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THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR (TAFI) IN DIFFERENT HEMORRHAGIC AND THROMBOTIC CONDITIONS

by

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Stockholm 2003

In the mother's body, a fetus understands the whole Universe.
After being born, the baby forgets all it once understood.

Jewish proverb

I take the liberty to add:

All the efforts we humans make are just attempts to
regain this understanding ... and find the truth.

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To two persons named PAVLE

My father, who taught me what is valuable in life
My son, who makes my life valuable

Thrombin Activatable Fibrinolysis Inhibitor (TAFI) in Different Hemorrhagic and Thrombotic Conditions

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Until recently the coagulation and fibrinolytic systems were usually considered as separate entities. Accounts of a fibrinolysis inhibitor that is generated by thrombin shed more light on this field and changed the concept by giving thrombin, the pivotal enzyme in hemostasis, another important role in the down-regulation of fibrinolysis. Thrombin Activatable Fibrinolysis Inhibitor (TAFI), also known as procarboxypeptidase B and procarboxypeptidase U, is a relatively recently described inhibitor of fibrinolysis that can be converted into its activated form which is a carboxypeptidase B-like enzyme, by the thrombin/thrombomodulin complex. In this study, the precursor of TAFI is denominated pro-TAFI (instead of TAFI or procarboxypeptidase U). Since this "inhibitor" of fibrinolysis is functional only in its active form, we reserve the term TAFI for the active form of the enzyme and suggest the term TAFIi for the inactive form. Although the role of TAFI in pathology is not completely known, it is reasonable to expect that the level of TAFI is altered in various thrombotic and hemorrhagic diseases.

Objective: The aim of the study is to investigate possible changes in different forms of TAFI and their influence on fibrinolysis in different clinical conditions associated with hypocoagulable states and decreased thrombin generation (e.g. hemophilia A and von Willebrand disease (VWD)) as well as in hypercoagulable states such as APC resistance and conditions associated with complicated pregnancy and diabetes mellitus.

Methods: Different forms of TAFI were determined in patients with APC resistance due to FV Leiden mutation, in patients with hemophilia A and VWD, in patients with diabetes mellitus type I and in women with preeclampsia. Data were obtained on total thrombin activatable fibrinolysis inhibitor antigen (including pro-TAFI, its active form TAFI and its inactive form TAFIi), pro-TAFI activity, TAFI/TAFIi antigen, overall hemostatic potential (OHP) and overall fibrinolytic potential (OFP) in plasma, and clot lysis time (CLT) derived from this test. OHP and TAFI-dependent fibrinolysis were also determined *in vitro* in variously deficient plasmas after addition of different concentrations of rVIIa (NovoSeven).

Results: A decrease in pro-TAFI, accompanied by no change in total TAFI antigen, was found in APC resistant patients.

A significant decrease in TAFI antigen and impaired fibrinolysis that was not TAFI dependent were observed in women with preeclampsia compared to those with a normal pregnancy.

Pro-TAFI levels did not differ between diabetic patients with or without microvascular complications and controls. TAFI antigen was lower (though not significantly so) in both groups of diabetic patients, while OHP was increased (significantly in the group with microvascular complications). Neither fibrinolysis itself nor TAFI-dependent fibrinolysis differed between the two groups of diabetic patients compared to controls.

Pro-TAFI was decreased in hemophilia A, hemophilia B and VWD patients, together with no changes in total TAFI antigen. An increase in TAFI/TAFIi antigen was found in both hemophilia and VWD patients compared to controls.

rFVIIa in a concentration of 2.4μg/mL improved the overall hemostasis in FV, FVIII and FIX deficient plasmas. Not even very high concentrations of rFVIIa (9.6μg/mL) induced hypercoagulation in deficient plasmas or in normal pooled plasma (NPP). It seems that fibrinolysis is also regulated by factors other than TAFI but higher concentrations of rVIIa do, at least partly, induce (most probably through increased thrombin generation) a TAFI-dependent down-regulation of fibrinolysis in FVIII and FIX deficient plasmas.

Conclusions: TAFI contributes to an impairment of fibrinolysis in patients with APC resistance. TAFI does not induce a further down-regulation of initially impaired fibrinolysis in preeclampsia, most probably because it is reduced as a result of renal and hepatic disturbances. In patients with type I diabetes mellitus and microvascular complications, TAFI does not induce an impairment of fibrinolysis despite the presence of increased overall coagulation and hemostasis. The transformation of pro-TAFI into TAFI and TAFIi in hemophilia and VWD is most probably induced by plasmin and could partly counterbalance the up-regulation of fibrinolysis in these conditions. rVIIa improves overall hemostasis and fibrinolysis in hemophilia patients and in FVIII and FIX deficient plasma, but the improved down-regulation in overall fibrinolysis is only partly TAFI-dependent. The OHP assay seems to be a handy and inexpensive tool not only for the determination of overall hemostasis, coagulation and fibrinolysis but also for indirect estimation of TAFI-dependent fibrinolysis. TAFI obviously has a role as a link between coagulation and fibrinolysis but since it is activated by both thrombin and plasmin and is also inactivated by plasmin, both enzymes could regulate TAFI's role and influence the TAFI-dependent regulation of fibrinolysis. The definitive role of TAFI therefore remains to be proven.

This thesis is based on the following papers, which are referred to in the text by their Roman numerals: (I-VI)

I Thrombin activatable fibrinolysis inhibitor (TAFI) antigen and TAFI activity in patients with APC resistance caused by factor V Leiden mutation.
Antovic JP, Blombäck M.
Thromb Res. 2002; 106: 59-62.

II Does thrombin activatable fibrinolysis inhibitor (TAFI) contribute to impairment of fibrinolysis in patients with preeclampsia and/or intrauterine fetal growth retardation?
Antovic JP, Rafik Hamad R, Antovic A, Blombäck M, Bremme K.
Thromb Haemost. 2002; 88:644-7.

III Thrombin activatable fibrinolysis inhibitor (TAFI) and hemostatic changes in patients with type I diabetes mellitus with and without microvascular complications.
Antovic JP, Yngen M, Östenson CG, Antovic A, Wallen NH, Jörneskog G, Blombäck M.
Blood Coag Fibrinol. 2003 in press.

IV Total thrombin activatable fibrinolysis inhibitor (TAFI) antigen and pro-TAFI in patients with hemophilia A.
Antovic J, Schulman S, Eelde A, Blombäck M.
Haemophilia 2001; 7: 557-60.

V Does enzyme other than thrombin contribute to unexpected changes in the levels of the different forms of thrombin activatable fibrinolysis inhibitor (TAFI) in patients with hemophilia A, B and Von Willebrand disease?
Antovic JP, Schulman S, An SSA, Greenfield RS, Blombäck M.
J Thromb Haemost. 2003 in revision.

VI Does recombinant factor VIIa apart from overall hemostasis regulate TAFI dependent fibrinolysis? – *in vitro* analysis using overall hemostasis potential (OHP) assay.
Antovic JP, Antovic A.
Manuscript

CONTENTS

ABBREVIATIONS

ε-ACA	epsilon amino caproic acid
ALT	alanine amino transferase
APC	activated protein C
AST	aspartate amino transferase
AT	antithrombin
BMI	body mass index
BP	blood pressure
Ca^{++}	calcium ions
CLT	clot lysis time
D-D	D-dimer
DIC	disseminated intravascular coagulation
DM	diabetes mellitus
DVT	deep vein thrombosis
ELISA	enzyme-linked immunosorbent assay
Fb	fibrin
Fbg	fibrinogen
FII-XIII	coagulation factors II-XIII
FVL	factor V Leiden
HbA1c	hemoglobin A1c
HDL	high density lipoproteins
IUFGR	intarauterine fetal growth retardation
LDL	low density lipoproteins
NPP	normal pooled plasma
OCP	overall coagulation potential
OFP	overall fibrinolytic potential
OHP	overall hemostatic potential
PAI-1, 2	plasminogen activator inhibitors 1 and 2
PC	protein C
PE	pulmonary embolism
PI	plasmin inhibitor (alpha-2 antiplasmin)
PL	phospholipids
Pro-TAFI	pro-thrombin activatable fibrinolysis inhibitor (procarboxypeptidase B, proenzyme, zymogen)
PS	protein S
PTCI	potato tuber carboxypetidase inhibitor – specific inhibitor of TAFI
rVIIa	recombinant factor VIIa (NovoSeven)
TAFI	thrombin activatable fibrinolysis inhibitor (carboxypeptidase B, enzyme, active form)
TAFIi	inactive form of thrombin activatable fibrinolysis inhibitor (inactive enzyme)
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TM	thrombomodulin
t-PA	tissue plasminogen activator
VWF	von Willebrand factor
F(X)DP	fibrin degradation products

INTRODUCTION

Hemostasis is one of the physiological systems that contribute to the maintenance of homeostasis in the human organism. Its function is to maintain the normal fluidity of blood in the blood vessels on the one hand and to arrest bleeding after injury to the blood vessels on the other hand.

The components involved in normal hemostasis include blood vessels (primarily endothelium and subendothelium), blood cells (especially platelets) and systems for blood coagulation and fibrinolysis.

The key event in hemostasis is the formation of fibrin. Through the series of steps in which plasma zymogens of serine proteases are transformed into active enzymes, the coagulation system leads to formation of the thrombin enzyme that catalyzes the transformation of fibrinogen into fibrin. Fibrin, the final product of coagulation, is the main substrate for the fibrinolytic system, the role of which is to locate fibrin clots at the site of an injury and dissolve them.

Due to the properties of endothelium and the presence of inactive forms of proteins, coagulation is normally not activated in the blood stream. Injury to blood vessels and endothelial damage lead, however, to the exposure of tissue factor (TF) into the circulation. TF forms a complex with FVIIa and this complex, TF-FVIIa, initiates an extrinsic or TF pathway that activates FX to Xa either directly or via activation of FIX. The intrinsic pathway or contact activation pathway starts after activation of FXII by kallikrein. FXIIa activates FXI, which activates factor IX to FIXa. Together with FVIII as a cofactor on phospholipid surfaces, FIXa activates FX to FXa and the latter, with FVa as a cofactor on phospholipid surfaces, produces so-called prothrombinase, which converts prothrombin (FII) into thrombin (FIIa). Thrombin, the key enzyme in hemostasis, transforms fibrinogen to fibrin; besides activating FXIII to FXIIIa, it activates platelets, FV and FVIII, FXI to FXIa, as well as protein C to activated PC (APC). Apart from the APC system, there are several inhibitors of coagulation: tissue factor pathway inhibitor (TFPI) which inhibits the TF-FVIIa-FXa complex, antithrombin (AT) which inhibits thrombin as well as FIXa, Xa and XIa, and C1-inhibitor which inhibits FXIIa. The coagulation system is far more complex; initiation, propagation and a full burst of thrombin generation are necessary for its function and the most of reactions occur on the different cell surfaces. Negatively charged intact endothelium prevents platelets binding and activation. Thrombomodulin expressed on endothelial cells binds thrombin and has a role both in coagulation and fibrinolysis regulation. After injury platelets bind to exposed subendothelium, through WVF, fibrinogen and platelets glycoprotein interactions. Activated platelets secrete FVa which is a binding site for Xa, while FXIa also binds to platelets by other receptors. Activated monocytes express TF, and have receptors for factor Xa.

The formation of fibrin triggers the activation of the fibrinolytic system, which is also based on the conversion of zymogen to enzyme, with feedback activation and inhibition. The initial period of fibrin clot formation is accompanied by the release of plasminogen activators. Endothelial cells release tissue plasminogen activator (t-PA), which (like urokinase (u-PA)) activates plasminogen (especially plasminogen bound to fibrin) to plasmin. Plasmin lyses fibrin to the fibrin degradation products. Plasmin inhibitor (PI) (previously named alpha-2 antiplasmin) is an inhibitor of fibrinolysis, as are plasminogen activator inhibitors (PAI-1 and 2). A simplified scheme of blood coagulation and fibrinolysis is presented in Figure 1.

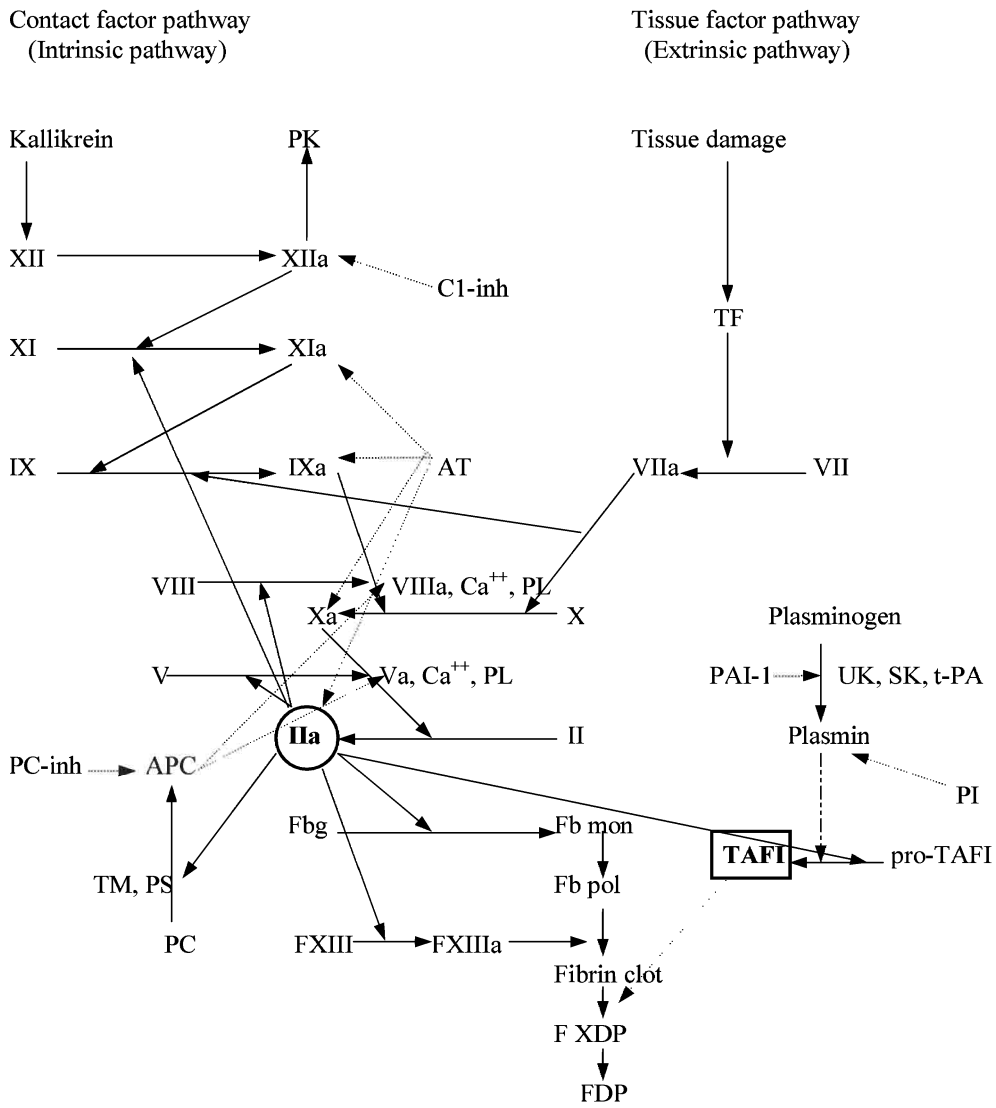

Figure 1. Simplified presentation of blood coagulation and fibrinolysis

→ Activation or stimulation

⋯→ Inhibition or degradation

– – – → Possible activation of pro-TAFI by plasmin

Abbreviations: TF – tissue factor, HK – high molecular weight kininogen, PK – prokallikrein, IIa – thrombin, Fbg – fibrinogen, Fb – fibrin, AT – antithrombin, PC – protein C, PS – protein S, TM – thrombomodulin, PC-inh – protein C inhibitor, C1-inh – C1 inhibitor, TFPI – tissue factor pathway inhibitor, TAFI – thrombin activatable fibrinolysis inhibitor, UK – urokinase, SK – streptokinase, PAI-1 – plasminogen activator inhibitor, PI (plasmin inhibitor, alpha-2 antiplasmin), FDP - fibrin degradation products, PL – phospholipids.

Until recently, coagulation and fibrinolysis were considered as separate entities, linked by fibrin (the final product of the coagulation system which serves as a substrate for the fibrinolytic system). Accounts of thrombin activatable fibrinolysis inhibitor (TAFI) shed more light on this field and presented another link between the two systems.

DISCOVERY OF TAFI, ITS STRUCTURE AND NOMENCLATURE

In 1989 Hendriks et al described a labile carboxypeptidase activity that interferes with carboxypeptidase N activity and is not present in blood but occurs after clotting. Campbell and Okada (1989) showed an increase in arginine carboxypeptidase activity during coagulation. Bajzar et al purified (1995) the same enzyme and showed that it could be activated by thrombin and is therefore capable of inhibiting fibrinolysis. Although the findings of these groups, as well as those of Eaton and al (1991), who described the same enzyme, have been considered to be novel, in a letter Kluft (2001) pointed out that in the late sixties (1968) Helle had described how calcium-dependent activation of coagulation induces a fibrinolysis-inhibiting effect which he named coagulation dependent inhibition (CDI). While he did not identify a specific protein and/or enzyme, his can be said to be the first description of a link between coagulation and fibrinolysis.

Different groups accordingly described the same enzyme during a short period of time and they named it differently.

It has been shown that the enzyme belongs to the metallocarboxypeptidase B family, which hydrolizes C-terminal peptide bonds, preferably with basic amino residues (Hendriks et al 1989a, b, Campbell and Okada 1989). It differs from carboxypeptidase N as regards stability, pH and substrate specificity. The instability of the enzyme at 37°C prompted Hendriks et al to name it carboxypeptidase U (unstable) (CPU).

Campbell and Okada (1989) used the name carboxypeptidase R (CPR), where R stands for arginine (the enzyme cleaves arginine residues). Eaton et al (1991) suggested the name plasma carboxypeptidase B (CPB) on account of the similarity with pancreatic carboxypeptidase B.

Bajzar et al (1995) showed that the enzyme functions as an inhibitor of fibrinolysis activated by thrombin and therefore named it thrombin activatable fibrinolysis inhibitor (TAFI).

Although it cannot be said that these names are wrong, they all have drawbacks. CPU is not appropriate because there may be other unstable carboxypeptidases. Since the enzyme also cleaves lysine residues, CPR is inadequate and although plasma CPB may seem acceptable, this name was used in earlier literature for CPN, which could cause confusion. The most exact nomenclature for classifying the enzyme is the numerical system: EC 3.4.17.3 but this is not convenient for everyday use.

The term TAFI, on the other hand, does seem appropriate because it combines activation of the enzyme and its function. While procarboxypeptidase is an adequate name for the proenzyme (zymogen), it does not seem appropriate to refer to the inactive form as TAFI (thrombin activatable fibrinolysis inhibitor) because the inhibitor could be present only in the active form (the current recommendation is TAFIa for the active and TAFIai for the inactive form).

Our suggested nomenclature for thrombin activatable fibrinolysis inhibitor (TAFI) corresponds with the widely accepted terminology for coagulation proenzymes and their active forms and provides a link between the enzyme's name and function. The precursor of TAFI *(zymogen)* can then be denominated pro-TAFI (instead of TAFI or procarboxypeptidase U). Since this inhibitor of fibrinolysis is functional only in its active form, there is no point in using the term TAFIa (carboxypeptidase U). Instead we suggest that the term TAFI is reserved for the enzyme's active form. The inactive form of TAFI is also present and we suggest the term TAFIi for it. We have used this nomenclature here, although we are aware that it does have disadvantages, primarily because plasmin as well as thrombin may be involved in the activation of the proenzyme. We believe that a final decision on the nomenclature of TAFI will be reached at a future meeting of the Scientific Standardization Committee (SSC) of the International Society of Thrombosis and Haemostasis (ISTH).

form	**E.C**	**enzyme**	**usual nomencl.**	**our nomencl.**
zymogen	-	procarboxypeptidase U, R, B	TAFI	pro-TAFI
active form	3.4.17.3	carboxypeptidase U, R, B	TAFIa	TAFI
inactive form	-	inactive carboxypeptidase U, R, B	TAFIai	TAFIi

Table 1. Different forms of TAFI and their nomenclature

SYNTHESIS, CHARACTERISATION AND PURIFICATION OF TAFI

Pro-TAFI is a glycoprotein synthesized in the liver as a prepropeptide consisting of 423 amino acids with a molecular weight of 55 kD (Eaton et al 1991). Its plasma concentration has been established as being 4-15 μg/mL (Bajzar et al 1996a, Mosnier et al 1998) and it circulates bound to plasminogen (Wang et al 1994). Pro-TAFI has recently also been identified in platelets at a concentration of about 50ng/1x10^9 platelets, while m-RNA analysis indicates that platelets' pro-TAFI is synthesised in the megakaryocytes (Mosnier et al 2003).

The presence of TAFI in mouse, pig, guinea-pig, rat and dog has been described (Schatteman et al 1999a, Marx et al 2000a, Kato et al 2000). Large inter-species differences in TAFI activity have been found, ranging from 20% in mouse to 500% in pig compared to humans, while TAFI's main characteristics (including instability) are the same in all the investigated species.

Recombinant pro-TAFI has been expressed in insect and baby hamster kidney cells (Boffa et al 1998, Zhao et al 1998) and it can be activated with the thrombin/thrombomodulin complex to TAFI with characteristics that are similar to those of the natural variant of TAFI.

Purification of pro-TAFI is based on its high affinity to plasminogen. Plasma is applied to a lysine-Sepharose column, which removes plasminogen and proteins bound to plasminogen. This is followed by affinity chromatography on plasminogen-Sepharose and consequent elution of pro-TAFI by epsilon amino capronic acid (εACA) (Eaton et al 1991, Bajzar et al 1995). Ion exchange chromatography is also frequently used (Broze and Higuchi 1996, Marx et al 2000b) and so, to a lesser extent, is immunoaffinity chromatography with antibodies against pro-TAFI (Marx et al 2000b). Moreover, purification of recombinant pro-TAFI could be based on cation exchange at pH 6.8 (Zhao et al 1998).

GENOMIC ORGANIZATION AND TAFI POLYMORPHISM

The human pro-TAFI cDNA was isolated and characterized in 1991 (Eaton et al) and the pro-TAFI gene was characterized as having 48 kb of genomic DNA and consisting of 11 exons (Boffa et al 1999), and mapped to chromosome 13q14.11 (Tsai and Drayna 1992, Vanhoof et al 1996). Mouse and rat cDNA sequences have also been isolated and characterized (Sato et al 2000, Kato et al 2000). Characterization of the human pro-TAFI promoter showed a lack of common TATA sequences (Boffa et al 1999).

Expression of TAFI has been postulated to be influenced by an inflammation mechanism. Stimulation by LPS injection was followed by increased m-RNA expression of pro-TAFI (Sato et al 2000) and a correlation has been shown between pro-TAFI and C-reactive protein in humans (Silveira et al 2001). However, this was not confirmed in a recent study and furthermore, IL-1 and 6 suppress the expression of pro-TAFI m-RNA in HepG2 cells (Boffa et al 2003).

A pro-TAFI deficiency has not yet been described in humans, which may indicate that it is either of no significance or would be incompatible with life. Knock-out mice for the pro-TAFI gene were developed; no embryonic lethality was observed, while deficient mice developed normally, reached adulthood and were fertile with normal pregnancies. Pro-TAFI deficiency did not lead to an increased bleeding tendency, neither did it influence a thrombosis model (Nagashima et al 2002 a, b).

Several forms of the pro-TAFI gene have been described. Alanine-threonine substitution on position 147 does not lead to significant changes in TAFI's function (Zhao et al 1998), neither does it increase the risk of deep vein thrombosis in factor V Leiden carriers (Morange et al 2001).

Seven examples of polymorphism (5 in the promoter: C-2599G, -2345 2G/1G, A-1690G, G-1102T and G-438A, and 2 in the 3' region: C+1542G and T+1583A) have been found to be associated with the level of TAFI antigen and individually contribute to a large fraction of the TAFI antigen level (Henry et al 2001). Four new forms associated with the 5' untranslated region have also been described and likewise associated with different TAFI levels (-152A/G, -530C/T, -1053T/C and -1925T/C) (Franco et al 2001).

Another new form (-1040C/T) in the coding region of TAFI results in threonine-isoleucine substitution (Brouwers et al 2001), which leads to increased antifibrinolytic activity (Schneider et al 2002) without adding to the risk of myocardial infarction (Morange et al 2002).

The roles of different forms of TAFI and their influence on the level of TAFI, and especially on clinical conditions, have not yet been established for certain.

ACTIVATION OF PRO-TAFI

Pro-TAFI is activated by trypsin, plasmin and thrombin (Eaton et al 1991, Mao et al 1999) after cleavage at Arg92 to yield 15 kD activation peptide and 35 kD activated enzyme TAFI. Thrombin is a poor activator of pro-TAFI, with K_m of 2.14 μmol/L and k_{cat} of $0.0021s^{-1}$. Thrombomodulin enhances the thrombin-dependent activation of pro-TAFI 1250-fold, primarily through the increase in k_{cat} (Bajzar et al 1996c). Release of the activation peptide uncovers the binding site of Arg143 of TAFI and enables its binding to the C-terminal carboxyl group of the substrate (Bouma et al 2001).

Plasmin activates pro-TAFI eight times more efficiently than thrombin, with K_m of 55 nmol/L and k_{cat} of $0.00044s^{-1}$ (Mao et al 1999). But even this is much weaker compared to the thrombin/thrombomodulin complex, indicating that this complex should be the main physiological activator of pro-TAFI. However, recent work has confirmed that plasmin is involved both in pro-TAFI activation and in TAFI inactivation (Marx et al 2002) and it has also been shown that TAFI activity displays a biphasic pattern, peaking first during coagulation and then during the fibrinolytic phase, what implies that both thrombin and plasmin may generate TAFI (Leurs et al 2003). Unfractioned heparin may further increase the catalytic efficacy of plasmin to 1/10 of the efficacy of the thrombin/thrombomodulin complex (Bajzar et al 1996c, Mao et al 1999).

It has also been shown that pro-TAFI may be activated by neutrophile elastase (Kawamura et al 2002), which could contribute to hemostatic disturbances in inflammatory disorders.

INACTIVATION OF TAFI

TAFI is highly unstable, with a half-life of only 10 minutes at 37°C (Boffa et al 1998). The instability of TAFI has been attributed to proteolytic cleavage and a spontaneous temperature-dependent process (Eton et al 1991, Wang et al 1994, Bajzar et al 1996c). Trypsin, plasmin and thrombin could cleave TAFI at Arg330 (Eton et al 1991) and it was speculated that this cleavage is responsible for inactivation of TAFI. However, site-directed mutagenesis, in which Arg330 was replaced with Gln, did not preclude inactivation by the thrombin/thrombomodulin complex, indicating that this site is not responsible for the inactivation (Boffa et al 2000). Neither does mutation at Arg320 prevent inactivation (Boffa et al 2000).

TAFI is also cleaved at Arg302 into 25 and 11 kD polypeptides (Marx et al 2000b). Mutagenesis at this site and replacement of arginine with glutamine prevented proteolysis, leading to the conclusion that Arg302 is a major cleavage site (Marx et al 2000b, Boffa et al 2000). But as this mutant is still inactivated, it seems that inactivation of TAFI to TAFIi is a consequence of conformational instability (Wang et al 1994, Boffa et al 1998, Marx et al 2000b).

Lowering the temperature increases the stability of TAFI from 10 minutes at 37°C to 45 minutes at 30°C and several hours at 22°C, while it is stable at 0°C (Boffa et al 1998). Epsilon amino caproic acid (εACA) and the arginine analogue, 2-guanidinoethylmercaptosuccinate (GEMSA),

prevent both the thermal instability of TAFI and proteolytic cleavage at Arg302 (Tan and Eaton 1995, Boffa et al 2000).

The thermal instability of TAFI at 37°C in guinea-pig, rat and rabbit is the same as in humans. However, increased thrombin generation was able to prevent TAFI decay in these animals, whereas it stimulated TAFI decay in human serum (Komura et al 2002).

Activation of pro-TAFI and inactivation of TAFI are complex processes and it seems that thrombin, thrombin/thrombomodulin and plasmin may take part in both enzyme activation and inactivation, while thermal instability contributes to TAFI inactivation. The most potent activator of pro-TAFI is thrombin/thrombomodulin (Schatteman et al 2000).

TAFI AND FIBRINOLYSIS

The fibrinolytic process starts after binding of t-PA and plasminogen to fibrin. This binding is enabled by C-terminal lysine residues at partially degraded fibrin and lysine-binding sites at plasminogen and t-PA (Christensen 1985, Miles et al 1991, Fleury and Angles-Cano 1991). Plasminogen bound to fibrin is a better substrate for t-PA than free plasminogen, because it is protected from rapid inactivation by plasmin inhibitor (alpha-2 antiplasmin) (Hoylaerters et al 1982, Sakharov and Rijken 1995).

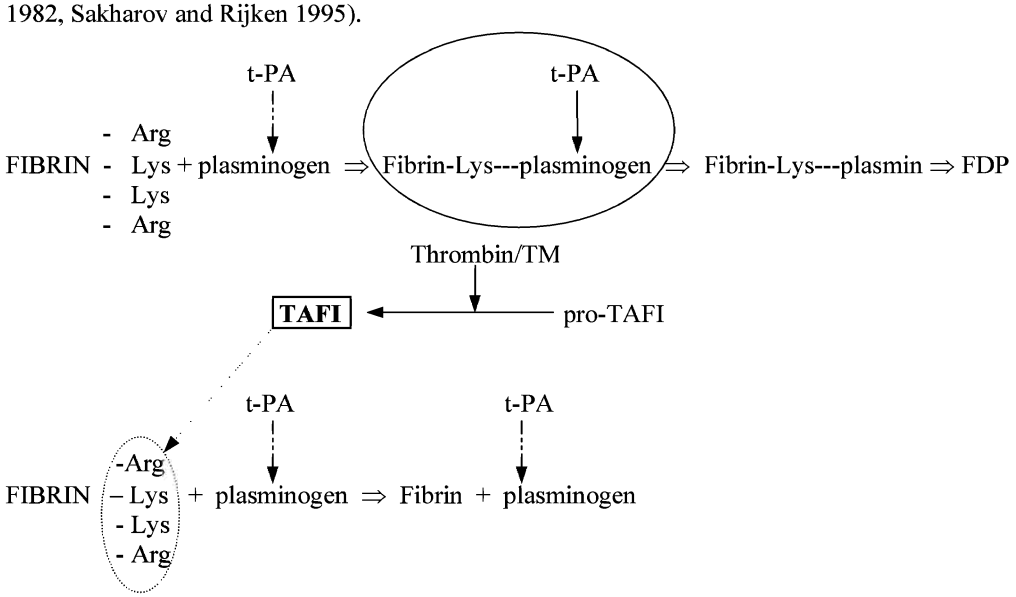

Figure 2. Simplified presentation of TAFI's role in fibrinolysis (modified from Bouma et al 2001)

Activation or stimulation

Degradation (cleaving off Arg, Lys carboxy terminal residues by TAFI)

Weak activation of non- bound plasminogen by t-PA

--- non covalent bounds

Abbreviations: t-PA - tissue plasminogen activator, Arg – arginine, Lys - lysine

TAFI cleaves off carboxy terminal arginine and lysine from fibrin and limits plasminogen binding (Sakharov et al 1997) as well as plasmin formation (Redlitz et al 1995, Wang et al 1998). TAFI may also directly inactivate plasmin at relatively high concentrations (Wang et al 1998). Glu-plasminogen is the primary target of TAFI-induced inhibition of fibrinolysis, while TAFI also inhibits conversion of Glu- to Lys-plasminogen (Bajzar et al 1995, Wang et al 1998). By preventing the conversion of fragment DD(E) of fibrin to fragment E and D-dimer and thus attenuating this fragment's activation of plasminogen, TAFI renders t-PA more fibrin specific (Stewart et al 2000).

It has been shown that FXIIIa may cross-link pro-TAFI for fibrin and thereby help to protect the newly-formed fibrin clot from premature plasmin degradation (Valnickova and Enghild 1998).

Briefly, the antifibrinolytic effect of TAFI amounts to the removal of C-terminal lysine residues from partially degraded fibrin and the prevention of plasminogen binding and the transformation of plasminogen into plasmin. This effect was confirmed in pro-TAFI deficient knock-out mice in which increased fibrinolysis has been observed (Swaisgod et al 2002). Recently, it has also been shown that inhibition of TAFI decreases the formation of microthrombi in a TF-induced fibrin deposition model in the rat as a consequence of up-regulated endogenous fibrinolysis (Muto et al 2003).

MEASUREMENT OF DIFFERENT FORMS OF TAFI

The instability of TAFI makes it difficult to measure the active form of the enzyme. A number of in-house and commercial assays have been developed for the measurement of different forms of TAFI.

Total TAFI antigen is measured by using ELISA with mono- (Mosnier et al 1998) or polyclonal antibodies (Chetaille et al 2000, Stromqvist et al 2001). Rocket immunoelectrophoresis is also used for the determination of TAFI (Van Tilburg et al 2000).

TAFI activity corresponds to the activatable amount of pro-TAFI. It is measured after in vitro activation of pro-TAFI with the thrombin/thrombomodulin complex. TAFI is determined, using substrate furoylacroleyl alanyl-arginine, by the change in absorbance at 336 nm (Hendriks et al 1990, Sakharov et al 1997). In another assay, TAFI induces release of hippuric acid from hippuril-arginin, which is detected by high-performance liquid chromatography (HPLC) (Schatteman et al 1999b), colorimetric quantification with cyanuric chloride (Mosnier et al 1998) and change in absorbance at 254 nm (Hendriks et al 1990, Bajzar et al 1995).

A quick homogeneous assay for the determination of pro-TAFI in microplates has recently been described (Schatteman et al 2001). After activation with thrombin/thrombomodulin, TAFI cleaves p-OH-hippuric acid from p-OH-Hip-Arg. Hippuricase converts the acid to glycine and p-hydroxybenzoic acid, which, after oxidative coupling with 4-aminoantipyrine, develops quinoneimine dye. Absorbance of dye is read at 506 nm. This assay shows a good correlation with the HPLC assay.

Commercial kits for the determination of pro-TAFI are also based on its in vitro activation by the thrombin/thrombomodulin complex and measurement of the difference between the amounts of TAFI after activation and in non-activated plasma, which serves as control. The difference represents the amount of pro-TAFI in the sample (Van Thiel et al 2001).

The possibility of measuring the enzyme's active form is limited by TAFI's instability. TAFI is therefore determined indirectly by clot lysis assay initiated with thrombin (Broze and Higuchi 1996, Mosnier et al 1998) or tissue factor (Mosnier et al 2001d). Clot lysis time – defined as the time from the midpoint of the clear-to-maximum turbid transition (clotting time) to the midpoint of the maximum turbid-to-clear transition – and its shortening after addition of potato tuber carboxypeptidase inhibitor (PTCI), a specific inhibitor of TAFI, represent the amount of TAFI activity and its ability to down-regulate fibrinolysis. A good correlation was observed between the pro-TAFI activity test, the TAFI antigen test and the clot lysis assay with addition of PTCI (Mosnier et al 1998).

A commercial kit for the determination of active and inactive forms of the complex (TAFI and TAFIi) has been developed recently (Greenfield et al 2002) but its characteristics have yet to be fully estimated.

The determination and measurement of TAFI are still a challenge because it is conceivable that the amount of TAFI obtained after in vitro activation of pro-TAFI does not fully correspond with the amount of the active form of the enzyme that is present in plasma. The development and assessment of assays for the direct determination of TAFI in plasma are and should therefore continue to be a priority.

THE ROLE OF TAFI IN HEALTH AND DISEASE

Levels of TAFI in normal individuals reportedly vary over a broad range (Mosnier et al 1998, Chetaille et al 2000). Measured as TAFI antigen, the level varies from 41 – 259%. The level of TAFI antigen does not differ between men and women, while an increase with age has been described in females but not in males. TAFI antigen is significantly higher in black African male groups compared with Caucasian men (Chetaille et al 2000). In contrast to the inter-individual variation, individual TAFI levels are very stable (Chetaille et al 2000). Some authors report no changes in the TAFI level during pregnancy (Schatteman et al 1999b, Van Tilburg et al 2000, Meijers et al 2000a,) but one recent study found a moderate increase in TAFI antigen (Chabloz et al 2001).

As the generation of TAFI is obviously dependent on thrombin generation, clinical conditions involving increased or decreased thrombin generation could induce changes in the levels of different forms of TAFI.

In 1990, Bajzar and collaborators and De Fouw and collaborators independently reported that activated protein C (APC) shortens clot lysis time in a concentration-dependent, saturable manner. This mechanism was subsequently shown to be TAFI-dependent (Bajzar et al 1996b). Finally, these authors found that in the absence of TAFI, APC did not affect clot lysis and con-

cluded that an impaired TAFI-dependent profibrinolytic response to APC in patients with APC resistance due to factor V Leiden is an additional risk factor contributing to a thrombotic tendency in these individuals (Bajzar et al 1996c). These experimental findings were indirectly confirmed in children with factor V Leiden mutation who had the poor fibrinolytic response after venous occlusion (Nowak-Göttl et al 1997). It was also shown in a mouse model that APC prevents thromboembolism by interrupting the thrombin-induced mechanism that down-regulates fibrinolysis and makes fibrin clots more resistant to lysis (Gresele et al 1998). Finally, the roles of protein C inhibitor and protein S in TAFI-dependent fibrinolysis further support the role of the protein C system in the regulation of fibrinolysis through the TAFI-dependent mechanism (Mosnier et al 2001a, b).

The links between APC and TAFI generation are obvious but complex. Through TAFI and APC, the thrombin/thrombomodulin complex has a dual role in coagulation and fibrinolysis. On the one hand it induces APC generation, which is anticoagulant and profibrinolytic, while on the other hand it induces TAFI generation, which is antifibrinolytic.

It has been shown that epidermal growth factor (EGF) domains at thrombomodulin are required for activation of both APC and TAFI (Kurosawa et al 1988, Kokame et al 1998, Wang et al 2000). Thrombin binds to EGF-like domains 5 and 6 (Kurosawa et al 1988). EGF-like domain 4 is essential for the activation of APC (Kokame et al 1998) and domain 3 is essential for the activation of TAFI (Kokame et al 1998, Wang et al 2000). Different domains on thrombin are involved in TAFI and APC activation. Residues E25, D51 and R89/R93/E94 are important for TAFI activation, while residues R178/R180/D183, E229, R233 are involved in APC activation (Hall et al 1999).

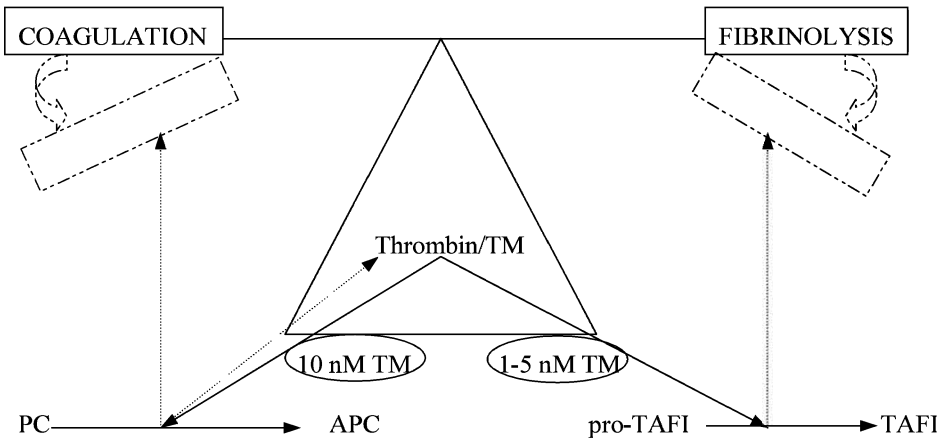

Figure 3.Simplified presentation of thrombin/thrombomodulin's effect on coagulation and fibrinolysis (modified from Bouma et al 2001)
Activation
Inactivation
Down-regulation
Abbreviations: TM – thrombomodulin, PC – protein C, APC – activated protein C, TAFI – thrombin activatable fibrinolysis inhibitor

Activation of TAFI and APC by the thrombin/thrombomodulin complex through different domains could occur simultaneously, inducing both pro- and antifibrinolytic effects. In one study,

however, low concentrations of thrombomodulin (1-5 nM) stimulated TAFI activation, while a higher concentration (10 nM) depressed TAFI activation through the APC-induced down-regulation of thrombin generation (Mosnier et al 2001c). This could indicate that low concentrations of thrombomodulin are antifibrinolytic, while higher concentrations are profibrinolytic. That could be even more important in that the concentration of thrombomodulin rises from large to smaller blood vessels and is highest in capillaries. It has also been shown that both soluble and cellular thrombomodulin could activate TAFI (Bajzar et al 1998).

Defective thrombin generation induces a bleeding tendency in patients with inherited coagulation factor deficiency and von Willebrand's disease (Keultars et al 2000). The efficacy of antifibrinolytic treatment in patients with these disorders supports the notion that enhanced fibrinolysis is an important factor in bleeding disorders (Ramström and Blombäck 1975, Piot et al 2002). It is also known that patients with factor XI deficiency, which is, in principle, a mild disorder, are prone to bleeding from tissues with higher fibrinolytic activity (Asakai et al 1991, Berliner et al 1992). The accounts of TAFI may provide an acceptable explanation for this up-regulation of fibrinolysis in patients with impaired thrombin generation. It has been shown that clots formed from FVIII, IX, X and XI deficient plasmas lyse prematurely and that factor supplementation corrects this defect and at the same time increases the rate and extent of carboxypeptidase U (TAFI) activation (Broze and Higuchi 1996). Addition of factor VIII and also of TAFI restored fibrinolysis in hemophilia A plasmas at low tissue factor concentrations (Mosnier et al 2001d). Since high amounts of thrombin are necessary for the generation of TAFI, it seems that a normal intrinsic pathway is also required for full TAFI generation and down-regulation of fibrinolysis.

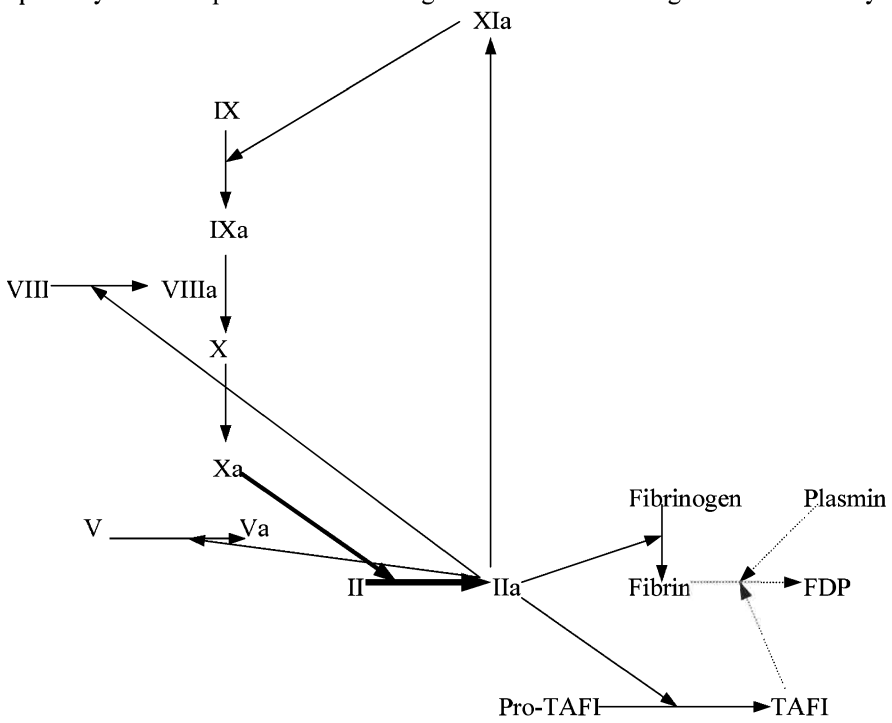

Figure 4. FXI loop – full thrombin generation through the intrinsic pathway (modified from Bouma et al 2001)

Activation

Inhibition and degradation

In a revised model of blood coagulation, exposure of tissue factor (TF) plays a key role in the initiation of coagulation (Rao and Rapaport 1988). TF binds VIIa and this complex can activate both IX to IXa and X to Xa on TF-bearing cells (i.e. monocytes). TF-VIIa-Xa form a complex with TFPI, which quickly inactivates them (Broze 1992). At high TF concentrations, this inhibition could be overestimated and X is transformed to Xa by this complex. But at low TF concentrations, inhibition with TFPI prevents the synthesis of the full amount of thrombin, so just a small amount of thrombin is generated. This amount initiates coagulation and activates FVIII to VIIIa, FV to Va and platelets. This leads in turn to amplification and synthesis of FIXa and FXa, which results in the transformation of prothrombin to thrombin (Hoffman and Monroe 2001). Although this amount of thrombin is sufficient for the transformation of fibrinogen to fibrin and the clotting of fibrin, the formation of thrombin continues inside the clot (Von dem Borne et al 1997) and most of the generation of thrombin occurs after clot formation (Rand et al 1996) through the intrinsic pathway in an FXI-dependent manner (Von dem Borne et al 1995). This amount of thrombin is sufficient for the generation of TAFI and the down-regulation of fibrinolysis, and it has accordingly been shown that TAFI generation is FXI-dependent (Mosnier et al 1998).

Increased thrombin generation induced by the intrinsic pathway and increased TAFI generation with a consecutive down-regulation of fibrinolysis could, at least partly, explain the increased thrombotic risk in patients with elevated FVIII, IX and XI (Koster et al 1995, Vlieg et al 2000, Meijers et al 2000c).

The role of TAFI in thrombotic disease could be a consequence of its increase after the up-regulation of thrombin generation, but measurements of different forms of TAFI indicate that TAFI *per se* could also contribute to the thrombotic tendency.

Elevated TAFI antigen levels have been described as a mild risk factor for deep vein thrombosis (DVT) (Van Tilburg et al 2000); apart from this, the sole presence of high levels of pro-TAFI further increases the risk of DVT in patients with factor V Leiden mutations (Libourel et al 2002). On the other hand, Schroeder et al recently showed that TAFI antigen levels did not differ between patients with and without acute pulmonary embolism (2003).

Increased pro-TAFI levels that correlate with FVII and C-reactive protein have been found in patients with ischemic heart disease and stable angina (Silveira et al 2000). The French group observed that conventional cardiovascular risk factors (i.e. age, blood pressure, waist to hip circumference) had no influence on the level of TAFI antigen and concluded that the high inter-individual variability called for genetic studies (Juan-Vague et al 2000). The same group observed an unexpected decrease of TAFI antigen in patients with myocardial infarction (MI) and a higher frequency of alleles of the TAFI gene was associated with a decrease in TAFI. A conceivable protective effect of TAFI in MI could be a consequence of its role in the stabilisation of fibrin plaque and prevention of its rupture (Juhan Vague et al 2002). In contrast to this, an increase in TAFI antigen has been described in venous and especially coronary artery blood in patients with coronary artery disease and it correlated with the level of acute phase reactants (Schroeder et al 2002). Recently the French group also identified an increase in TAFI antigen as a risk factor for angina pectoris in France, while the incidence of Ala 147Thr polymorphism is higher in patients compared to controls (Morange et al 2003). Increased level of TAFI antigen has been described in acute ischemic stroke, and its level correlates with the level of neurological detoriation (Montaner et al 2003).

Pro-TAFI is increased in hyperlipidemic subjects with hypercholesterolemia (Puccetti et al 2001), while both pro-TAFI and TAFI antigen are increased in patients with type 2 diabetes mellitus and obesity (Hori et al 2002), and the latter is increased in diabetic patients with microalbuminuria compared to those with normoalbuminuria (Yano et al 2003). Elevated pro-TAFI and total TAFI antigen levels have been found in patients with nephrotic syndrome (Malyszko et al 2002), while total TAFI antigen was elevated in renal transplantant recipients (Hryszko et al 2001a). Hypolipemics, e.g. fluvastatin, significantly reduce TAFI antigen in renal transplant patients (Malyszko et la 2003), while another hypolipemic, simvastatin, lowered this antigen in patients on peritoneal dialysis (Malyszko et al 2001), who, unlike patients on hemodialysis, have increased levels of both pro-TAFI and total TAFI antigen (Hryszko et al 2001b). The above findings could indicate an important role for TAFI in the development of atherothrombotic changes in patients with metabolic disturbances associated with hyperlipidemia and hypercholesterolemia, although recent findings indicate that an increased TAFI level is not independently associated with the metabolic markers of insulin resistance syndrome in obese patients, but may reflect the specific pathway of TAFI regulation at the level of hepatic synthesis (Aubert et al 2003).

TAFI antigen increased during the use of oral contraceptives and counteracted increased fibrinolysis in these individuals (Meijers et al 2000a), while oestradiol + trimegestone lowered pro-TAFI, whereas oestradiol alone or together with dydrogesteron did not influence pro-TAFI levels (Post et al 2002).

Both pro-TAFI and TAFI antigen are decreased in patients with liver cirrhosis and correlate with the severity of the disease (Van Thiel et al 2001); this seems to be a logical consequence of the liver being the site of pro-TAFI synthesis. It is noteworthy, however, that a decrease in total TAFI antigen is not associated with increased plasma fibrinolysis, most probably because of a reduced synthesis of profibrinolytics (Lisman et al 2001).

Both pro-TAFI and total TAFI antigen decreased in disseminated intravascular coagulation (DIC) due to increased consumption, while a further decrease in patients with infection and organ failure suggests a role in the mechanism of organ failure in DIC-associated sepsis (Watanabe et al 2001). A decrease in pro-TAFI without any fall in total TAFI antigen has been reported in acute promyelocytic leukaemia; it was explained as a possible consequence of increased plasmin activity and could further contribute to the bleeding tendency in these patients (Meijers et al 2000b).

TAFI is affected by antithrombotic therapy. Heparin diminished TAFI generation, up-regulated fibrinolysis and reduced the stability of the developing clot (Lisman and De Grot 2003), while it did not influence fibrinolysis of stable mature clots; this implies that fibrin-bound thrombin plays the most important role in the generation of TAFI (Collucci et al 2002). Argatroban thrombin inhibitor decreases TAFI generation and up-regulates fibrinolysis (Nagashima 2002, Hashimoto et al 2002). Inhibition of thrombin generation is the possible mechanism by which melagatran – a direct thrombin inhibitor that binds both free and fibrin-bound thrombin – decreases the transformation of pro-TAFI to TAFI and up-regulates fibrinolysis (Mattsson et al 2002).

Finally, the role of TAFI in fibrinolysis could enhance effects of treatment and reduce the risk of bleeding in patients undergoing therapeutic thrombolysis. PTCI – a specific inhibitor of TAFI – significantly improved t-PA induced fibrinolysis without adverse effects in a rabbit thrombosis model (Klement et al 1999, Nagashima et al 2000, Hashimoto et al 2002). Besides the direct in-

hibition of TAFI, some authors suggest that inhibition of FXI through the indirect down-regulation of TAFI could improve effects of t-PA induced thrombolysis without an increased bleeding risk (Minnema et al 1998). This therapeutic approach has not yet been tested in human studies.

The definitive role of TAFI, not only in thrombolytic treatment but also in many other clinical conditions associated with increased or decreased thrombin generation, or in atherothrombotic disease, has also yet to be proven.

The role of plasmin in TAFI generation is another issue that should be emphasized in the future.

That, together with efforts to develop accurate and precise methods for the *ex vivo* determination of TAFI (the active form of the enzyme) and the standardization of its nomenclature, should be the main goals of TAFI investigation in the near future.

AIM OF THE STUDY

The general aim of this study was to investigate changes in different forms of TAFI and their possible influence on fibrinolysis in different clinical conditions associated with hyper- and hypocoagulability.

Specific aims of the study were:

- Estimation of TAFI's role in patients with hypercoagulable states associated with increased thrombin generation in thrombotic disorders (patients with APC resistance due to factor V Leiden mutation) using *ex vivo* measurement of different forms of TAFI.

- Estimation of possible changes in TAFI and their influence on the risk of thrombosis in conditions associated with hypercoagulable states (i.e. diabetes mellitus and preeclampsia).

- Estimation of TAFI's role in patients with hypocoagulable states associated with impaired thrombin generation in bleeding disorders (e.g. hemophilia and Von Willebrand disease) using *ex vivo* measurement of different forms of TAFI.

- *In vitro* estimation of effects of different doses of recombinant factor VIIa (NovoSeven) on changes in TAFI and their influence on blood fibrinolysis in plasmas deficient in various coagulation factors.

PATIENTS, MATERIALS AND METHODS

PATIENTS

Thirteen healthy volunteers (laboratory staff), mean age 42 years (range 19-58), were used as controls in papers I, IV and V.

Seventeen female patients with APC resistance due to heterozygous FV Leiden mutation and at least one episode of deep vein thrombosis and/or pulmonary embolism, mean age 37 years (range 24-65) years, were included in paper I.

Thirty-two patients with hemophilia A (12 severe, 8 moderate and 12 mild), 4 patients with hemophilia B (1 severe and 3 mild) and 21 patients with VWD (8 with type 1, 3 with type 2A and 10 with type 3), mean age 42 (range 19-76) years, were included in paper V; none of them had an inhibitor against factor VIII (FVIII) and/or factor IX (FIX). Seventeen of the thirty-two hemophilia A patients were also included in paper IV.

Thirty-eight patients with type I diabetes mellitus of >10 years duration, with or without microvascular complications, selected from a register at the Department of Endocrinology & Diabetology at the Karolinska Hospital, were included in paper III. Eighteen of these patients had microvascular complications – non-proliferative diabetic retinopathy according to the ETDRS-Airlie House Classification; the remaining twenty patients had no history of microvascular complications or cardiovascular disease. The patients were matched for sex, age, duration of disease and metabolic control. Patients with severely impaired metabolic control (HbA1c > 9.0%) were excluded. The patients were compared to twenty healthy individuals, matched for sex and age, recruited among the laboratory staff.

Forty-six patients with preeclampsia (PE) and/or intrauterine fetal growth retardation (IUFGR) (21 with severe and 17 with mild PE, 8 with IUFGR combined with proteinuria but without hypertension) (mean age 35.4 (range 24-40) years) and sixteen women with a normal pregnancy (mean age 29.3 (range 22-37) years) as controls were included in paper II. Blood samples were taken in the third trimester in both groups.

Local ethics committee permission was obtained for all the studies.

MATERIALS:

Factor II, V, VII, VIII, IX, XI, XII deficient plasmas (concentration of the respective factor < 0.01 U/mL), purchased from Helena Bioscience, Sunderland, UK, and normal pooled plasma (NPP) obtained from 30 healthy individuals, were used in paper VI.

The rVIIa, obtained as a generous gift from Professor Ulla Hedner, Novo Nordisk (Bagsvaerd, Denmark), was added, in final rVIIa concentrations of 0.6, 1.2, 2.4, 4.8 and 9.6 μg/mL, to the deficient plasmas and to NPP 15 minutes before the determination of OHP in paper VI.

BLOOD COLLECTION

Plasma samples were prepared from citrated (trisodium-citrate 0.129mol/L, pH 7.4,) whole blood (ratio 1 + 9) by centrifugation at room temperature and 2000 x g for 20 minutes, then divided into aliquots and frozen at –70°C until test performance in papers I, II, IV and V.

In paper III, plasma samples were prepared from citrated (trisodium-citrate 0.129mol/L, pH 7.4) whole blood (ratio 1 + 9) by centrifugation at +4°C and 1400 x g for 10 minutes, after which the plasma was aliquoted and frozen at –70°C until test performance.

METHODS

Pro-TAFI assay

Concentrations of pro-TAFI were determined with an Actichrome plasma TAFI activity kit from American Diagnostica Inc, Greenwich, USA, based on a chromogenic assay that measures TAFI after activation with specific activator and non-activated plasma, which serves as control. The difference between amount of TAFI in activated and non-activated plasma was calculated and represents the amount of pro-TAFI activity in the sample. The test was performed according to the manufacturer's instructions. The results (obtained after comparison with a standard curve using standards from the manufacturer) correspond to the amount of TAFI obtained after in vitro quantitative conversion of pro-TAFI to TAFI and are presented as μg/mL. Both inter- and intra-assay CVs are < 7%. This assay was used in papers I, III, IV and V.

TAFI antigen assay

TAFI antigen was determined with an ELISA kit from Affinity Biologicals Inc, Ontario, Canada, which consists of affinity purified sheep anti-TAFI IgG captured antibody and prediluted HRP-conjugated affinity purified sheep anti-TAFI detecting antibody. This test measures total TAFI antigen, including pro-TAFI (procarboxypeptidase U), TAFI (carboxypeptidase U) and TAFIi (inactivated carboxypeptidase U). All the necessary buffers and reagents were prepared according to the manufacturer's instructions. Test plasma samples were diluted 1/200. Normal pooled plasma (NPP) obtained from 30 healthy donors was used as a standard. The results (all run in

duplicate) are shown as a percentage of NPP values. Intra-assay and inter-assay CVs are 5.8 and 8.0, respectively. This assay was used in papers I, II, III, IV and V.

TAFI-TAFIi antigen assay

TAFI-TAFIi antigen was determined with an IMUBIND® TAFIa/ai Antigen ELISA kit from American Diagnostica Inc (Greenwich, CT, USA). This ELISA measures TAFI and TAFIi antigens that are present in plasma. The assay uses potato tuber carboxypeptidase inhibitor – a specific inhibitor of TAFI (PTCI)-coated microtiter wells as the solid phase absorbent and an anti-TAFI-IgG-horseradish peroxidase reagent for detection of bound TAFI-TAFIi antigen. All the necessary buffers and reagents were prepared according to the manufacturer's instructions. Each sample was run in duplicate and results are reported as μg/mL. Both intra-assay and inter-assay CVs were <7%. This assay was used in paper V.

Determination of overall hemostatic potential (OHP)

Overall hemostatic potential (OHP) in plasma was determined as previously described (He et al 2001). Briefly, the method is based on the determination of a fibrin aggregation curve. For the overall coagulation potential (OCP), $CaCl_2$ (final conc. 35 mmol/l) and thrombin (final conc. 0.09 IU/ml) were added to Tris buffer (66 mmol/l Tris and 130 mmol/l NaCl, pH 7.4). For the overall hemostatic potential (OHP), t-PA (final conc. 660 ng/ml) was also added to the buffer for OCP. Sixty microlitres of plasma in each well was mixed with 50 μl of the respective buffer in the assay of OCP or OHP (final conc. of $CaCl_2$ 17 mmol/ml and thrombin 0.04 IU/ml in both, plus t-PA 300 ng/ml in the latter). Absorbance (Abs) at 405 nm was recorded each minute for 40 min to construct the two fibrin-aggregation curves, OCP and OHP. The area under the curve (AUC) was expressed by summing the Abs values (Abs-sum). The difference between the two areas reflects the overall fibrinolysis potential (OFP), calculated as OFP=(OCP-OHP)/OCP. Intra-assay and inter-assay CVs are 8.6 and 5.1%, respectively, for OHP and 3.1 and 4.2% for OCP. This assay was used in papers II and III, while in paper VI, since clotting is seriously impaired and almost absent in FVIII and FIX deficiency, we developed a modification of the original method with the addition to each plasma sample of a platelet reagent, which is a platelet membrane preparation derived from washed fresh normal human platelets (final conc. 10^6 mL^{-1}), as a source of phospholipids.

Clot lysis time

Clot lysis time (CLT) was measured using a fibrin aggregation curve for the determination of OHP. It was defined as the time from the midpoint of the clear-to-maximum turbid transition (defined as clotting time) to the midpoint of the maximum turbid-to-clear transition. CLT was also measured after in vitro addition of PTCI, final concentration 50μg/ml. Intra-assay and inter-assay CVs are 4.8 and 6.1% respectively. This assay was used in papers II, III and VI.

OTHER PARAMETERS AND TESTS

Blood pressure, serum albumin, ALT, AST and serum creatinine as well as proteinuria were determined in paper II.

Blood pressure, body mass index, plasma fibrinogen, glucose and insulin, serum triglyceride, cholesterol, HDL and LDL cholesterol, HbA1c and microalbuminuria were determined in paper III.

STATISTICS

The results are presented as mean ± SD in all papers except those in paper III, where they are presented as mean ± SEM.

The two-tailed Mann-Whitney test for non-parametric values was used for the determination of statistical significance in papers I and IV.

Student's t-test was used for the determination of statistical significance in papers II and V.

Correlation was used in paper II.

The Kruskal-Wallis test with Dunn's post-test computing was used for the determination of statistical significance in papers III and V.

The Graph Pad program, version 3.02, was used for statistical calculations and the presentation of the results.

RESULTS

PRO-TAFI AND TOTAL TAFI ANTIGEN IN PATIENTS WITH APC RESISTANCE DUE TO FACTOR V LEIDEN MUTATIONS

No significant differences in the levels of TAFI antigen were observed between controls (98.87 ± 37.42%) and patients with APC resistance (83.40 ± 38.85%) (p=0.31) (Figure 1, paper I). TAFI activity was significantly reduced in the APC resistant patients (12.79 ± 5.06μg/mL) compared to controls (17.85 ± 4.62μg/mL) (p=0.02) (Figure 2, paper I).

TOTAL TAFI ANTIGEN AND TAFI-DEPENDENT FIBRINOLYSIS IN PREECLAMPSIA

TAFI antigen in patients with PE and/or IUFGR was significantly lower (48.89±26.83%) than in women with a normal pregnancy (116.30±74.10%) (p<0.0001) (Figure 1, paper II).

The level of TAFI antigen and the concentration of protein in urine were negatively correlated (R=-0.41) in the 10 preeclampsia women in whom urine samples were taken.

CLT was longer (23.7±3.8 min.) in 5 preeclampsia patients than in 10 normal pregnancies (21.4±3.4 min), although the difference is not statistically significant, while OFP was lower in the patients (0.23±0.10) than in the normal pregnancies (0.37±0.13), with borderline statistical significance (p=0.046) (Table 2, paper II).

OFP did not increase after the addition of PTCI to patients' samples (0.22±0.07).

OVERALL HEMOSTASIS, TAFI AND TAFI-DEPENDENT FIBRINOLYSIS IN DIABETES MELLITUS TYPE I

The pro-TAFI levels in patients with type I diabetes mellitus with or without vascular complications did not differ significantly from controls (p=0.64) (Table 2, paper III).

Total TAFI antigen tended to decrease in both patient groups (59.7±7.2% and 73.4±8.9% with and without microvascular complications, respectively) compared to controls (91.9±12.2%) (p=0.12) (Table 2, paper III).

Neither OFP nor CLT differed in patients with type I diabetes with and without microvascular complications compared to controls (Table 2, paper III).

After the addition of PTCI, some shortening of CLT was observed in the diabetic patients without microvascular complications (1.2 min.) but not in those with complications, while a very slight shortening was found in the control group (0.3 min.).

OHP in the diabetic patients with complications was significantly higher than in the healthy controls (8.9±0.9 vs. 6.7±0.4; $p<0.05$); it was also higher in the diabetics without complications (7.8±0.6) but here the difference from the healthy controls did not reach statistical significance ($p>0.05$) (Figure 1, paper III).

OCP in the diabetic patients with complications was likewise statistically higher than in the healthy controls (12.8±1.1 vs. 10.2±0.5; $p<0.05$); it was also higher in the diabetics without complications (11.6±0.7) but again the difference did not reach statistical significance ($p>0.05$) (Figure 2, paper III).

PRO-TAFI, TAFI-TAFIi ANTIGEN AND TOTAL TAFI ANTIGEN IN PATIENTS WITH HEMOPHILIA A, HEMOPHILIA B AND VWD

The level of total TAFI antigen did not differ significantly between controls (98.87 ± 37.42%) and patients with hemophilia A (83.02 ± 34.49%) ($p=0.28$), although it was slightly lower in the hemophilia patients (Figure 1, paper IV). Pro-TAFI was significantly reduced in the hemophilia patients (13.09 ± 3.81 μg/mL) compared to controls (17.85 ± 4.62 μg/mL) ($p=0.01$) (Figure 1, paper IV).

When additional hemophilia A patients were included, as well as patients with hemophilia B and VWD, the total TAFI antigen level did not differ between any of the patient groups and the controls (Figure 1, paper V). Neither did total TAFI antigen differ either within the group of hemophilia A patients in relation to severity (mild, moderate and severe) or within the group of VWD patients in relation to subtype (types 1, 2A and 3).

A significant decrease in pro-TAFI was found in all the bleeding disorders (10.72 ± 4.57 μg/mL ($p<0.001$); 8.00 ± 2.35 μg/mL ($p<0.01$) and 8.98 ± 2.33 μg/mL ($p<0.001$) in hemophilia A, hemophilia B and VWD, respectively) compared to controls (17.85 ± 4.61 μg/mL) (Figure 2, paper V). Pro-TAFI levels did not differ either between hemophilia A patients with different severity or between VWD patients with different subtypes.

TAFI-TAFIi antigen was significantly increased in hemophilia A patients (1.05 ± 1.01 μg/mL) ($p<0.05$) and in VWD patients (0.96 ± 1.01 μg/mL) ($p<0.05$) compared to controls (0.55 ± 0.36 μg/mL) (Figure 3, paper V). The TAFI-TAFIi levels did not differ either between hemophilia A patients with different severity or between VWD patients with different subtypes.

OVERALL HEMOSTASIS AND FIBRINOLYSIS AND TAFI-DEPENDENT FIBRINOLYSIS IN DIFFERENT COAGULATION FACTOR DEFICIENT PLASMAS AFTER ADDITION OF DIFFERENT DOSES OF rFVIIa (NovoSeven)

rFVIIa did not induce an increase in either OCP or OHP in NPP, even at the very high concentration (Table 1 and 2, paper VI).

OCP and OHP were markedly increased in FV deficient plasma with the lowest concentration of rFVIIa; OHP reached approximately one-half of the values in NPP (Table 2, paper VI). rFVII progressively increased fibrinolysis (increase in OFP and shortening of CLT) in a dose-dependent manner in FV deficient plasmas (Tables 3 and 4, paper VI).

The lowest concentration of rVIIa markedly increased OCP and OHP in FVIII and FIX deficient plasmas; OHP reached a plateau at a concentration of 2.4 μg/mL with values of approximately 2/3 of those in NPP (Table 1 and 2, paper VI). Higher concentrations of rVIIa (2.4-9.6 μg/mL) down-regulated fibrinolysis, shown as a decrease in OFP and a prolongation of CLT, to the NPP levels in FVIII deficient plasma or even lower in FIX deficient plasma (Tables 3 and 4, paper VI).

OCP and OHP in FXII deficient plasma were higher than in NPP and further increased after addition of rFVIIa (Tables 1 and 2, paper VI). Initially impaired fibrinolysis in FXII deficient plasma increased slightly with the low concentration of rFVIIa and then was further down-regulated at higher concentrations (Tables 3 and 4, paper VI).

TAFI's effect on CLT and fibrinolysis down-regulation was present in FV deficient plasmas before addition of rFVIIa (Figure 1a, paper VI) but not in either FVIII or FIX deficient plasmas, while some effect in the latter plasmas was present after addition of higher concentrations of rFVIIa (2.4-9.6 μg/mL) (Figures 1c and 1d, paper VI). There was an influence of TAFI in FXI deficient plasma which increased after addition of higher concentrations of rFVIIa (2.4-9.6 μg/mL) (Figure 1f, paper VI). TAFI's effect on fibrinolysis and CLT was constant in FX and FXII deficient plasmas and addition of rFVIIa did not seem to exert any marked influence (Figures 1e and g, paper VI).

DISCUSSION

TAFI AND APC RESISTANCE DUE TO FACTOR V LEIDEN MUTATION
Paper I

Resistance to activated protein C (APC resistance) due to a single point mutation in the gene for factor V, the so called FV Leiden mutation, caused by the replacement of arginine with glutamine at position 506 (Bertina et al 1994), is the most common cause of thrombophilia, with a carrier frequency prevalence of 3-7% or up to 15% in some selected groups (Price and Ridker 1997), giving a total relative risk of thrombosis in carriers of 2.8 (Simioni et al 1999). Increased thrombin generation has been demonstrated in patients with APC resistance due to the factor V Leiden mutation (Zoller et al 1996). At the same time, APC has been found to have profibrinolytic effects, supposedly as a possible consequence of reduced TAFI activation caused by an APC-induced decrease in thrombin generation (Bajzar et al 1996a). In the absence of TAFI, APC did not affect clot lysis and the authors concluded that impaired TAFI-dependent fibrinolysis makes an additional contribution to the thrombotic tendency in subjects with factor V Leiden mutation (Bajzar et al 1996b).

Our results can constitute indirect evidence of an association between increased thrombin generation and an increase in TAFI. We observed a decrease in pro-TAFI activity accompanied by no difference in the total TAFI antigen level. The cause could lie in an activation of pro-TAFI to TAFIa and an equilibrium shift towards an increase in TAFI. That might indicate that TAFI is increased as a consequence of the elevated generation of thrombin in patients with APC resistance with factor V Leiden mutation. An increase in TAFI down-regulates fibrinolysis and can be an additional risk factor for thrombosis in these patients.

Recent findings from our laboratory with the OHP assay have demonstrated a reduction of fibrinolysis (decreased OFP) in patients with APC resistance caused by FV Leiden mutation (Antovic A, He S, Bremme K and Blombäck M – unpublished data), which can further support our results and their explanations.

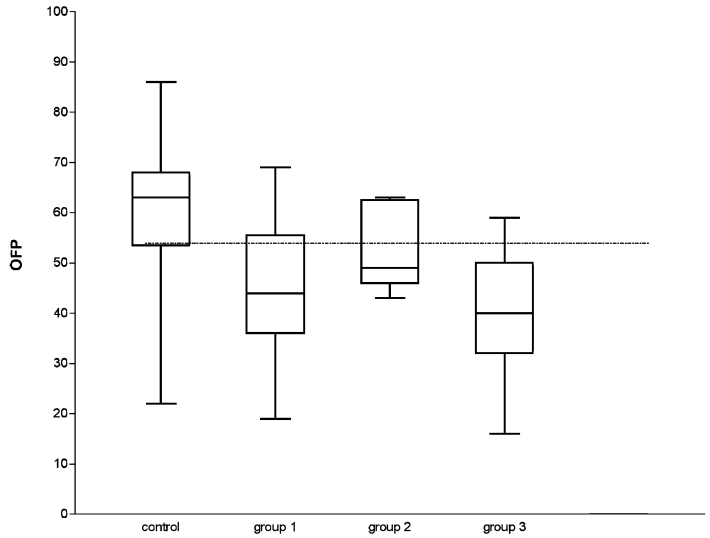

Figure 5: OFP (presented as a percentage of OHP) in patients with previous episode of DVT during pregnancy. Group 1 – patients without APC resistance and FVL, group 2 – patients with APC resistance without FVL, group 3 – patients with both APC and FVL. Control compared with groups 1 and 3 – $p< 0.0001$.

These findings could be indirect proof that an increase in TAFI down-regulates fibrinolysis and constitutes an additional risk of thrombosis in these patients.

TAFI AND PREECLAMPSIA
Paper II

Both preeclampsia with intrauterine fetal growth retardation (IUFGR) and IUFGR alone are important pathological syndromes of pregnancy, induced by an impaired utero-placental circulation. Preeclampsia is characterized by increased blood pressure and proteinuria and affects 3-5% of all pregnancies (Roberts and Cooper 2001). Activation of coagulation is another common finding in preeclampsia (Perry and Martin 1992, Schjetlein et al 1997) and in IUFGR (Sheppard and Bonnar 1999). A hypofibrinolytic state has been described that is due to an increase in plasminogen activator inhibitor (PAI-1) in both plasma and placenta (Sheppard and Bonnar 1999, Gilabert et al 1995, He et al 1995) and an increase of PAI-2 in placenta (Kanfer et al 1996). Due to hypercoagulability, this impaired fibrinolysis could further increase clotting in the microvascular circulation.

Contrary to previous findings in thrombotic disease and to our expectations, TAFI was significantly lower in our patients with preeclampsia and/or IUFGR compared to normal pregnancy. This decrease could have to do with an excessive loss of TAFI in urine. The molecular mass of pro-TAFI (55 kD) is even lower than that of albumin (64 kD), which is lost excessively in proteinuria. As the molecular masses of TAFI and its inactivated form are around 35 kD, it seems

logical that TAFI could be lost due to proteinuria, which was present in our patients with PE as well as in those with IUFGR, together with a decrease in serum albumin. In the patients in whom urinary proteins were measured quantitatively, an inverse correlation was found between TAFI antigen levels in plasma and urinary proteins, which further supports this hypothesis. Pro-TAFI forms a complex with plasminogen that could prevent its loss. Meanwhile, increased thrombin generation, which is present in PE and IUFGR, could lead to conversion into the active form (TAFI), which on account of a lower molecular mass is more susceptible to urine loss in renal impairment. Increased levels of liver enzymes, also shown in our patients, indicate a moderate hepatic dysfunction, which may explain the decrease in pro-TAFI, since this is synthesized in the liver. So it seems that both renal and hepatic impairment could contribute to decreased TAFI in preeclampsia patients.

The decrease in TAFI could be the reason why, in spite of depressed overall fibrinolysis, we have not observed a further down-regulation of fibrinolysis by TAFI. It seems that the amount of TAFI was not sufficient for this.

We can therefore speculate that a mechanism – in principle pathological – such as a decrease in protein due to nephropathy and impaired liver function in preeclampsia, can prevent a further impairment of fibrinolysis and a further thrombotic tendency and complications in these women.

OVERALL HEMOSTASIS AND TAFI IN DIABETES MELLITUS TYPE I
Paper III

Diabetes mellitus (DM) is considered to be a prothrombotic condition and is associated with an increased risk of cardiovascular complications (Kannel et al 1990). Increased levels of PAI-1 are frequently observed in type II DM and the disease is associated with decreased endogenous fibrinolysis activity (Nordt and Bode 2000). Although an activation of coagulation (e.g. increases in the prothrombin F1+2 fragment, the thrombin–antithrombin complex, FVII:C and D-dimer) (Reverter et al 1997, Giusti et al 2000) is present in type I DM, it seems that neither impaired fibrinolysis nor increased PAI-1 are features of this disorder (Vicari et al 1992, Mahmoud et al 1992).

Although elevated levels of both pro-TAFI and total TAFI antigen were recently described in obese patients with type II DM and insulin resistance (Hori et al 2002), we found no difference in the level of pro-TAFI, while total TAFI antigen tended to decrease in patients with DM type I. No difference in OFP and CLT supports previous findings of an intact endogenous fibrinolytic potential in patients with type I diabetes mellitus. The addition of PTCI shortened CLT to some extent in diabetic patients without microvascular complications and did not affect CLT in diabetic patients who had such complications, while the shortening of CLT in controls was very slight. From these data we speculate that TAFI plays a minor role in the regulation of fibrinolysis in healthy controls, while its importance increases with increased endogenous thrombin generation, which is observed in diabetic patients. It seems that even slightly subnormal levels of TAFI antigen are sufficient to induce a small down-regulation of fibrinolysis in the diabetic patients

without microvascular complications. Levels of total TAFI antigen (consisting of pro-TAFI, TAFI and TAFIi) tended to be even lower in the patients who had microvascular complications, which may have to do with a loss of the lower molecular weight forms (TAFI and TAFIi (35kD)) on account of renal impairment and/or a consumption of TAFI secondary to a more widespread vasculopathy in these patients. The amount of active TAFI present in plasma in these patients is probably not sufficient for the down-regulation of fibrinolysis despite signs of an increased generation of thrombin (i.e. an increased OHP).

On the other hand, hypercoagulability (increased OCP and OHP) was present mainly in our group of patients with complications, i.e. the patients with more advanced disease. We found no difference between the groups in the plasma levels of F1+2. Thus, compared to F1+2, OHP may be a useful and more sensitive method for detecting hemostatic changes early in diabetic microangiopathy, especially considering that all our patients were well treated, with a low level of complications. However, from the present study it cannot be determined whether hypercoagulability is a consequence rather than a cause of disturbances at the microvascular level.

TAFI AND HYPOCOAGULABLE CONDITIONS (HEMOPHILIA A, B AND VWD)
Papers IV and V

It has been suggested that TAFI levels are altered in hemophilia A (Mosnier et al 2001d). Moreover, premature clot lysis is a characteristic of hemophilic plasma (Broze and Higuchi 1996). Thrombin generation is seriously impaired in hemophilia (Keultars et al 2000), while a secondary burst of thrombin is necessary for the generation of TAFI (Von dem Borne et al 1997). Impaired thrombin generation may therefore result in decreased TAFI activation and up-regulation of fibrinolysis, which in turn increases the bleeding tendency in patients with hemophilia.

We have confirmed the absence of a difference in the level of total TAFI antigen in hemophilia patients (Guo et al 2000), while we found that pro-TAFI is decreased in hemophilia A (paper IV). At that time the cause of the decreased pro-TAFI was not clear to us. Activation of pro-TAFI to TAFI and an equilibrium shift towards an increase in TAFI do not seem to be an acceptable explanation since impaired thrombin generation in hemophilia is most probably not able to potentiate this activation compared to controls. One possible cause of the decrease in pro-TAFI could therefore be an inactivation of procarboxypeptidase B by plasmin or some other protease.

When additional patients with hemophilia A were included, as well as patients with hemophilia B and VWD, our findings were similar (paper V). Using a novel ELISA test for quantitating TAFI-TAFIi antigen in plasma, we found increased levels of this antigen in patients with hemophilia A and VWD. Thus, the findings of decreased pro-TAFI levels along with increased TAFI-TAFIi antigen levels would be consistent with an enhanced rate of pro-TAFI activation in these bleeding disorders.

The finding of elevated levels of TAFI-TAFIi antigen in patients with hemophilia A and VWD may, at least partly, explain the discrepancy between the observation of normal ELISA-

determined total TAFI antigen levels and reduced levels of functional pro-TAFI (proenzyme) in these patients.

As thrombin generation is seriously impaired and almost absent in hemophilia A, hemophilia B and at least type 3 VWD, the mechanism by which TAFI activation is enhanced is not readily apparent. Elegant work by Marx and co-authors (2002) has shown that plasmin can have a role in both TAFI activation and inactivation. The finding of a biphasic pattern of TAFI generation in an in vitro clot lysis model (the second peak occurred in the fibrinolytic phase) (Leurs et al 2003) could further indicate that plasmin may play a role in TAFI activation. This is supported by studies showing that plasmin-plasmin inhibitor (plasmin-alpha 2 antiplasmin) complex is increased in hemophilia A and B patients compared to controls (Greenfield et al 2002). Similar findings have previously been described in acute promyelocytic leukemia (APL), where a decrease in pro-TAFI was demonstrated together with normal total TAFI antigen. Since APL is associated with increased fibrinolysis due to augmented generation of plasmin, it can be postulated that the prolonged activation of pro-TAFI by plasmin causes its consumption (Meijers et al 2000b).

It can be speculated that cleavage of pro-TAFI to TAFI by plasmin leads to the down-regulation of fibrinolysis, which reduces the bleeding tendency in hemophilia A, hemophilia B and VWD. Plasmin can also proteolytically cleave pro-TAFI at an alternative site, leading to the generation of a 44.3 kD non-functional form. This can explain our finding that pro-TAFI was only partly transformed to TAFI and/or TAFIi - 35.8 kD forms. However, plasmin proteolytic generation of the 44.3 kD form can result in depletion of TAFI in plasma and induce an impairment of TAFI-dependent inhibition of fibrinolysis, which may promote the bleeding tendency in these patients. In this way, the TAFI activation pathway may play a critical role in the hemostatic balance in hemophilia and VWD.

OVERALL HEMOSTASIS AND TAFI-DEPENDENT FIBRINOLYSIS IN DIFFERENT DEFICIENT PLASMAS AFTER ADDITION OF VARIOUS DOSES OF rFVIIa (Novoseven) Paper VI

Recombinant factor FVIIa (rFVIIa) (Novo Seven) has been shown to be an efficient and safe agent for the treatment of patients with hemophilia A and B with inhibitors and for patients with FVII deficiency (Hedner and Glazer 1992, Negrier and Hay 1999, Scharrer 1999).

rFVIIa restores impaired thrombin generation and enables a secondary burst of thrombin, which is necessary for clot formation as well as for clot stability (Kjalke et al 2001). Restoring thrombin generation may also restore TAFI generation and induce a down-regulation of fibrinolysis (Lisman et al 2002)

From this *in vitro* study it seems that the OHP assay may be a promising tool for monitoring rVIIa, as previously indicated in a small *ex vivo* study (Antovic et al 2002).

There is no effect of rFVIIa on FXI deficient plasma, but OCP and OHP are lower in these samples compared to NPP. This could indicate that initial thrombin generation exists but that a full

thrombin burst is precluded by a lack of FXI that has to do with the generation of thrombin along the intrinsic pathway via factor FXI (Von dem Borne et al 1995).

Clotting did not occur in the FII deficient plasma, while OCP and OHP were low in FX deficient plasma, but addition of rFVIIa did not induce any response in these deficient plasmas. This confirms previous findings that prothrombin and FX are necessary for hemostatic effects of rFVIIa (Kawaguchi et al 2002).

The lowest dose of rFVIIa increased OCP and OHP in FVIII and FIX deficient plasmas. The increase in OHP reached a maximum in both plasmas after addition of 2.4μg/mL, while a further increase in the rFVIIa concentration did not lead to a further increase in OHP. This could indicate that therapeutic doses of rFVIIa should be adjusted to obtain this concentration of rFVIIa in plasma.

Interestingly, OCP and OHP in FXII deficient plasmas were higher than in NPP and increased further after addition of rFVIIa. It seems that OCP and OHP may be useful for the determination of hypercoagulable conditions associated with FXII deficiency (Mannhalter et al 1987), especially as other routine tests (e.g. aPTT) show the same pattern for both hypocoagulable conditions in FVIII, IX and XI deficiencies and hypercoagulability in FXII deficiency. Impaired fibrinolysis reportedly contributes to the thrombotic tendency in FXII deficiency (Rodeghiero et al 1991, Levi et al 1991) and our study also showed a down-regulation of fibrinolysis.

rFVIIa, even at very high concentrations, did not induce hypercoagulability in NPP. This finding confirms previous observations (Gallistil et al 1999) and could indicate that it is safe to use rFVIIa in patients without bleeding disorders.

Higher concentrations of rFVIIa (2.4 - 9.6 μg/mL) induced a down-regulation of fibrinolysis, making this similar to that in NPP, in FVIII and FXI deficient plasmas or even weaker in FIX deficient plasma. In FX deficient plasma, OFP and CLT failed to reach the values obtained in NPP.

TAFI has little or no effect on the down-regulation of fibrinolysis in FVIII and FIX deficient plasmas either before or after the addition of low concentrations of rFVIIa. This indicates that factors other than TAFI contribute to the regulation of fibrinolysis. It seems, however, that rFVIIa could down-regulate fibrinolysis through both TAFI-dependent and TAFI-independent mechanisms (e.g. clot stability due to increased generation of FXIIIa). It also seems that higher concentrations of rFVIIa augment the generation of TAFI and down-regulate fibrinolysis, in keeping with some previous in vitro studies (Lisman et al 2002) on rFVIIa inhibition of fibrinolysis through TAFI-mediated effects.

CONCLUSIONS

- TAFI contributes to an impairment of fibrinolysis in patients with APC resistance due to FV Leiden mutation; a decrease in pro-TAFI accompanied by normal total TAFI antigen could be an indirect proof of this.

- TAFI does not induce a further down-regulation of initially impaired fibrinolysis in pre-eclampsia, most probably due to its decrease induced by renal and hepatic impairment.

- TAFI does not induce an impairment of fibrinolysis, despite the presence of increased overall coagulation and hemostasis, in patients with type I diabetes mellitus and micro-vascular complications.

- The transformation of pro-TAFI to TAFI and TAFIi in hemophilia and von Willebrand's disease is most probably induced by plasmin and could partly counterbalance the up-regulation of fibrinolysis in these conditions.

- rVIIa improves overall hemostasis and fibrinolysis in hemophilia patients and in FVIII and FIX deficient plasma, but this improvement in overall fibrinolysis is only partly dependent on TAFI.

- The OHP assay seems to be a handy and inexpensive tool not only for the determination of overall hemostasis, coagulation and fibrinolysis but also for indirect estimation of TAFI-dependent fibrinolysis.

- TAFI obviously plays a role as a link between coagulation and fibrinolysis but since it is activated by both thrombin and plasmin and is also inactivated by plasmin, it seems that both enzymes could regulate TAFI's role and influence the TAFI-dependent regulation of fibrinolysis. TAFI's definitive role has therefore not yet been established.

- The determination of all *in vivo* forms of TAFI (pro-TAFI, TAFI, TAFIi and the 44.3 kD plasmin cleavage product), along with *ex vivo* measurement of clot lysis, will provide a much fuller understanding of TAFI-related processes in hypo- and hypercoaguable conditions, while the development of precise and accurate assays for *ex vivo* determination of the active form of the enzyme (TAFI) should be the top priority in the near future.

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