Introduction

Thrombin activatable fibrinolysis inhibitor (TAFI) is a zymogen present in plasma that can be activated (TAFIa) by thrombin or plasmin (1-4). Thrombomodulin (TM) acts as a cofactor stimulating over 1000-fold the activation by thrombin (5-7). Plasmin not only acts as an activator but can also inactivate TAFI and TAFIa by proteolytic cleavage at Arg302, Lys327 or Arg330 (8). TAFIa can cleave C-terminal lysine and arginine residues that become available when plasmin partially degrades fibrin (9-13). These residues are binding sites for plasminogen and plasmin and essential for the upregulation of fibrinolysis as the activation of plasminogen is accelerated (14-16) and fibrin bound plasmin becomes more resistant to inactivation by plasmin inhibitor (PI), previously $\alpha_2$-antiplasmin (17, 18). TAFIa blocks this upregulation and causes, possibly in this way, a prolongation of lysis times (9, 10, 12).

Study of seven plasminogen activators in an internal clot lysis model

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Summary

TAFIa was shown to attenuate fibrinolysis. In our in vitro study, we investigated how the inhibitory effect of TAFIa depended on the type and concentration of the plasminogen activator (PA). We measured PA-mediated lysis times of plasma clots under conditions of maximal TAFI activation by thrombin-thrombomodulin in the absence and presence of potato carboxypeptidase inhibitor. Seven different PAs were compared comprising both tPA-related (tPA, TNK-tPA, DSPA), bacterial PA-related (staphylokinase and APSAC) and urokinase-related (tcu-PA and k2tu-PA) PAs. The lysis times and the retardation factor were plotted against the PA concentration. The retardation factor plots were bell-shaped. At low PA concentrations, the retardation factor was low, probably due to the limited stability of TAFIa. At intermediate PA concentrations the retardation factor was maximal (3-6 depending on the PA), with TNK-tPA, APSAC and DSPA exhibiting the strongest effect. At high PA concentrations, the retardation factor was again low, possibly due to inactivation of TAFIa by plasmin or to a complete conversion of glu-plasminogen into lys-plasminogen. Using individual plasmas with a reduced plasmin inhibitor activity (plasmin inhibitor Enschede) the bell-shaped curve of the retardation factor shifted towards lower tPA and DSPA concentrations, but the height did not decrease. In conclusion, TAFIa delays the lysis of plasma clots mediated by all the plasminogen activators tested. This delay is dependent on the type and concentration of the plasminogen activator, but not on the fibrin specificity of the plasminogen activator. Furthermore, plasmin inhibitor does not play a significant role in the inhibition of plasma clot lysis by TAFI.

Keywords
TAFI, internal lysis, plasminogen activator, plasmin inhibitor

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Given what is currently known about the mechanism of action of TAFI, we speculated that TAFIa would lead to variable inhibition of plasminogen activator (PA)-induced fibrinolysis, depending not only on the concentration of the PA but also on the type of the PA. Different PAs operate through distinct mechanisms that rely to a varying extent on fibrin binding, on the C-terminal lysine-residues, on the conversion of glu- to lys-plasminogen and on the cofactor effect of soluble fibrin degradation products including (DD)E (19-24).

Up to now, tissue-type PA (tPA) has been the PA of choice to study the effect of TAFI on fibrinolysis. TAFI-related retardation of tPA-mediated lysis has been described in purified systems (25), in clots prepared from diluted (10, 12, 26-28) and undiluted plasma (11), in whole blood clots (29) and in several animal models (9, 30-32). Walker et al. (23) have recently studied the DSPA cofactor activities of TAFIa-treated soluble fibrin degradation products but still more insight is needed to understand the mode of action of TAFI with different plasminogen activators. Under our in vitro model conditions, we investigated how internal plasma clot lysis is affected by the PA concentration and how TAFI changes the lytic activities of different PAs. Additional models, such as a model of external clot lysis, will be necessary to fully understand the multiple inter-linked mechanisms of TAFI and allow extrapolations to clinical practice of thrombolytic therapy.

A variety of plasminogen activators was tested in this study, from tPA-related plasminogen activators such as tPA itself, Tenecteplase (TNK-tPA) and Desmodus rotundus salivary plasminogen activator (DSPA) to bacteria-related PAs such as staphylokinase (STA) and anisoylated plasminogen-streptokinase activator complex (APSAC), and urokinase-related PAs such as two-chain urokinase-type PA (tcu-PA) and Amediplase (k2tu-PA). The properties of these plasminogen activators have been described before (33, 34). To study the role of plasmin inhibitor in the TAFI-related retardation of lysis we used citrated plasma from a homozygous and a heterozygous individual for plasmin inhibitor Enschede (35, 36). This is a dysfunctional plasmin inhibitor molecule, which exhibits no inhibition of plasmin.

We demonstrated that TAFI delays clot lysis for all the PAs tested. No contribution of the plasmin inhibitor to the maximal TAFI-related retardation was found.

Materials and methods

**Materials**

The tcu-PA (Ukidan) preparation was purchased from Serono. Staphylokinase (recombinant Sak42D) was a gift from Dr. H. R. Lijnen (University of Leuven). Other PAs were kindly supplied by the companies listed below: tPA (Actilyse), by Boehringer Ingelheim; TNK-tPA, by Genentech Inc; k2tu-PA (Amediplase), by Menarini; DSPA, by Schering AG; Anistreplase (Eminase), by Tramedico BV. Anistreplase concentration in mg/ml was calculated by using 1 IU/mg as specific activity. The tcu-PA concentration was determined spectrophotometrically using A_{280} = 100 %, 1 cm = 13.6 and its specific activity was 175,000 IU/mg. Human thrombin and potato carboxypeptidase inhibitor (PCI) were acquired from Sigma and Calbiochem, respectively. Rabbit lung TM, with a specific activity towards thrombin of 1.2 units/µg, was supplied by American Diagnostica Inc. Citrated platelet-poor plasma from ten healthy volunteers was obtained from the blood bank. A plasma pool was prepared and used in all normal plasma experiments. Citrated platelet-poor plasmin inhibitor (PI) deficient plasma from both a heterozygous and a homozygous individual with plasmin inhibitor Enschede was used when indicated.

**TAFI activation by thrombin-TM complex**

Pooled blood bank plasma was dialysed against 50 mM Hapes, 100 mM NaCl, 20 mM tri-sodium citrate, pH 7.4 to remove phosphate ions and 100 µl were added to the wells of a microtitre plate containing 25 µl of a mix per well. The mix was composed of thrombin (3.3 NIH units/ml), tPA (0.20 µg/ml), CaCl₂ (20 mM), PCI (0 or 30 µg/ml) and variable TM concentrations (0-1.8 units/ml) in a 50 mM Hapes buffer, pH 7.4 containing 0.1% w/v BSA. The concentrations between brackets refer to the final concentrations in the clotted plasma. The clots, prepared at room temperature, were immediately covered with 50 µl paraffin oil (Merck - 107162) and the microtitre plate was placed in the incubation chamber pre-warmed at 37°C of a TECAN Sunrise Microplate-reader. The optical density was measured continuously at 405 nm. Lysis time is defined as the time point corresponding to a 50% decrease in optical density. The lysis time was determined by fitting lysis profiles with a sigmoidal regression equation.

**TAFIa effect on PA-mediated clot lysis**

Dialysed pooled blood bank plasma was added to the wells of a microtitre plate according to the procedure described above. A final concentration of 0.60 u/ml of TM was chosen in order to obtain maximal retardation of clot lysis under the assay conditions and the concentration of each of the seven PAs used was varied. The antifibrinolytic effect of TAFIa was described with the retardation factor, defined as lysis time in the absence of PCI divided by the lysis time in the presence of PCI. All the data result from the mean values of 3 independent measurements.

**TAFIa effect on PA-mediated clot lysis in plasmin inhibitor Enschede plasma**

Citrated plasmas from a heterozygous and a homozygous individual with PI Enschede were used in the procedure described above using both tPA and DSPA as plasminogen activators.
### Plasmin inhibitor activity

Plasmin inhibitor activity was determined as described by Billing Clason et al. (37) and expressed in % pooled plasma.

### TAFI antigen concentration

A sandwich-type enzyme-linked immunosorbent assay (ELISA) was used to determine TAFI antigen levels in dialysed pooled blood bank plasma and in plasma of PI Enschede individuals. This ELISA made use of sheep polyclonal antibodies against TAFI (Affinity Biologicals Inc.). The levels were expressed in % pooled plasma, using as calibrator a citrated plasma pool composed of 40 normal individuals.

### Results

#### TAFI activation by thrombin-TM complex

The tPA-mediated lysis of plasma clots prepared in the presence of variable TM concentrations is depicted in Figure 1A. Prolongation of lysis times by TM was dose-dependent in the absence of PCI. Even the lowest TM concentration tested (0.01 unit/ml) produced a considerable prolongation of lysis time. At a TM concentration of 0.06 unit/ml the maximum effect was achieved, resulting in a 3-fold prolongation of lysis time (Fig. 1B). A small retardation of clot lysis by PCI was observed in the absence of TM that is probably due to TAFI activation by thrombin. In the presence of PCI there was no effect of the TM concentration on lysis time (Fig. 1B). The prolongation of lysis time that could be quantitatively inhibited by PCI was attributed to TAFIa. The TAFI antigen concentration of the plasma pool was 96.4 ± 13.7%.

#### TAFIa effect on PA-induced lysis of plasma clots

Figure 2 shows the dependence of lysis times on the PA concentration in the internal clot lysis model with maximal TAFI activation. Seven different PAs were tested in the presence and absence of PCI (Fig. 2A-G). For all the PAs, a decrease in lysis time with increasing PA concentrations was observed. The difference between the dose-response curves, in the absence and presence of PCI, depicts the potency of TAFIa antifibrinolytic activity, which was quantified through the retardation factor (Fig. 2H-N). At low PA concentrations, long lysis times (100-500 min) were found and low retardation factors were derived. At intermediate PA concentrations the retardation factor became maximal. The retardation factor declined again at high PA concentrations, in particular when short lysis times (<10 min) were obtained. All PAs generated bell-shaped retardation factor curves over variable concentration ranges. The maximal retardation factors were between 3-6. A powerful TAFI-related retardation (factor 5.8) was observed for TNK-tPA. The APSAC and DSPA-mediated plasma clot lysis was also considerably influenced by TAFIa (factor 5.1 and 5.0, respectively). tPA, the most common PA used to study the effects of TAFI on clot lysis, and tcu-PA showed an intermediate maximal TAFIa-dependent retardation factor (factor 3.4 for both). The weakest effect of TAFIa was observed for STA and k2tu-PA (factor 3.1 and 2.9, respectively).

#### The effect of TAFIa on PA-mediated clot lysis in plasmin inhibitor Enschede plasma

By using plasmin inhibitor Enschede plasma the role of plasmin inhibitor in the antifibrinolytic effect of TAFI was examined.
Figure 2: Effect of TAFI on the internal lysis of plasma clots induced by seven PAs in the absence (■) and in the presence (□) of potato carboxypeptidase inhibitor (PCI). A. Tissue-type PA (tPA); B. Tenecteplase (TNK-tPA); C. Desmodus rotundus salivary PA (DSPA); D. Staphylokinase (STA); E. Anisoylated plasminogen-streptokinase activator complex (APSAC); F. Two-chain urokinase-type PA (tcu-PA); G. Amediplase (k2tu-PA). Retardation factor variations with PA concentration. H. tPA; I. TNK-tPA; J. DSPA; K. STA; L. APSAC; M. tcu-PA; N. k2tu-PA. Lysis time was defined as the time point corresponding to a 50% decrease in OD 405 nm and the retardation factor as the lysis time in the absence of PCI divided by the lysis time in the presence of PCI. (line) Retardation factor baseline.
Citrated plasma from both a heterozygous and a homozygous individual for PI Enschede was used. The PI functional activity was 7 ± 4% for the homozygous individual and 51 ± 2% for the heterozygous individual (% normal pool plasma). TAFI antigen levels did not differ significantly for the two individuals (73 ± 4 and 79 ± 9%, respectively). Figure 3 shows that replacement of the pooled normal plasma by the PI Enschede heterozygous or homozygous individual plasma resulted in a shift towards shorter lysis times, both in the presence and in the absence of PCI and both for tPA (Fig. 3A) and for DSPA (Fig. 3B). Employment of the PI Enschede plasmas also resulted in a shift of the bell-shaped curves of the retardation factors towards lower tPA concentrations (inset, Fig. 3A and 3B). The maximal retardation factor remained constant when pooled normal plasma was replaced by PI Enschede heterozygous plasma for both tPA and DSPA. When PI Enschede homozygous plasma was used the maximal retardation factor increased somewhat for both PAs tested (3 to 4.5 for tPA and 5 to 8 for DSPA). These results indicated that PI does not play a major role in the retardation of the clot lysis by TAFI.

**Discussion**

TAFIa, the activated form of thrombin activatable fibrinolysis inhibitor (TAFI), a plasma basic procarboxypeptidase, which is able to cleave C-terminal lysine and arginine residues from partially degraded fibrin (1-4). These C-terminal lysine residues act as binding sites for plasminogen, and as fibrin is degraded by plasmin their number increases, enhancing plasminogen activation. TAFIa can delay lysis by preventing this enhancement of plasminogen activation. It has also been shown that the binding of plasmin to fibrin renders plasmin less susceptible to inactivation by plasmin inhibitor (17, 18). Yet, it is not known whether TAFI can delay lysis by preventing the protection of plasmin from inactivation by plasmin inhibitor.

Information concerning the effect of TAFI on clot lysis with different PAs is limited as most observations were done in systems with tPA-mediated lysis. Therefore, we performed this study to assess the antifibrinolytic potential of maximally activated TAFI in the lysis of plasma clots mediated by several types and concentrations of plasminogen activators. In addition, we were also interested in the role of plasmin inhibitor in TAFIa-related retardation of the internal lysis of a plasma clot.

Maximal TAFI activation was achieved by clotting plasma with thrombin and an optimal concentration of thrombomodulin in the presence of calcium ions (Fig. 1) (11). Seven different PAs (tPA, TNK-tPA, tcu-PA, APSAC, DSPA, k2tu-PA and STA) were tested with this model revealing that the presence of TAFIa prolonged lysis times for all PAs. The maximal retardation factor varied between 3- and 6-fold depending on the plasminogen activator. Moreover, we found that the effect of TAFIa was dependent on the PA concentration (bell-shaped curves in Fig. 2H-N).

The effect was small at low PA concentrations (lysis times > 100 min), which we attributed to the restricted stability of TAFIa. Indeed, we have previously investigated the stability of TAFIa activity under similar conditions (11). TAFIa activity declined rapidly, falling back to baseline levels within 60 min. Recently, low concentrations of PCI were shown to stabilize TAFIa, in particular at low tPA concentrations (27, 38). However, here we used a considerably higher PCI concentration which fully inhibits TAFIa. Altogether, this leads us to conclude
that at low PA concentrations other fibrinolysis inhibitors play a more prominent role in our plasma clot lysis system than TAFI.

At high PA concentrations (lysis times < 10 min) the effect of TAFIa was also low. We hypothesised that at these high PA concentrations elevated amounts of plasmin were generated. In our system, TAFI is activated by the thrombin/TM, which makes TAFIa available for proteolytic inactivation by plasmin (8). Another possibility is that at high PA and high plasmin concentrations glu-plasminogen is fully converted into lys-plasminogen. Lys-plasminogen is produced by plasmin cleavage of the NH₂-terminal part of glu-plasminogen and presents a more open conformation than native glu-plasminogen. Moreover, the rate of activation of lys-plasminogen to plasmin is greater than that of glu-plasminogen. TAFIa seems to be specific to the glu-form of plasminogen (13). The finding that in the absence of plasmin inhibitor activity and hence higher plasmin concentration the decrease in the effect of TAFIa occurred at lower tPA and DSPA concentrations corroborates the attribution of this decrease to a high plasmin activity (Fig. 3).

The strongest TAFIa effects were observed for TNK-tPA and DSPA, two highly fibrin specific plasminogen activators. A moderate effect was, however, found for STA, which is also highly fibrin specific. Moreover, APSAC, which has low fibrin specificity, was amongst the PAs that displayed the strongest effect. This suggests that the magnitude of the inhibitory effect of TAFIa on internal plasma clot lysis does not depend strongly on the fibrin specificity of the plasminogen activator used.

Plasmin bound to C-terminal lysine residues is partially protected from inhibition by PI. TAFIa eliminates these binding sites and might therefore be able to modulate plasmin activity. Hence, TAFIa might rely on the rapid inhibition of unbound plasmin by PI to achieve its maximal lysis retardation effect. If this is the case, decreasing PI activity would lead to an increase in the plasmin concentration and to a faster lysis as well as a smaller TAFIa effect.

Keeping this in mind, we studied the role of PI in the mechanism of TAFIa retardation of clot lysis using plasmas from a heterozygous and a homozygous individual for PI Enschede. This dysfunctional PI molecule displays complete immunological identity with normal PI and has normal plasminogen-binding properties, but an abnormal functional activity resulting in a fully defective inhibition of plasmin (35, 36).

The bell-shaped retardation factor curves shifted towards lower PA concentrations for both tPA and DSPA (Inset, Fig. 3A and 3B, respectively). The maximal TAFIa-related retardation factor remained constant for the heterozygous PI Enschede individual and increased somewhat for the homozygous individual. So, we can conclude that PI does not play a significant role in the inhibition of plasma clot lysis by TAFI.

The increase in maximal TAFIa-related retardation factor might be explained if this individual was also homozygous for the more stable and active variant of TAFI (325 Ile/Ile) (39). Therefore, TAFI antigen for the PI Enschede individuals was determined by two immunological assays previously described by Gils et al. (40) (not shown). The results showed that both the homozygous and the heterozygous PI Enschede individuals were not homozygous for Ile325. Another possible explanation would consist of TAFIa being able to remove the C-terminal lysine present in PI, interfering in this way with the rapid inactivation of plasmin (41). However, recent work has demonstrated that Lys 436 in the plasmin inhibitor and not the C-terminal lysine residue (Lys 452) is necessary for the interaction with plasmin (42). This implies that the increase in retardation factor remains still unexplained.

In conclusion, TAFI was found to affect the lysis of plasma clots mediated by all the PAs tested in broad concentration intervals. Also, the extent of the inhibitory effect of TAFI in the internal lysis of plasma clots was not determined by the fibrin specificity of the plasminogen activator. Moreover, the inhibitory effect of TAFIa on clot lysis did not depend on the presence of PI.

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Abbreviations

The abbreviations used are: TAFI, thrombin activatable fibrinolysis inhibitor; TAFIa, activated thrombin activatable fibrinolysis inhibitor; TM, thrombomodulin; PCI, potato carboxypeptidase inhibitor; PA, plasminogen activator; tPA, tissue-type plasminogen activator; TNK-tPA, tenecteplase; DSPA, Desmodendus rotundus salivary plasminogen activator; STA, staphylokinase; APSAC, anisoylated plasminogen-streptokinase activator complex; tcu-PA, two-chain urokinase-type plasminogen activator; k2tu-PA, amediplase; PI, plasmin inhibitor, previously α₂-antiplasmin.
References


