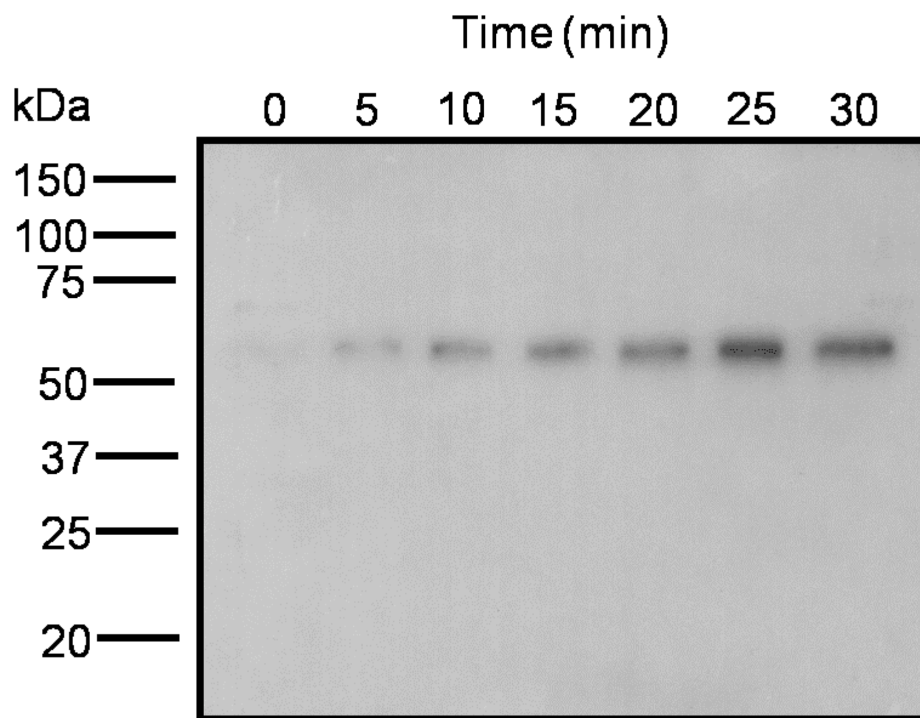

Figure 3-3. Time course of secretion of TAFI from thrombin-activated human platelets.

The presence of TAFI in the medium of human platelets activated with thrombin was analyzed by western blot. Isolated platelets in HTB (1 x 10^{10} platelets/mL) were activated with 5 nM thrombin for 30 minutes at room temperature. At the times indicated above the blot, platelets were centrifuged and aliquots of the platelet medium were supplemented with 5 μM PPAck and subjected to 10% SDS-PAGE under reducing conditions followed by western blot analysis using a TAFI-specific polyclonal antibody. The western blot was subsequently incubated with rabbit anti-sheep IgG and immunoreactive bands were visualized by chemiluminescence. Positions of the molecular mass markers are shown to the *left* of the blot.

of platelet-rich thrombus formation, activated platelet-derived TAFI could reach levels sufficient to attenuate the fibrinolytic cascade.

3.4 Lysis of Platelet-Rich Thrombi

To assess the potential antifibrinolytic effect of activated platelet-derived TAFI, platelet-rich thrombi were generated in the absence of plasma-derived TAFI. Retracted fluorescent thrombi were prepared from washed human platelets, TAFI-deficient plasma, and fluorescently-labeled fibrinogen as described in Section 2.2.7. The fluid surrounding the retracted thrombi was then supplemented with various concentrations of t-PA (0-10 nM) to induce the fibrinolytic cascade (Figure 3-4). Lysis was observed as fluorescence units released into the surrounding medium of retracted fluorescent thrombi. As expected, the rate of lysis was dependent on the initial concentration of t-PA in the system (Table 3-1). Minimal lysis was observed in the absence of t-PA over the 105-minute time course, indicating the increase in fluorescence was dependent on plasminogen activation and not a result of thrombus instability under the lysis conditions. Because the isolated platelets used to augment the TAFI-deficient plasma for thrombus formation were free of plasma-derived TAFI, the sole source of TAFI in these retracted thrombi can be considered to be the platelets. Based on the TAFI quantification experiments outlined above, the platelet counts used in the generation of the thrombi were sufficient to secrete ~4 nM activatable platelet-derived TAFI into the medium following stimulation by thrombin.

3.5 Inhibition of Thrombus Lysis by Recombinant TAFI

To determine whether thrombolysis could be inhibited by activated TAFI under these conditions, the medium of retracted thrombi formed in triplicate was supplemented with 10 nM thrombomodulin and 20 nM recombinant TAFI and lysis was induced by t-PA (Figure 3-5). A 1.7-fold reduction in lysis rate was observed in the presence of active TAFI relative to the lysis

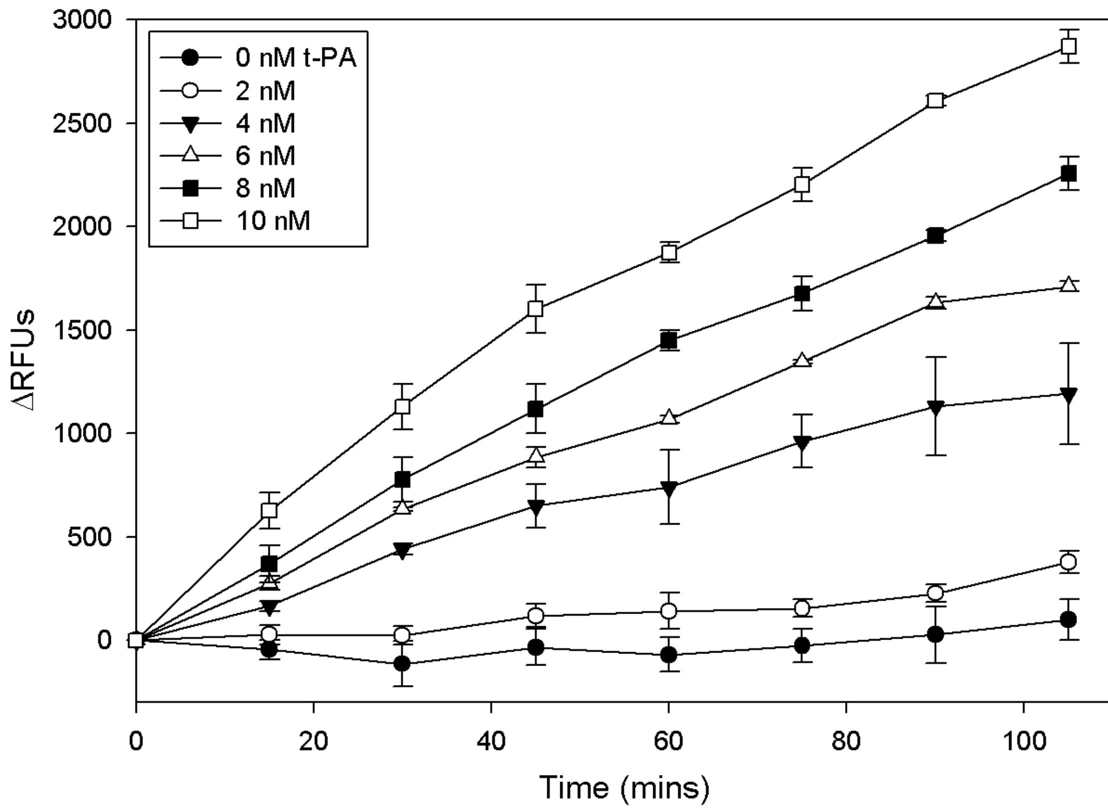

Figure 3-4. Lysis of fluorescent platelet-rich thrombi by addition of t-PA.

Fluorescent thrombi were generated as described in Section 2.2.7. The medium of retracted fluorescent thrombi was supplemented with various concentrations of t-PA (0-10 nM) to induce the fibrinolytic cascade. Lysis of thrombi was monitored at 37˚C as fluorescence units released. Individual plots represent the mean fluorescence released from duplicate thrombi. Error bars represent the data range (n = 2).

Table 3-1. Lysis rates of platelet-rich thrombi at various t-PA concentrations.

Lysis of retracted fluorescent thrombi was accomplished by addition of t-PA to the thrombus medium. Lysis rates are expressed as release of fluorescence (RFU min^{-1}) and determined by linear regression of the plots in Figure 3-4 (each plot is the mean of n = 2).

t-PA (nM)	Lysis Rate (RFU min^{-1})
0	1.0
2	3.2
4	11.8
6	16.7
8	21.2
10	26.6

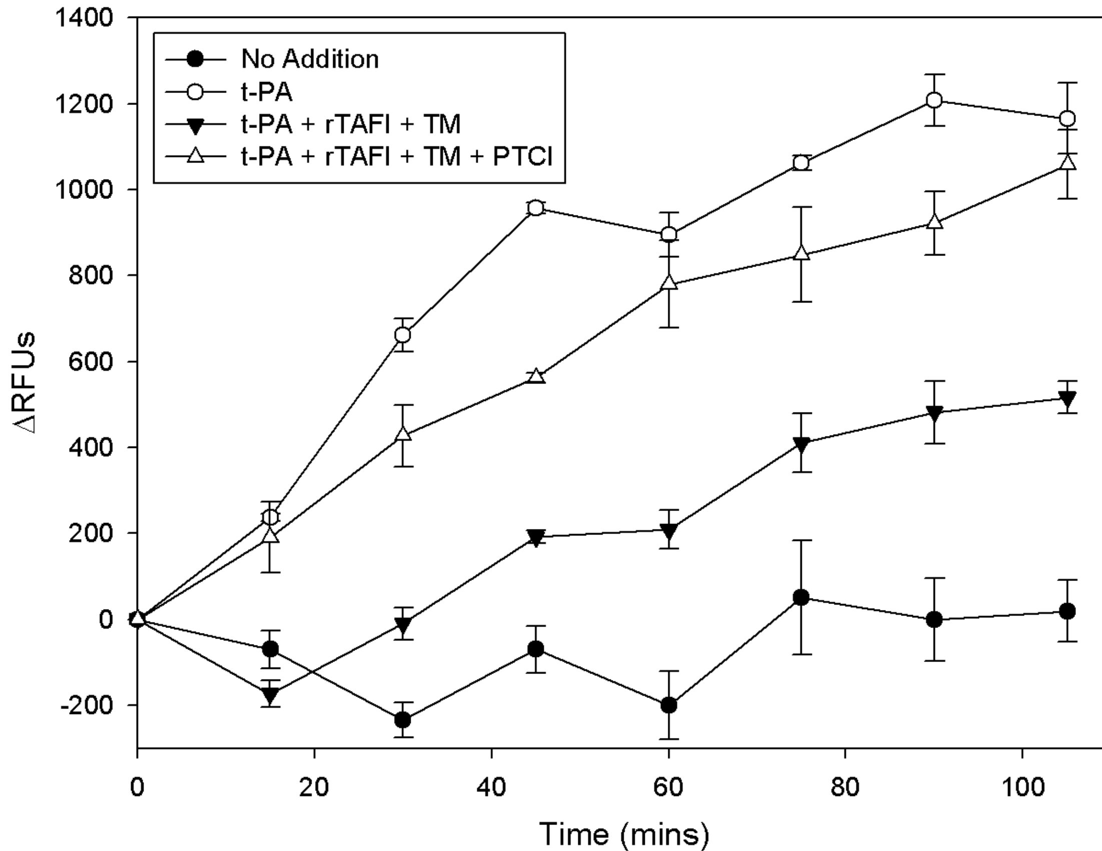

Figure 3-5. Inhibition of platelet-rich thrombolysis by recombinant TAFI.

Fluorescent thrombi were generated as described in Section 2.2.7. The medium of retracted fluorescent thrombi was supplemented with t-PA (4 nM) to induce the fibrinolytic cascade in the presence and absence, where indicated, of 10 nM TM, 20 nM rTAFI and 25 µg/mL PTCI. Lysis of thrombi was monitored at 37°C as fluorescence units released. Individual plots represent the mean fluorescence released in three separate thrombi. Error bars represent ± SEM (n = 3).

rate induced by the addition of t-PA alone, indicating that TAFIa is capable of attenuating the fibrinolytic cascade in this system (Table 3-2). This reduction in lysis rate was confirmed to be a TAFIa-dependent effect by the inclusion of 25 μg/mL PTCI during thrombus formation (i.e. prior to addition of TM, rTAFI, and t-PA), which restored lysis rates to a level similar to that observed upon addition of tPA alone.

3.6 Inhibition of Thrombus Lysis by Platelet-Derived TAFI

Because activated platelets are the only source of TAFI, in the absence of added recombinant TAFI, in the medium of retracted thrombi used in the thrombolysis assay, we attempted to detect inhibition of the fibrinolytic cascade by platelet-derived TAFI alone without supplementary recombinant TAFI. Here, the medium of retracted thrombi formed in triplicate was supplemented with 10 nM thrombomodulin and lysis was induced by t-PA (Figure 3-6). A 2.3-fold reduction in lysis rate was observed when thrombomodulin was added to the thrombus medium relative to the lysis rate observed upon addition of t-PA alone (Table 3-3). This result confirms that activated platelet-derived TAFI can attenuate platelet-rich thrombus lysis independently of plasma TAFI *in vitro*. The reduction in lysis rate was confirmed to be a platelet-derived TAFIa-dependent effect by the inclusion of 25 μg/mL PTCI during thrombus formation (i.e. prior to addition of TM and t-PA), which restored lysis rates to a level similar to that observed upon addition of t-PA alone.

3.7 Differential Inhibition of Thrombus Lysis by Platelet-Derived TAFI and Supplementary Recombinant TAFI

To determine whether activated platelet-derived TAFI could augment inhibition of the fibrinolytic cascade achieved by TAFIa already present in the medium surrounding platelet-rich thrombi, retracted fluorescent thrombi were lysed in the presence and absence of various

Table 3-2. Attenuation of lysis rates by activated recombinant TAFI.

Lysis of retracted fluorescent thrombi was monitored in the presence or absence of 20 nM rTAFI, 10 nM TM, and 25 µg/mL PTCI, where indicated. Lysis rates are expressed as release of fluorescence (RFU min^{-1}) and determined by linear regression of plots in Figure 3-5 (each plot is the mean of n = 3).

t-PA	rTAFI	TM	PTCI	Lysis Rate (RFU min^{-1})
-	-	-	-	1.1
+	-	-	-	11.2
+	+	+	-	6.5
+	+	+	+	10.0

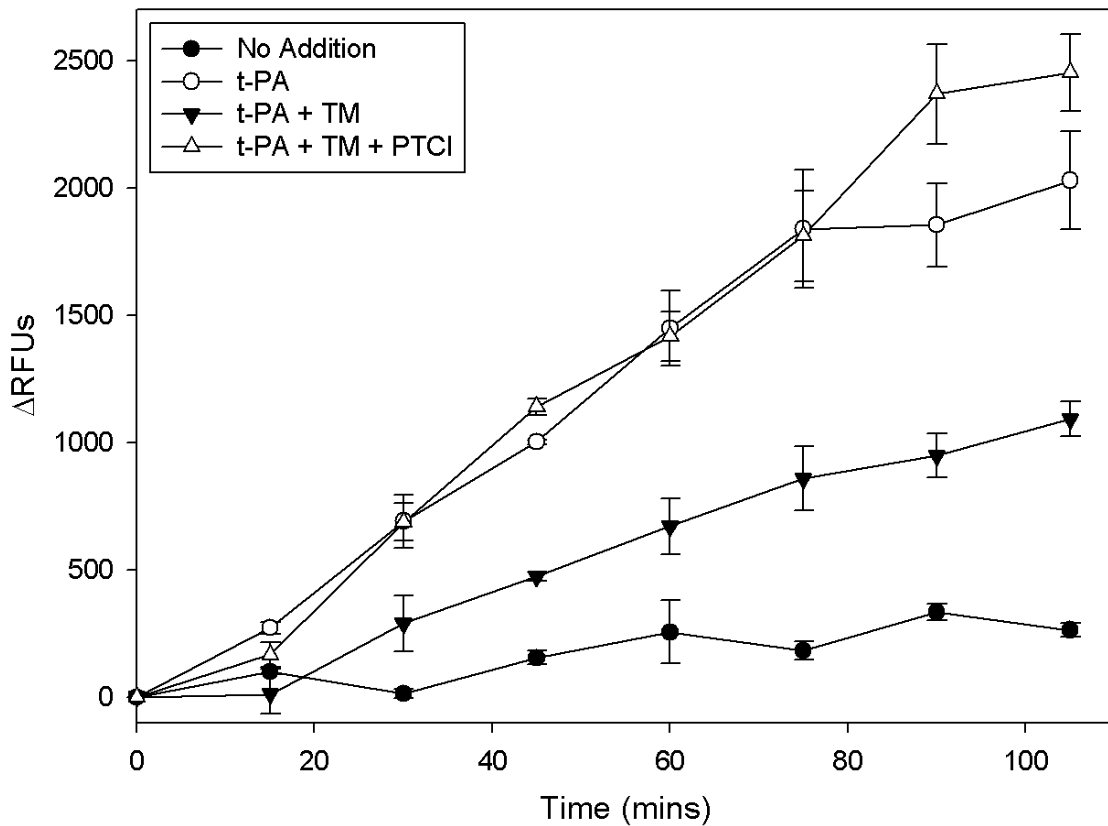

Figure 3-6. Inhibition of platelet-rich thrombolysis by platelet-derived TAFI.

Fluorescent thrombi were generated as described in Section 2.2.7. The medium of retracted fluorescent thrombi was supplemented with t-PA (4 nM) to induce the fibrinolytic cascade, in the presence or absence, where indicated, of 10 nM TM and 25 μg/mL PTCI. Lysis of thrombi was monitored at 37˚C as fluorescence units released. Individual plots represent the mean fluorescence released in three separate thrombi. Error bars represent ± SEM (n = 3).

Table 3-3. Attenuation of lysis rates by activated platelet-derived TAFI.

Lysis of retracted fluorescent thrombi was monitored in the presence of absence of 10 nM TM and 25 µg/mL PTCI, where indicated. Lysis rates are expressed as release of fluorescence (RFU min^{-1}) and determined by linear regression of plots in Figure 3-5 (each plot is the mean of n = 3).

t-PA	TM	PTCI	Lysis Rate (RFU min^{-1})
-	-	-	3.0
+	-	-	24.3
+	+	-	10.6
+	+	+	21.4

concentrations of rTAFI (Figure 3-7). Here, the medium of retracted fluorescent thrombi formed in duplicate was supplemented with various rTAFI concentrations (0-40 nM) and 10 nM TM to activate all TAFI present in the thrombus medium (platelet-derived and recombinant). Activation of platelet-derived TAFI by 10 nM TM resulted in a 1.3-fold reduction in lysis rate relative to lysis rate observed upon addition of t-PA alone (Table 3-4). Addition of 4 nM supplementary rTAFI resulted in 1.7-fold reduction in lysis rate, in turn indicating that platelet-derived and recombinant TAFIa combine to enhance the inhibition of the fibrinolytic cascade in this system. Addition of rTAFI at 10, 20, and 40 nM resulted in very similar reductions in lysis rate, corresponding to a maximal inhibition of thrombus lysis above 10 nM supplementary rTAFI (14 nM total TAFI) in the system. Taken together, these observations indicate that the secretion of platelet-derived TAFI can augment concentrations of TAFI already present in plasma to enhance inhibition of the fibrinolytic cascade.

3.8 Purification of Platelet-Derived TAFI from Human Platelet Releasates

To purify platelet-derived TAFI, bulk platelet releasates were subjected to immunoaffinity chromatography followed by centrifugal ultrafiltration and buffer exchange. Platelet-derived TAFI was obtained at a final concentration of approximately 33 μg/mL (~560 nM) as determined by measurement of absorbance at 280 nm. The isolated sample of platelet-derived TAFI was observed to be free of contaminating platelet proteins on silver stained SDS-PAGE gels (Figure 3-8). Based on the quantification experiments of total TAFI secreted by thrombin-stimulated platelets outlined in Section 3.3, estimates of overall yield were in the 10-20% range. Purified platelet-derived TAFI was completely activatable by thrombin and TM, which resulted in appearance of the characteristic 35 kDa TAFIa band on SDS-PAGE. A comparison of mobility between plasma-derived TAFI and purified platelet-derived TAFI

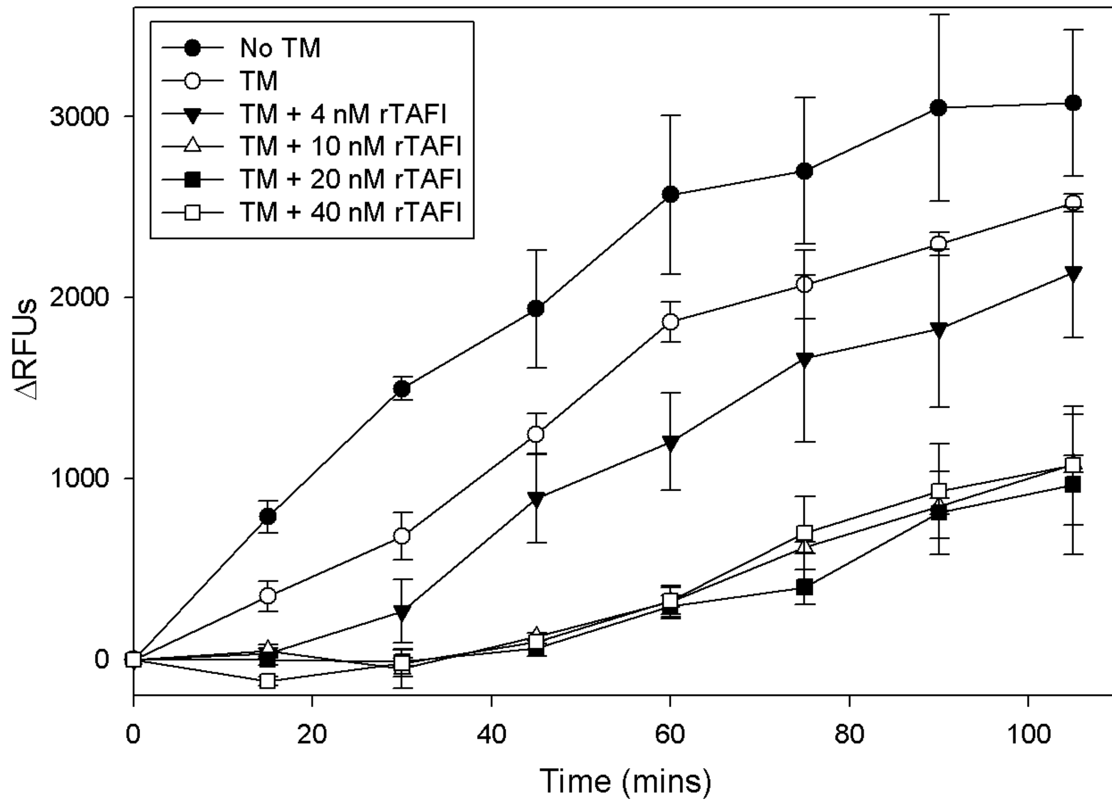

Figure 3-7. Differential attenuation of thrombolysis by activated platelet-derived and recombinant TAFI.

Fluorescent thrombi were generated as described in Chapter 2.2.7. The medium of retracted fluorescent thrombi was supplemented with t-PA (4 nM) to induce the fibrinolytic cascade, in the presence or absence, where indicated, of 10 nM TM and various concentrations of rTAFI. Lysis of thrombi was monitored at 37˚C as fluorescence units released. Individual plots represent the mean fluorescence released in duplicate thrombi. Error bars represent the data range (n = 2).

Table 3-4. Attenuation of lysis rates by activated platelet-derived TAFI with supplementary recombinant TAFI.

Lysis of retracted fluorescent thrombi was monitored in the presence and absence of 10 nM TM and various concentrations of rTAFI, where indicated. Lysis rates are expressed as release of fluorescence (RFU min^{-1}) and determined by linear regression of plots in Figure 3-7 (each plot is the mean of n = 2).

rTAFI (nM)	TM	Lysis Rate (RFU min^{-1})
0	-	34.8
0	+	26.0
4	+	20.1
10	+	8.3
20	+	7.1
40	+	8.6

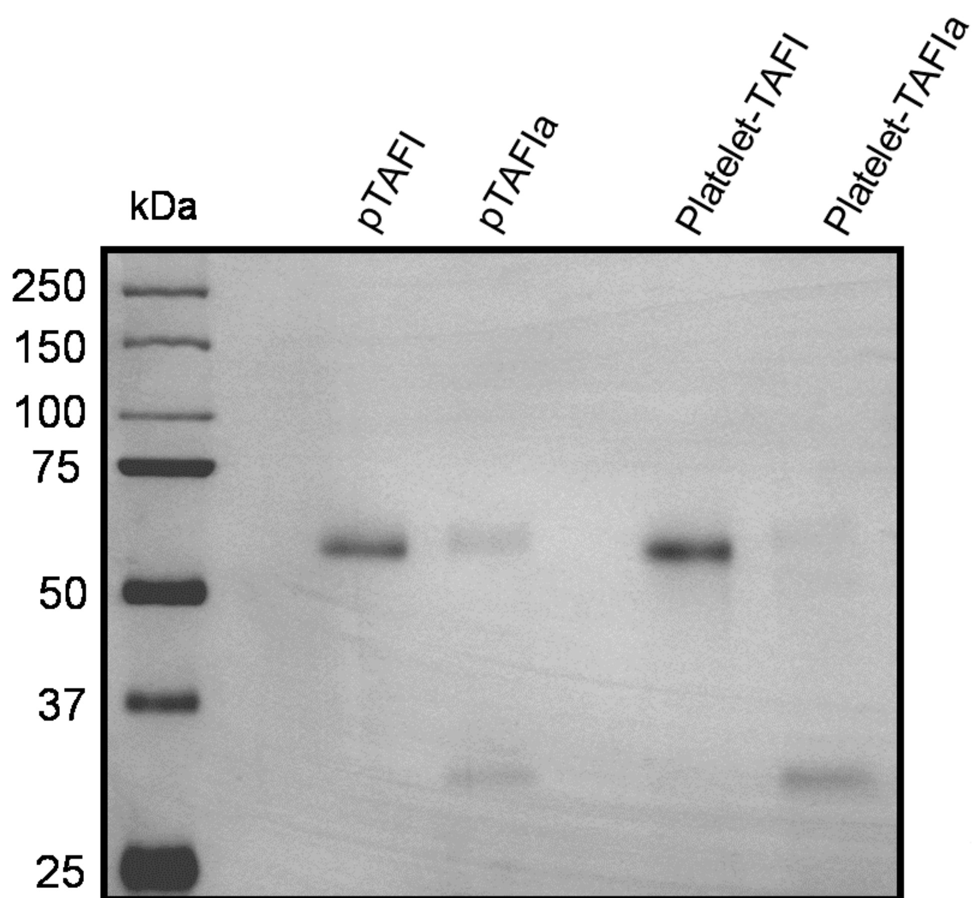

Figure 3-8. Isolation and activation of purified platelet-derived TAFI.

Platelet-derived TAFI was isolated and purified from bulk platelet releasates as described in Chapter 2. Plasma-derived and platelet-derived TAFI were activated with 25 nM thrombin and 100 nM TM (pTAFIa and Platelet-TAFIa). Samples of the zymogens (Platelet-TAFI and pTAFI) and active enzymes were subjected to 10% SDS-PAGE under reducing conditions. Proteins and markers were visualized by silver staining.

indicates again that any difference in size between the two isoforms is not nearly as significant as that observed by Mosnier *et al.* (*95*).

Chapter 4
Discussion

4.1 The Mobility of Platelet-Derived TAFI on SDS-PAGE

In their report on the subject, Mosnier and colleagues observed platelet-derived TAFI as a 50-kDa species on SDS-PAGE (*95*). In the present study, purified platelet- and plasma-derived TAFI migrated at ~60 kDa on SDS-PAGE. Though the molecular weight of platelet- and plasma-derived TAFI cannot be assumed to be identical on the basis of migration on SDS-PAGE, the results presented here do indicate that any difference in size is not as significant as that reported previously. This discrepancy is likely a result of variations in the platelet isolation protocols used by the two groups. In contrast to the modified Mustard method used here (*100*), Mosnier *et al.* employed gel-filtration chromatography to isolate human platelets from plasma. Although it is an effective technique in maintaining platelets in a resting state and removing contaminating plasma proteins from the final suspension, platelet gel filtration requires buffers to contain a concentration of bovine serum albumin (BSA) that we observed to interfere with the mobility of TAFI on SDS-PAGE. Indeed, the platelet isolation protocol cited by Mosnier and coworkers includes 0.2% BSA in the Tyrode's buffer used throughout the gel filtration process (*101*). When we included BSA at this concentration in the CFTB used for initial platelet isolation, substantial variation in the electrophoretic mobility of platelet-derived TAFI was observed. This variation took the form of substantial gel-to-gel differences in the observed mobility of platelet TAFI as well as distorted bands. Because BSA migrates as a 67-kDa species on SDS-PAGE, if present in sufficient quantities it directly interferes with the mobility of proteins of similar molecular weight. In the case of the western blots used for initial detection of TAFI in platelet releasates

(e.g. Figure 3-1), the mobility of the immunoreactive material (i.e. TAFI) was observed to increase with increasing concentrations of BSA included in the isolation buffers (Figure 4-1).

To confirm that a gel filtration procedure did not enable detection of a different platelet-derived TAFI isoform than the one detected here, we also isolated human platelets by gel filtration and the releasates were subjected to immunoprecipitation with an anti-TAFI antibody. Likewise, releasates of platelets isolated by the modified Mustard method were also subjected to TAFI immunoprecipitation in the presence of 0.2% BSA. In both cases, the immunoprecipitated material migrated at 60 kDa on SDS-PAGE (data not shown). This result suggests that platelet-derived TAFI obtained from either gel-filtered platelets or platelets isolated by centrifugation are identical species. Again, this observation indicates that platelet-derived TAFI from gel-filtered platelets is likely not a 50-kDa species, but was observed as such due to electrophoretic mobility interference by BSA.

The observation of TAFI in platelet releasates as a 60-kDa species on SDS-PAGE necessitated confirmation that the detected material was indeed platelet-derived TAFI and not contaminating plasma-derived TAFI from the platelet donor packs. Because neither TAFI nor VWF was detected by western blot in the final suspensions following platelet isolation, the isolation procedure was considered to be effective in the clearance of contaminating plasma proteins. Additionally, the absence of the platelet-specific protein PF4 in all of the wash steps as well as the final platelet suspension suggests that the isolation procedure was also effective in the maintenance of platelets in a resting state. Taken together, these results indicate that the TAFI detected in platelet releasates here and purified thereafter was *bona fide* platelet-derived TAFI and not contaminating plasma-derived TAFI.

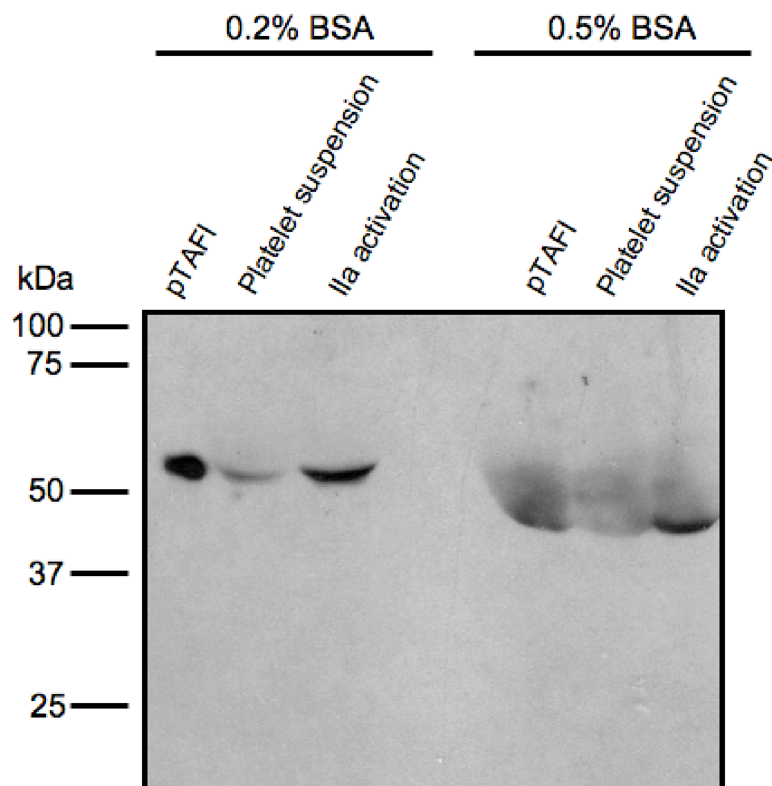

Figure 4-1. Effect of variable BSA concentration on the electrophoretic mobility of TAFI.

The effect of increasing BSA concentrations on the electrophoretic mobility of platelet- and plasma-derived TAFI was analyzed by western blot. Isolated human platelets in HTB containing 0.2% and 0.5% (w/v) BSA were activated with 5 nM thrombin for 30 minutes at room temperature. Samples of the platelet suspension prior to and following activation were subjected to 10% SDS-PAGE under reducing conditions followed by western blot analysis using a TAFI-specific polyclonal antibody raised in sheep. The western blot was subsequently incubated with rabbit anti-sheep IgG and immunoreactive bands were visualized by chemiluminescence. Samples of plasma-derived TAFI in HTB containing 0.2% and 0.5% (w/v) BSA were included on the gel as positive controls (pTAFI). Positions of the molecular mass markers are shown to the *left* of the blot.

4.2 The Origin of Platelet-Derived TAFI

Based on the observation of TAFI mRNA in intermediate and late stage megakaryocytic cell lines, it was hypothesized by Mosnier *et al.* that platelet-derived TAFI originates in megakaryocytes (*95*). This assertion was strengthened by the apparent difference in molecular weight between platelet- and plasma-derived TAFI that was subsequently attributed to variation in glycosylation patterns. The observation here that platelet-derived TAFI is scarcely distinguishable from plasma TAFI in apparent molecular weight could be interpreted as evidence against a megakaryocyte source for platelet TAFI. Notably, however, data from our laboratory (obtained by Joellen Lin) indicate that megakaryocytic cell lines both express TAFI mRNA and secrete TAFI protein, in turn suggesting TAFI might be actively sequestered in platelet granules following endogenous mRNA translation. Additionally, this megakaryocyte-derived TAFI was observed to be a 60-kDa species on SDS-PAGE. Furthermore, the cDNA sequence of megakaryocyte-derived TAFI was observed to be identical to that expressed in the liver, thereby ruling out alternative splicing and also suggesting that the protein sequence of this megakaryocyte-derived TAFI is identical to that of plasma-derived TAFI.

Despite its apparent expression in megakaryocytes *in vitro*, uptake of TAFI from plasma into megakaryocytes or platelets *in vivo* cannot be ruled out. Other platelet-derived proteins like fibrinogen, VWF, and factor V are known to be endocytosed from plasma by megakaryocytes prior to packing into α-granules (*102*). Interestingly, factor V is truncated to a physically and functionally distinct cofactor following its uptake from plasma (*30*, *31*). Based on the mobility of both the platelet-derived TAFI zymogen and the active enzyme on SDS-PAGE, if TAFI is in fact endocytosed from plasma into megakaryocytes *in vivo,* it does not appear to undergo significant modifications prior to storage in secretory granules.

Analysis of purified platelet-derived TAFI obtained in this study by mass spectrometry could determine its molecular weight and the character of *N*-linked glycans, thereby providing insight into its source. Since platelet-derived TAFI potentially originates in megakaryocytes, the character of *N*-linked glycans could differ from that of plasma-derived TAFI. Indeed, recombinant TAFI from mammalian and insect cell lines differs from plasma-derived TAFI with respect to the size and composition of *N*-linked glycans (*56*, *103*). Despite this variation though, in the case of mammalian recombinant TAFI, extensive comparisons with TAFI purified from plasma indicate these two proteins are similar in terms of activation by thrombin-thrombomodulin and other activators, thermal stability, inhibition of fibrinolysis and other enzymatic and functional properties (*56*). While deglycosylated platelet- and plasma-derived TAFI have similar mobility on SDS-PAGE, identical function cannot necessarily be inferred from this result. It has been suggested that specific *N*-linked glycans could influence TAFI functionality, on the basis of mutagenesis of individual glutamine residues that are the sites of *N*-linked glycosylation (*104*), or might participate in stabilization of the dynamic flap region in the TAFI structure discussed in detail in Section 1.4 (*67*, *105*). Thus, if platelet-derived TAFI originates in megakaryocytes, differences in functionality due to subtle differences in the nature of the glycans are a possibility despite the similar electrophoretic mobility of platelet- and plasma-derived TAFI.

As an alternative means to determine its origin, platelet-derived TAFI could be characterized in patients who have received bone marrow transplants. In this case, platelet-derived TAFI would reflect the genotype of the donor, not the recipient, if platelet-derived TAFI is expressed in the megakaryocyte and if the genotypes are different between the donor and recipient. There are two common single nucleotide polymorphisms that result in amino acid substitutions that could be detected by mass spectrometry (Thr/Ala147 and Thr/Ile 325).

Alternatively, the 2-fold increased stability of the TAFIa variant bearing the Ile at position 325 could be exploited in this regard.

4.3 Localization and Secretion of Platelet-Derived TAFI

The detection of TAFI in the releasates of isolated thrombin-stimulated platelets is in agreement with the previously reported localization of TAFI in the secretory granules of human platelets. It is likely that platelet-derived TAFI is localized in the α-granules of platelets along with the many other coagulation and fibrinolysis factors contained there (*102*), although this concept has yet to be directly tested. Indeed, the secretion of TAFI upon platelet activation is coincident with the release of other α-granular proteins like VWF and PF4 (Figure 3-2). Alternative localization is a possibility, though dense granules tend to contain ions, nucleotides, and transmitters as opposed to proteins (*102*). To confirm the presence of TAFI in platelet α-granules, epifluorescence or electron microscopy should be employed to observe the co-localization of TAFI with *bona fide* α-granular proteins by immunofluorescence or immunogold staining, respectively.

Experiments analyzing the temporal release of TAFI indicate that maximal secretion occurs within 25 minutes of stimulation by thrombin (Figure 3-3). Hence, thrombus formation in the thrombolysis assay was allowed to proceed for 30 minutes in order to facilitate a maximal TAFI secretion and clot retraction response. It was expected that TAFI would be secreted rapidly in this scenario (i.e. within the first 5 minutes), as granule secretion is known to occur immediately after platelets are activated *in vivo* (*102*). That TAFI was released gradually in the temporal secretion analysis is likely the result of the experimental design, as a high concentration of platelets (1×10^{10} platelets/mL) was activated with a relatively small amount of thrombin (5 nM) at ambient temperature without mixing to closely parallel the design of the thrombolysis assay. When physiological concentrations ($\sim 3 \times 10^{8}$ platelets/mL) were activated by thrombin at

37˚C with gentle agitation, a substantially more rapid activation response was observed. Indeed, when platelet degranulation was induced in the latter scenario, TAFI could be detected in the platelet medium within 1 minute of the characteristic aggregation response indicative of platelet activation (data not shown). Still, the qualitative observation of increasing amounts of TAFI appearing in the medium of thrombin-stimulated platelets over the activation time course provided further evidence that TAFI is indeed localized in the secretory granules of platelets. Furthermore, this gradual secretion of TAFI from platelets confirmed that the protein detected by western blot was not contaminating plasma-derived TAFI that remained in the medium following platelet isolation.

There is a growing body of evidence that the contents of platelet α-granules are heterogeneous. Recent observations, for example, suggest that angiogenic regulators are subject to differential packing within α-granules (*106*). Whether coagulation and fibrinolytic factors are subject to this type of sorting is unknown, but the potential for localization of TAFI in specifically procoagulant or antifibrinolytic granules is an interesting scenario that should be investigated.

4.4 The Antifibrinolytic Effect of Platelet-Derived TAFI

In their preliminary report, Mosnier and colleagues determined that activated platelet-derived TAFI is able to cleave both a small substrate (hippuryl-arginine) as well as a larger one (minimally degraded fibrin). Here, we have shown for the first time that activated platelet-derived TAFI is able to attenuate the fibrinolytic cascade *in vitro* using a novel thrombus lysis assay. This observation validates the hypothesis that in the context of platelet-rich thrombus formation, platelets secrete TAFI at levels sufficient to inhibit thrombolysis. Additionally, we have shown that activated TAFI secreted from platelets can combine with a concentration of TAFIa already present in plasma and contribute to enhanced inhibition of fibrinolysis. This may have

significance *in vivo,* as TAFI secreted by platelets could augment the plasma pool of TAFI, in turn resulting in a local increase in TAFI concentration at the site of platelet-rich thrombus formation. Since the concentration of TAFI in plasma is near or below the K_m for its activation by thrombin, thrombin-thrombomodulin, or plasmin (*61*), secretion of TAFI from platelets could impact the amount of activated TAFI in this milieu and influence clot lysis time.

When increasing concentrations of recombinant TAFI were included in the fluid surrounding retracted platelet-rich thrombi, a maximal inhibition of thrombus lysis was observed at supplementary rTAFI concentrations above 10 nM. This is consistent with the observation that the antifibrinolytic effect of TAFIa reaches a plateau (*82*, *83*). Because platelet-derived TAFIa appears to combine with a concentration of TAFIa already present to achieve maximal inhibition, a similar scenario might occur *in vivo*. That is, in the presence of activated platelets, maximal inhibition of fibrinolysis could be achieved more readily than in their absence due to the putative ability of platelet-derived TAFI to augment the total plasma pool.

The antifibrinolytic potency of platelet-derived TAFI relative to its plasma-derived counterpart remains unknown. To determine if differential potency exists between the two isoforms, the antifibrinolytic potential of purified platelet-derived TAFI should be determined in a typical *in vitro* clot lysis assay along with an assessment of its activation kinetics and thermal stability. Platelet-derived TAFI purified from outdated platelet concentrates was observed to be completely activatable by thrombin/thrombomodulin (Figure 3-8), suggesting that its stability and activatability is not compromised in the time between collection and eventual platelet isolation and activation (typically 5-7 days post-collection date). This observation suggests that an accurate assessment of the antifibrinolytic effect, thermal stability, activation kinetics, and other enzymatic properties could be obtained from platelet-derived TAFI purified in the manner outlined in this study.

4.5 The Thrombolysis Assay: A Novel Approach to the Inhibition of Fibrinolysis

The formation of fluorescent platelet-rich thrombi was adapted from protocols described by Mutch and coworkers (*107*, *108*). Instead of bathing whole-blood thrombi in normal human plasma as these investigators did, a scenario was designed in which retracted platelet-rich thrombi were formed in the absence of any plasma-derived TAFI. This was accomplished by generating thrombi with washed, isolated platelets free of contaminating plasma proteins and plasma that was immunodepleted of TAFI. Additionally, because thrombi were not transferred to a different plasma bath following their formation, all proteins secreted from platelets were retained in the thrombus and the surrounding fluid. These key modifications to the methods of Mutch and colleagues allowed for the direct assessment of the ability of platelet-derived TAFI to attenuate the fibrinolytic cascade. As discussed above, the antifibrinolytic effect of platelet-derived TAFI could have been assessed using the purified protein in *in vitro* clot lysis assays, however the novel system employed here resembles the *in vivo* scenario in which TAFI is secreted directly from platelets following their activation. Moreover, platelet-containing thrombi are more representative of arterial thrombi formed *in vivo* than clots consisting of fibrin alone that are generated in typical clot lysis assays (*5*).

Although the thrombolysis assay is a better model of certain *in vivo* scenarios than clots consisting of fibrin alone, the inclusion of platelets in the fluorescent thrombi necessitated thorough optimization of the initial thrombus formation method. In particular, the volatility of resting platelets in the plasma mixture and the adhesive nature of activated platelets contributed to substantial variability in thrombus structure. Prolonged incubation of platelets in the plasma mixture would periodically result in premature activation, in turn resulting in inconsistent thrombus structure. Additionally, thrombi would occasionally adhere to the sides of the tubes in which they were formed, resulting in delayed clot retraction and substantial variation in thrombus

size and density. Early in the development of the assay it was observed that lysis rates could vary significantly depending on the physical structure of the thrombus, consistent with the work of Collet and coworkers (*47*). Hence, it was necessary to include 0.05% Tween in the initial platelet/plasma mixture to generate clots of consistent character and structure.

Lysis rates of fluorescent thrombi were determined to be dependent upon the initial amount of t-PA added to the system following thrombus formation and clot retraction. Notably however, lysis rates induced by t-PA exhibited substantial interassay variability, presumably due to inconsistency in thrombus structure and secretion of platelet-derived regulatory factors. This variation necessitated a pre-optimization step prior to each thrombolysis assay, in which a t-PA concentration that elicited a modest lysis rate was determined. This step proved essential in the setup of the assay, as low concentrations of t-PA did not induce lysis whereas high concentrations of t-PA were observed to overcome a TAFIa-dependent effect on lysis rate (data not shown). As discussed in Sections 1.1.2 and 1.3, absence of lysis in the presence of t-PA is likely the result of platelet secretion of PAI-1 and α_2-antiplasmin (*108*, *96*), as well as reduced availability of t-PA binding sites in platelet-rich thrombi (*47*). Variable contribution of these factors likely accounts for the different lysis rates of platelet-rich thrombi observed at identical t-PA concentrations used in Figures 3-4, 3-5, 3-6, and 3-7.

As discussed in Section 1.4, TAFI can be activated *in vitro* by thrombin, plasmin, and most efficiently by the thrombin-thrombomodulin complex. Under the conditions of our thrombolysis assay, the activator of TAFI is likely to be thrombin complexed with added thrombomodulin. Nevertheless, in the context of our assay, as well as *in vivo*, other potential routes of TAFI activation can be considered. Aside from its exogenous addition to the fluid surrounding retracted thrombi in specific experiments, there is evidence that thrombomodulin is expressed in platelets (*109*, *110*). Thus, in the context of thrombus formation in the thrombolysis

assay, it might be expected that thrombin generated endogenously through prothrombin activation or exogenous thrombin used to initiate clot formation would also complex with platelet-surface thrombomodulin to activate any TAFI present. Alternatively, endogenous activation of prothrombin by the intrinsic pathway could also activate TAFI at levels sufficient to attenuate fibrinolysis *in vitro* (*61*). In these scenarios, activated TAFI in the fluid surrounding retracted thrombi would inhibit fibrinolysis following the addition of t-PA in the absence of supplementary thrombomodulin. Though both of these activation scenarios are a possibility in our thrombolysis assay, substantial activation of TAFI in the absence of exogenous thrombomodulin is unlikely, as the inclusion of PTCI did not increase lysis rates above those achieved by the addition of t-PA alone (Tables 3-2 and 3-3). Thus, we conclude that significant TAFI activation does not occur prior to addition of exogenous thrombomodulin and t-PA under our conditions.

It should be noted that the concentration of platelets used in the thrombolysis assay (1×10^{10} platelets/mL) was about 20-fold higher than the typical platelet concentration in human plasma ($\sim 3 \times 10^{8}$ platelets/mL). Nevertheless, previous studies by Robbie *et al.* observed that generation of thrombi *in vitro* containing physiological levels of PAI-1 required whole blood to be augmented with platelets at concentrations identical to those used in our thrombolysis assay (*111*). Hence, to generate thrombi *in vitro* that resemble thrombi formed *in vivo* with respect to content of platelet-derived inhibitors of fibrinolysis, plasma augmented with platelets at the concentrations used in our assay were considered appropriate.

4.6 Potential Physiological Relevance of Platelet-Derived TAFI

The apparent similarity in both size and available concentration of plasma and platelet TAFI raises a critical question: does platelet-derived TAFI serve a distinct or complementary physiological role? A thorough discussion of the possible implications of platelet-derived TAFI is necessary so as not to underestimate its potential influence on fibrinolysis in certain physiological

or pathophysiological contexts. As such, the ratio of plasma- and platelet-derived TAFI as well as the spatiotemporal context of platelet activation and secretion during thrombus formation must be considered.

In typical human platelet-rich plasma, platelet-derived TAFI constitutes about 0.1% of the total plasma TAFI pool (*95*). When platelets aggregate during thrombus formation, however, activated platelets form close, stable contacts with each other (*112*). Given this dense arrangement of activated platelets within the thrombus, the associated secretion of platelet granules could increase the local TAFI concentration at the site of injury. Indeed, enrichment of granular proteins in vascular lesions has been observed, presumably due to limited diffusion and accumulation between the gaps of activated platelets (*46*). Quantification experiments from our study suggest the intraplatelet concentration of activatable TAFI that is secreted following stimulation by thrombin is about 40 nM. Since nanomolar increases in TAFI activity have been observed to prolong clot lysis time (*71*), this auxiliary source of TAFI may be significant in the context of vessel damage and platelet plug formation.

The distribution of platelets at sites of vascular injury is vastly different from their concentration in normal human plasma (*5*). That is, platelet abundance in human thrombi is dependent on vascular localization and severity of injury. Arterial thrombi are known to contain large aggregates of activated platelets and the number of individual platelets in a thrombus is correlated with the size of the damaged region and shear stress. Thus, the quantity of platelet-derived protein secreted locally at an injured site likely varies widely depending on the context of vessel damage. Still, the observation here that activated platelets incorporated into thrombi secrete TAFI at levels sufficient to attenuate fibrinolysis *in vitro* suggests that platelet-derived TAFI could influence fibrinolysis *in vivo*.

Since platelets activate and aggregate directly at sites of vascular damage, the spatial context of TAFI secretion could also be significant. When platelets activate in the vicinity of the endothelium during platelet plug formation, it is possible that TAFI secreted from these platelets is in close proximity with the endothelial cell surface cofactor thrombomodulin. As discussed in Section 1.4, TAFI is activated most efficiently with thrombin in complex with thrombomodulin, and large amounts of active thrombin are known to accumulate in and around the thrombus (*113*). Therefore, a zone of increased TAFI concentration could be generated at the periphery of a developing thrombus, in turn resulting in enhanced TAFI activation facilitated by thrombin and thrombomodulin at the thrombus-endothelium interface. This is an intriguing possibility, particularly in light of observation that thrombin-stimulated platelets enhance plasminogen activation in a carboxyl-terminal lysine-dependent manner (*114*). Moreover, treatment of activated platelets with carboxypeptidase B reduced the affinity of plasminogen binding. Thus, activated platelet-derived TAFI in close proximity with the developing thrombus could serve a regulatory role in platelet- and fibrin-dependent plasminogen activation.

The process of clot retraction extrudes a bulk of the fluid phase plasma from the developing thrombus (*35*). Still, as discussed in Section 1.3, platelet-derived proteins are thought to accumulate in the protected spaces between activated platelets. Presumably then, some platelet-derived TAFI is retained in these gaps along with other granular proteins and soluble agonists. Furthermore, limited diffusion of plasma-derived TAFI into the thrombus could result in the enrichment of exclusively platelet-derived TAFI in these protected spaces. Thus, a conceivable scenario is that platelet-derived TAFI prevents premature lysis internally, from within the thrombus, while plasma-derived TAFI exerts its antifibrinolytic effect externally, from the vascular milieu. Indeed, retracted platelet-rich clots are resistant to fibrinolysis (*18*), however a thorough analysis of the basis of this resistance has not been performed. Activated platelet-

derived TAFI within the thrombus could play a regulatory role in this regard, in addition to the reduced accessibility of t-PA binding sites in the fibrin network following clot retraction (discussed in detail in Section 1.3). Aside from the potential for platelet-derived TAFI to inhibit fibrinolysis from within the thrombus, platelet-rich thrombi may also contain unidentified substrates for activated platelet-derived TAFI. Indeed, observation that TAFI can modulate vascular inflammation through cleavage of substrates other than fibrin is evidence of its multifunctionality.

The unique context of platelet-derived TAFI may also influence its function, even in the absence of substantive functional differences between the forms of TAFI themselves. Platelets secrete molecules from their granules that may modify the function of TAFI within the platelet-rich thrombus. Glycosaminoglycans are present on the platelet surface (*115*) and glycosaminoglycans such as heparin have been shown to influence both TAFI activation and TAFIa stability (*64*). Dense granules contain another polyanionic substance, polyphosphate (*116*), although the effects of this compound on TAFI function have yet to be investigated.

4.7 Summary and Conclusion

In the present study, we first confirmed previous reports that TAFI is secreted from human platelets following stimulation by thrombin. Secondly, using a novel thrombus lysis assay, we observed that platelet-derived TAFI is able to attenuate platelet-rich thrombolysis *in vitro* and contribute to enhanced TAFI-dependent inhibition of lysis in human plasma. Finally, we developed a purification protocol for platelet-derived TAFI that will enable a thorough characterization of the protein with respect to molecular weight, glycosylation patterns, thermal stability, and enzymatic properties. These analyses will provide insight into the origin and function of platelet-derived TAFI, which remain unclear.

By initiating clot retraction and secreting inhibitors like PAI-1 and α_2-antiplasmin, platelets prevent premature lysis of the haemostatic plug. The observation that TAFI is secreted from platelets adds another regulatory factor to the antifibrinolytic 'toolkit' contained in platelets that is released upon activation at regions of vascular damage. Although the concentration of TAFI in platelets is 1000-fold below the total plasma pool of TAFI, this does not necessarily preclude a distinct physiological role. It is clear that the dynamics of platelet activation, aggregation, and thrombus formation could result in platelet-derived TAFI concentrations at an injury site or regions of pathological thrombosis that have a significant impact on the fibrinolytic cascade. Like many other aspects of haemostasis, the contribution of individual regulatory proteins is often dependent on the context of vascular damage. The pool of platelet-derived TAFI is no exception, where a transient burst of TAFI released from human platelets at a site of thrombosis might have a significant effect on the fibrinolytic cascade.

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