Development of ELISAs Measuring the Extent of TAFI Activation

Erik Ceresa, Els Brouwers, Miet Peeters, Christina Jern, Paul J. Declerck, Ann Gils

Objectives—To date, quantitation of TAFI antigen levels has been mainly focused on “total” antigen levels and has been shown to yield ambiguous results because of the existence of different isofoms and various degrees of activation. Our objective was to develop assays that allow measuring the extent of TAFI activation.

Methods and Results—A variety of enzyme-linked immunosorbent assays (ELISAs) were evaluated for their preferential reactivity toward TAFI before and after activation, and toward the recombinantly expressed activation peptide. Three ELISAs with distinct reactivities were selected: recognizing either exclusively nonactivated TAFI, the released activation peptide, or exclusively TAFIa (activated TAFI). Evaluation of TAFI activation during clot lysis revealed that decreases of TAFI levels are associated with increases of the released activation peptide and TAFIa levels. In addition, antigenic measurement of TAFIa parallels activity measured by chromogenic assay. Analyzing plasma samples revealed that subjects with hyperlipidemia had significantly higher plasma levels of both the activation peptide (109.2 versus 95.5; P<0.001) and TAFIa (112.1 versus 103.3; P=0.03), and not of TAFI antigen (92.5 versus 87.9; P=0.07) (results in % of plasma pooled from normal lipoproteinemic subjects).

Conclusion—ELISAs that allow to measure the extent of TAFI activation were developed. These ELISAs constitute more sensitive markers in studies on the relationship between TAFI and cardiovascular diseases. (Arterioscler Thromb Vasc Biol. 2006;26:423-428.)

Key Words: fibrinolysis • monoclonal antibodies • TAFI • ELISA • extent of TAFI activation

The proenzyme thrombin activatable fibrinolysis inhibitor (TAFI; procarboxypeptidase U [proCPU]) can be activated to the active enzyme (TAFIa; carboxypeptidase U [CPU]) by trypsin-like enzymes such as thrombin, plasmin, or the thrombin/thrombomodulin complex (T/TM).1–3 On activation of TAFI (Phe1-Val401; 56 kDa), the activation peptide (AP) (Phe1-Arg92; 20 kDa, 4 N-linked glycosylations4) is released from the catalytic domain (TAFIa, Ala93-Val401; 36 kDa). Subsequently, TAFIa is inactivated through a conformational change to an inactive form (TAFIai), followed by proteolytic cleavage resulting in fragments of 25 and 11 kDa.5 TAFIa exerts an antifibrinolytic effect by removing C-terminal lysine residues from fibrin, resulting in a decreased plasmin formation and a retardation of clot lysis.6 TAFIa is highly unstable in vitro (half-life at 37°C between 8 to 15 minutes).7–10

Different clinical studies have investigated the possible relationship between TAFI and cardiovascular events. A positive correlation was found between TAFI levels and the risk for coronary artery disease,11 venous thrombosis,12 angina pectoris,13 and ischemic stroke.14 However, another study revealed that elevated TAFI may be protective against myocardial infarction.15 A possible explanation for these contradictory results was that the different studies used different methods for TAFI determination.16 Silveira et al used quantitative activation of the zymogen followed by determination of the total enzymatic activity with a chromogenic assay. Van Tilburg et al measured TAFI levels with an electroimmunoassay. A commercially available kit from Milan Analytica, based on affinity-purified sheep anti-TAFI IgG, was used in the studies of Morange et al and Juhan-Vague et al. Finally, Montaner et al obtained their results with a commercially available Zymutest TAFI antigen kit from Hyphen BioMed.14 Malyszko et al found that elevated TAFI (Actichrome TAFIa; American Diagnostica) might be a link in the pathogenesis of impaired fibrinolysis in diabetic nephropathy and thus atherosclerosis progression.17 Saibeni et al found that increased TAFI levels (enzyme-linked immunosorbent assay [ELISA]; Affinity Biologicals) might contribute to the prothrombotic state observed in inflammatory bowel syndrome through the induction of hypofibrinolysis.18 Eichinger et al showed that patients with higher TAFI levels (ELISA; American Diagnostica) have a higher risk for recurrent venous thromboembolism.19 To date, quantitation

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From the Laboratory for Pharmaceutical Biology and Phytopharmacology (E.C., E.B., M.P., P.J.D., A.G.), Faculty of Pharmaceutical Sciences, Katholieke Universiteit Leuven, Belgium; and The Institute of Clinical Neuroscience (C.J.), Sahlgrenska Academy, Göteborg University, Göteborg, Sweden.
Correspondence to Ann Gils, Laboratory for Pharmaceutical Biology and Phytopharmacology, Faculty of Pharmaceutical Sciences, Katholieke Universiteit Leuven, O&N2-PB824, Herestraat 49, B-3000 Leuven, Belgium. E-mail Ann.Gils@pharm.kuleuven.be
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423
of TAFI antigen levels, as a putative risk marker for cardiovascular events, has been mainly focused on “total” antigen levels (either by ELISA or by activity assays after full activation of TAFI), reporting levels of 4 to 15 μg/mL. However, it should be stressed that in most of these studies the used immunologic assays were not validated with respect to putative differential reactivities toward different isoforms and fragments of TAFI. Therefore, it cannot be excluded that apparently increased or decreased TAFI levels associated with particular disease states is not caused by differences in total TAFI protein levels but rather to differences in isoforms or differences in extent of activation. In this respect, it is important to note that recent studies, revealing a threshold or differences in extent of activation. In this respect, it is important to note that recent studies, revealing a threshold or differences in extent of activation (ie, either by measuring the released activation peptide or by measuring TAFIa) could represent a more relevant parameter.

In this study, we screened a variety (>1500) of monoclonal antibody (MA)-based ELISAs for their preferential reactivity toward TAFI before and after activation and toward the recombinantly expressed AP. This allowed us to select 3 ELISAs with distinct properties, recognizing either exclusively nonactivated TAFI, the released AP, or exclusively TAFIa.

Methods
Please see http://atvb.ahajournals.org for detailed Methods.

Results
Construction, Selection, and Characterization of Sandwich-Type ELISAs for Detection of Different TAFI Fragments
Forty monoclonal antibodies were pair-wise tested for their reactivity toward pTAFI before and after activation. Two groups of ELISAs could be distinguished: one group reacts only with intact, nonactivated TAFI and a second group reacts only with TAFI after it has been activated. Within this second group, 2 subgroups were identified: one subgroup reacted with the recombinant AP, whereas the other subgroup did not. Based on these preferential reactivities toward different TAFI fragments, 3 ELISA combinations were selected for further characterization: MA-T12D11/MA-T30E5-HRP, reacting with nonactivated TAFI, MA-T12D11/MA-T18A8-HRP, reacting with TAFI after activation as well as with the recombinant AP, and MA-T30E5/MA-T17D7-HRP, reacting with TAFI after activation but not with the recombinant AP.

Preliminary experiments, using Western blotting (data not shown), indicated that the epitopes of MA-T12D11 and MA-T18A8 reside in the region of the AP, whereas for MA-T17D7 the epitope resides in the 36-kDa fragment. Because MA-T30E5 does not work in blotting experiments, no information on the binding region is available.

For calibration of the selected ELISAs, 2 standards were developed, ie, So and S10 (cfr methods). The response of S10 in the MA-T12D11/MA-T30E5-HRP ELISA was 11 ± 3.2% compared with So whereas the response of So in the MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-T17D7-HRP ELISA was 0.7 ± 0.6% and 4.0 ± 2.1% compared with S10 respectively. Only MA-T12D11/MA-T18A8-HRP reacted with the recombinant AP: 100 μg/mL recombinant AP gave a response of 101 ± 11% compared with S10. Typical standard curves of the three ELISAs are shown in Figure 1.

TAFI fragments formed on activation of pTAFI with T/TM are shown in the inset of Figure 2A. At 5 minutes, the 56-kDa fragment of TAFI is completely converted to the 36-kDa fragment. Subsequently, at 10 minutes, the degradation products of 25 and 11 kDa are formed and eventually, and after 30 minutes, the 36 kDa catalytic domain of TAFI has a theoretical Mr of 19.4 kDa (with carbohydrate). In agreement with the literature, no information on the binding region is available.

Figure 1. Typical standard curves of the 2 developed standards S0 (●) and S10 (○). In MA-T12D11/MA-T30E5-HRP (A), in MA-T12D11/MA-T18A8-HRP (B), and in MA-T30E5/MA-T17D7-HRP (C). Y-axis represents the OD at 430 nm and X-axis represents the different plasma dilutions ranging from 1:2560 to 1:40 (of which 1:320, 1:160, 1:80, and 1:40 dilutions are indicated). (●) represents the reactivity of recombinant AP applied at a concentration equimolar to that in plasma (ie, 10 μg/mL TAFI contains 3.57 μg/mL AP). The 1/40 dilution of the standards corresponds with 90 ng/mL TAP.
Ala147Thr325 versus 15.9/0.2 minutes for TAFI-
maximal with a half-life of 14.0
MA-T30E5/MA-T17D7-HRP decreased after reaching a
Ala147Ile325) (see http://atvb.ahajournals.org). The response in
activation, reaching a minimum after 30 minutes (Figure 2B) in MA-T12D11/MA-T30E5-HRP decreases on
activation reaching a maximum at 5 minutes, which is maintained to 20
minutes (74 ± 26-fold increase, P < 0.0001 versus t = 0 minutes), followed by a slow decrease. The response in MA-T30E5/MA-T17D7-HRP increases on activation reaching a maximum at 5 minutes (70 ± 10-fold increase, P < 0.0001 versus t = 0 minutes) and then decreases reaching a minimum after 60 minutes ( < 2%). Similar results were obtained using plasmin to activate TAFI (data not shown) and using 2 recombinant TAFI isoforms (ie, TAFI-Ala147Thr325 and TAFI-Ala147Ile325) (see http://atvb.ahajournals.org). The response in MA-T30E5/MA-T17D7-HRP decreased after reaching a maximum with a half-life of 14.0 ± 0.2 minutes for TAFI-Ala147Thr325 versus 15.9 ± 0.3 minutes for TAFI-Ala147Ile325 (P < 0.05), respectively, in line with the increased stability of TAFIa of the Ile325 isoform.8

TAFIa activity in plasma (Figure 2B) reaches a maximum after 10 minutes and then decreases, reaching a minimum after 30 minutes. The response of pooled human plasma (Figure 2B) in MA-T12D11/MA-T30E5-HRP decreases on activation, reaching a minimum after 30 minutes ( < 15%, 7.6 ± 2.6-fold decrease, P < 0.0001 versus t = 0 minutes). In contrast, the response in MA-T12D11/MA-T18A8-HRP increases on activation reaching a maximum plateau after 30 minutes (60 ± 18-fold increase, P < 0.0001 versus t = 0 minutes). The response in MA-T30E5/MA-T17D7-HRP increases on activation reaching a maximum after 10 minutes (24 ± 14-fold increase, P = 0.002 versus t = 0 minutes), immediately followed by a decrease, reaching a minimum after 60 to 120 minutes ( < 25%).

Taken together, all aforementioned results demonstrate that MA-T12D11/MA-T30E5-HRP preferentially reacts with intact nonactivated TAFI, MA-T12D11/MA-T18A8-HRP reacts with the released AP and MA-T30E5-T17D7-HRP reacts with TAFIa and/or TAFIai.

**Quantitation of TAFI in Plasma**

A linear dose–response curve was observed in all 3 ELISAs when plasma (either nonactivated for the MA-T12D11/MA-T30E5-HRP ELISA or activated for the MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-T17D7-HRP ELISA) was diluted between 1/40-fold and 1/2560-fold. Within the linear portion of these curves, correlation coefficients exceeded 0.98.

For recoveries, intra-assay, inter-assay, and interdilution coefficients of variation and detection limits of the 3 ELISAs (please see http://atvb.ahajournals.org).

**Application of the Developed Assays In Vitro and In Vivo**

During clot formation and dissolution, TAFIa activity was measured using the chromogenic assay and antigen levels of TAFI and TAFI fragments were followed using the 3 described ELISAs (Figure 3).

As reported before,28 a biphasic pattern of TAFIa activity generation during in vitro clot lysis was observed. A first TAFIa activity peak was found immediately after clot formation, and a second peak during clot dissolution. Our data show that in the presence of 0.5 nM TM (Figure 3A), TAFI antigen levels (measured with MA-T12D11/MA-T30E5-
HRP) decreased, whereas released AP antigen levels (measured with MA-T12D11/MA-T18A8-HRP) increased concomitantly, both in a similar biphasic pattern. Antigen levels of TAFIa/ai (measured with MA-T30E5/MA-T17D7-HRP) increased during clot formation, followed by a decrease and increased slightly during clot dissolution, followed by a decrease. Under these conditions, TAFI antigen levels decreased, in a biphasic pattern, to 83±16% after clot formation and to 49±8.6% after clot dissolution; the released AP levels increased, in a biphasic pattern, to 25.1±7.0% after clot formation and to 47±5.3% after clot dissolution. TAFIa/ai antigen levels increased to 27±3.5% during clot formation and to 7.3±0.3% during dissolution. Strikingly, the biphasic changes as observed in the ELISAs coincide with the biphasic pattern observed in the TAFIa activity assay.

As reported before,8 higher TM concentrations (Figure 3A versus Figure 3B) increased the height and width of the first activity peak. With 5 nM TM (Figure 3B), the biphasic pattern was not observed. TAFI antigen levels immediately decreased, in a biphasic pattern, to 3.2±1.2% after clot formation, released activating peptide antigen levels immediately increased to 108±15.2% after clot formation. TAFIa/ai antigen levels increased transiently to 79±3.7% during clot formation. Again, these data demonstrate the differential reactivities of the ELISAs and their respective association with the activity assay. A decrease of intact TAFI antigen levels is associated with an increase in released AP antigen levels and with a transient increase of TAFIa/ai antigen levels.

The in vivo clearance of the released AP and TAFIa/ai was determined in female Balb/c mice using MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-T17D7-HRP, respectively. The biological half-lives of the released AP and TAFIa/ai were 27±5.4 and 11±3.9 minutes, respectively.

Plasma samples of 13 healthy individuals were analyzed in the different ELISAs, before and after 10 minutes of activation with T/TM (cfr chromogenic assay) (http://atvb.ahajournals.org).

Quantitation of TAFI and TAFI Fragments in a Swedish Study Population

Characteristics of the study population (n=300) are given in Methods and Materials (http://atvb.ahajournals.org). The plasma levels of TAFI and the released AP and TAFIa were 92.1% (median; interquartiles=78.8% to 103.2%), 106.5% (median; interquartiles=93.0% to 118.2%), and 110.9% (median; interquartiles=96.9% to 132.1%), respectively. TAFI and TAFI fragments (TAFI variables) did not show a significant correlation to age and there were no significant gender differences except a lower plasma level of the activation peptide in men compared with women (103.8% versus 110.7%; P=0.01). A significant correlation was observed between the activation peptide and intact TAFI (r=0.39, P<0.001), between TAFIa and the activation peptide (r=0.19, P=0.001), but not between TAFIa and intact TAFI. Strikingly, intact TAFI, TAFIa, and the activation peptide showed significant correlations to cholesterol (ie, r=0.15, P<0.05; r=0.12, P<0.05 and r=0.27, P<0.001, respectively) and triglycerides (ie, r=0.14, P<0.05; r=0.14, P<0.05; and r=0.23, P<0.001, respectively), whereas no significant associations were detected for high-density lipoprotein, insulin, blood pressure, or body mass index, with the exception for a correlation between the activation peptide and body mass index (r=0.13, P=0.03). Subjects with hyperlipidemia had significantly higher plasma levels of both the activation peptide (109.2 versus 95.5; P<0.001) and TAFIa (112.1 versus 103.3; P=0.03), whereas the difference for intact TAFI antigen did not reach the conventional level of statistical significance (92.5 versus 87.9; P=0.07). Twenty-eight subjects were on lipid-lowering therapy. If they were excluded from the analysis, similar correlations between the 3 TAFI variables and cholesterol as well as triglycerides were observed, and differences between subjects with hyperlipidemia and normolipidemia remained (activation peptide 109.9 versus 95.5, P<0.001; TAFIa 115.0 versus 103.3, P=0.01; intact TAFI 92.6 versus 87.9, P=0.03). There were no significant differences for any TAFI variable with regard to hypertension, diabetes, or smoking status (P>0.2 throughout). The activation peptide showed significant correlations to tissue-type plasminogen activator antigen (r=0.17, P<0.01), plasminogen activator inhibitor-1 (r=0.19, P<0.01), and fibrinogen (r=0.18, P<0.01), and an inverse correlation to tissue-type plasminogen activator activity (r=−0.17, P<0.01), whereas intact TAFI and TAFIa revealed no significant correlation.

Discussion

TAFIa is formed on activation of TAFI with T/TM or plasmin by release of the activation peptide and exerts an antifibrinolytic effect. Two polymorphisms (ie, Ala147Thr and Thr325Ile), resulting in the existence of 4 different isofoms, have been described.28,30 It has been suggested that increased TAFI plasma levels or increased activation is associated with cardiovascular events such as thrombosis, angina pectoris, and myocardial infarction.11–15 Therefore, different clinical studies have aimed to investigate the relationship between TAFI and cardiovascular events, based on the determination of TAFI levels in plasma. TAFI levels are determined either by immunologic assays, presumed to measure “total” TAFI antigen but not characterized in detail, or by activation of thezymogen followed by determination of total enzymatic activity reflecting total activatable TAFI levels.11–15 Alternatively, clot lysis experiments31 or the use of fluorescently labeled assays is partially dependent on the Thr325 polymorphism, it should be realized that interpretation of TAFI antigen levels as determined with noncharacterized immunologic assays is also quite ambiguous because TAFI can occur in different forms (TAFI, TAFIa, TAFIai, fragmentation products of TAFIai, released AP). Therefore, there is currently a strong need for well-characterized immunooassays for the evaluation of the levels of different TAFI forms. In addition, immunologic measurement of the extent of TAFI activation, either by measurement of the released AP or by measurement of TAFIa (ie, TAFIa and/or TAFIai), could be a more relevant parameter. Therefore, our objective was to develop well-
characterized assays that allow measurement of the extent of TAFI activation.

In this study, a panel of sandwich-type ELISAs was screened for their reactivity toward TAFI before and after activation. Three types of ELISAs could be distinguished. From the group of ELISAs, exclusively reacting with intact nonactivated TAFI, MA-T12D11/MA-T30E5-HRP was selected for further characterization. The response of purified TAFI or human plasma in MA-T12D11/MA-T30E5-HRP immediately decreases on activation, reaching a minimum at a time point corresponding with the time of exhaustion of activatable TAFI (Figure 2B). Taking all evidence together, including Western blotting experiments (data not shown), it can be concluded that this particular ELISA specifically reacts with intact, nonactivated TAFI.

From the group of ELISAs reacting with TAFIa, 2 ELISAs were selected, ie, MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-T17D7-HRP. These ELISAs reacted differentially on activation of TAFI (in plasma or purified). The response in MA-T12D11/MA-T18A8-HRP increases reaching a maximum plateau whereas the increased response on activation in the MA-T30E5/MA-T17D7-HRP is immediately followed by a strong decrease (Figure 2). This reveals that these 2 ELISAs reacted with different fragments from TAFI. To explore this ambiguity, we cloned, expressed, and purified the AP of human TAFI. This recombinant AP reacted only in MA-T12D11/MA-T18A8-HRP, although with a much lower reactivity compared with activated TAFI. The response of purified TAFI or human plasma in MA-T12D11/MA-T18A8-HRP increases on activation, reaching a maximum plateau value at a time-point corresponding with the time of exhaustion of activatable TAFI. All these data demonstrated that MA-T12D11/MA-T18A8-HRP specifically reacts with the released AP from TAFI. It should be mentioned that in contrast to purified TAFI, the response observed for plasma on activation remained stable after reaching the maximum. This is most likely caused by stabilization of the released AP in a plasma environment. This was confirmed by the observation that the response of activated pTAFI added to TAFI-depleted plasma remained stable (data not shown).

The response of human plasma (Figure 2B) in MA-T30E5/MA-T17D7-HRP increases transiently on activation, reaching a maximum after 10 minutes. Because the response decreases after reaching the maximum, we can conclude that this ELISA reacts with the 36-kDa fragment of TAFI and not with the 25-kDa and 11-kDa products. Strikingly, the increase in MA-T30E5/MA-T17D7-HRP occurs parallel with the increase in TAFIa enzymatic activity. The delay of the decreasing response in the ELISA compared with the decrease in enzymatic activity strongly indicates that MA-T30E5/MA-T17D7-HRP reacts with both TAFIa and TAFIai. This is also confirmed by the analysis of the fragmentation products by SDS-PAGE illustrating that the reactivity in the ELISA is associated with the presence of the 36-kDa fragment and decreases concomitantly with the appearance of the 25-kDa and 11-kDa products. Analysis of 2 recombinant isoforms (ie, TAFI-Ala147Thr325 and TAFI-Ala147Ile325) revealed similar responses in the 3 ELISAs. However, the response of the Ile325 isoform decreased significantly slower in the MA-T30E5/MA-T17D7-HRP ELISA. This is in agreement with the increased stability of Ile325 isoforms at 37°C.8

When using plasmin to activate TAFI, a similar response was observed except that the response in the MA-T30E5/MA-T17D7-HRP ELISA paralleled the time course of the generation and loss of TAFIa activity (data not shown). This is in agreement with Marx et al9 describing that on plasmin-mediated activation of TAFI, the appearance and disappearance of the 36-kDa fragment (ie, TAFIa/ai, followed by SDS-PAGE) matched the time course of generation and loss of TAFI activity. This provides further evidence that the MA-T30E5/MA-T17D7-HRP ELISA reacts with both TAFIa and TAFIai.

The 3 characterized ELISAs were also used to follow the antigen levels of TAFI and TAFI fragments during clot formation and clot dissolution. When using 5 nM TM, TAFI is completely activated during clot formation, whereas in the presence of 0.5 nM TM, there is partial activation during clotting followed by subsequent activation during fibrinolysis (Figure 3). This is also reflected by the antigen levels of the released AP and TAFIa. With 5 nM TM, released AP levels immediately increase to 100% and TAFIa levels transiently to 80%, whereas with 0.5 nM TM the released AP increases partially during clot formation and subsequently during clot lysis. In the presence of 0.5 nM TM, the antigen levels of TAFI follow the pattern of TAFIa activity with a first peak during clot formation and a second lower peak during clot lysis. These results are in agreement with the biphasic pattern of TAFIa generation during in vitro clot lysis in human plasma as described by Leurs et al.20 Leurs et al also found a thrombomodulin-dependent increase of the first activity peak during clot formation, which is also confirmed by our data (Figure 3).23

Analysis of 300 plasma samples revealed correlations between cholesterol and triglycerides for TAFI as well as TAFIa and AP. These findings are in agreement with the data of Silveira et al, who reported statistical correlations between TAFI and cholesterol contents of plasma and all lipoprotein fractions but high-density lipoprotein and a tendency of TAFI to correlate with the triglyceride contents of all lipoprotein fractions in healthy subjects.11 In the current study, higher plasma levels of both the activation peptide and TAFIa were observed for subjects with hyperlipidemia, compared with subjects with normolipidemia. In contrast, no association between total TAFI antigen levels and hyperlipidemia could be observed. This suggests that there is an increased activation state in hyperlipidemia. These findings provide evidence that indeed measurement of the extent of activation is a more relevant parameter in the search for an association between TAFI levels and risk markers for cardiovascular diseases. Moreover, evidence that measuring the extent of TAFI activation is more relevant is supported by the threshold phenomenon stating that it is not the total amount of TAFI protein but the amount of TAFIa that plays a critical role in fibrinolysis.23 The use of this kind of ELISA in large epidemiological studies might refute the contradictory results observed previously.11–15 Measuring the released activation peptide (MA-T12D11/MA-T18A8-HRP ELISA), indepen-
dent of intrinsic stability of TAFIα and also exhibiting the strongest correlation with hyperlipidemia, is the preferred assay.

In conclusion, we described the development of 3 different MA-based ELISAs. The MA-T12D11/MA-T30E5-HRP specifically reacts with nonactivated TAFI, the MA-T12D11/MA-T18A8-HRP specifically reacts with the released activation peptide of TAFI and the MA-T30E5/MA-T17D7-HRP specifically reacts with TAFIα and TAFIαl. Application in various settings (clot lysis, activation of plasma, clinical samples) has demonstrated that the 2 latter assays reliably provide a measure for the extent of TAFI activation and that the released activation peptide is strongly associated with hyperlipidemia.

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METHODS

Materials

Human thrombin, rabbit thrombomodulin and H-D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) were obtained from Sigma, American Diagnostica and Biomol Research Labs, respectively. Plasmin and aprotinin were purchased from Enzyme Research Labs and Fluka, respectively. Hippuryl-L-arginine and potato tuber carboxypeptidase inhibitor (PTCI) were obtained from Bachem and Calbiochem, respectively.

Blood samples (n=13) were taken on 4% citrate according to the guidelines of the blood transfusion centre (Red Cross, Leuven, Belgium), and were pooled for experiments with pooled human plasma.

TAFI was purified from human plasma (pTAFI) as described before with some modifications. Briefly, 1.2 L of citrated plasma was centrifuged at 44500 x g for 30 min. The supernatant was supplemented with 0.1 M ε-ACA and applied to a Sephadex G-25 column (Amersham Biosciences®) equilibrated with 50 mM Tris, 150 mM NaCl, pH=7.4 (TBS). Subsequently, the flow through was incubated (in batch, for 16 h at 4°C) with CNBr-activated Sepharose 4B beads (Amersham Biosciences®), to which a monoclonal antibody against TAFI (MA-T4E3) was bound, and which was equilibrated with 50 mM Tris, 150 mM NaCl, pH=7.4. Unbound and non-specifically bound proteins were removed by washing with TBS containing 0.5 M NaCl. The beads were poored into a column and bound proteins were eluted with 0.1 M glycine pH=2.7. Eluted fractions were collected in 1/10 (v/v) 1 M Tris pH=9.0, pooled and dialyzed towards 20 mM Tris pH=7.4 before application to a CNBr-activated sepharose column to which an antibody against human serum albumin was coupled, and which was equilibrated with 20 mM Tris pH=7.4. The flow through was applied onto a
protein A sepharose column (Amersham Biosciences®). The flow through of this column was then applied onto a SOURCE™ 15Q column (Amersham Biosciences®) equilibrated with 20 mM Tris pH=7.4. Bound proteins were eluted with a linear sodium chloride gradient (0-500 mM NaCl in 20 mM Tris pH=7.4). Fractions containing TAFI were pooled and dialyzed towards 20 mM Tris pH=7.4 and stored at -20°C.

Recombinant TAFI isoforms (Ala<sup>147</sup>Thr<sup>325</sup>, Ala<sup>147</sup>Ile<sup>325</sup>) and monoclonal antibodies (MA) raised against pTAFI were generated as described before.<sup>2</sup>

Lipofectamine 2000 and Optimem 1 medium containing glutamax were purchased from Invitrogen. Horseradish peroxidase (HRP)-conjugated Goat anti-mouse antibody was purchased from BioRad.

TAFI-depleted plasma was obtained by adsorption on MA-T4E3, raised against human plasma-derived TAFI, covalently coupled to CNBr-activated Sepharose 4B as described earlier.<sup>2</sup> The BCA protein assay kit (Pierce®) was used for determination of the concentration of the purified recombinant AP. The Glyco® enzymatic deglycosylation kit (ProZyme®) was used for N-deglycosylation of the recombinant AP.

**Cloning, expression and purification of the activation peptide**

The cDNA encoding the AP (Phe<sup>1</sup>-Arg<sup>92</sup>) was isolated from pcDNA3.1(+)-TAFI by PCR using the following primers: 5’CTTAAGCTTGTTACCATGAAGCTTTG3’, creating a KpnI site at the 5’ end and 5’GAGTCATTACCCGGTTCGCGGGGCTGACTGTGTCGTTGG3’, creating a AgeI site at the 3’ end. This cDNA was cloned in the pcDNA3.1/V5-HisA vector (Invitrogen®) using KpnI and AgeI restriction sites. The sequence of the construct was verified using the dideoxy chain termination sequencing method (A.L.F. DNA sequencer, Amersham Biosciences). The
recombinant pcDNA3.1/V5-HisA vector, containing the sequence for the AP, was transiently transfected in HEK293T cells using the Lipofectamine 2000 protocol (Invitrogen®). Media were harvested 96 hours after transfection, centrifuged, and the supernatant was collected. Purification was performed at 4°C. The collected supernatant was diluted (1:2) with PBS buffer (140 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH=7.4) and loaded on a Ni-NTA His-Bind® Resin (Novagen®) column (2 ml beads), equilibrated with 5 volumes charge buffer (50 mM NiSO₄) and 3 volumes washing buffer (0.5 M NaCl, 20 mM Tris-HCl, pH=7.9). The column was washed with 20 volumes washing buffer and bound protein was eluted with an imidazole gradient (0-200 mM imidazole in 0.5 M NaCl, 20 mM Tris-HCl, pH=7.9). Recombinant AP containing fractions were pooled, dialyzed against PBS and protein concentration was determined using the BCA Protein Assay Kit (Pierce®).

Evaluation of generated TAFI fragments upon activation

pTAFI (0.54 µM) was incubated with thrombin (20 nM), thrombomodulin (5 nM), and CaCl₂ (5 mM) in 60 µl Hepes buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 3 mM CaCl₂, pH=7.4) at 37°C for 0, 5, 10, 20, 30, 60 or 120 minutes. The reactions were stopped at the indicated time points by addition of 20 µl PPACK (30 µM final concentration). Sodium Dodecyl Sulphate (SDS, 1% final concentration) was added to the mixtures and the samples were heated during 30 seconds at 100°C. The different TAFI fragments, formed upon activation, were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), followed by Silver Staining.

Chromogenic assay for the measurement of TAFIa activity
TAFIa activity was measured as described before.\textsuperscript{3} pTAFI (0.09 µM) or pooled human plasma (1:4 diluted) was incubated with thrombin (20 nM), thrombomodulin (5 nM), and CaCl\textsubscript{2} (5 mM for pTAFI and 17 mM for plasma) or with plasmin (100 nM) and CaCl\textsubscript{2} (5 mM) in 60 µl Hepes-BSA buffer (Hepes buffer containing 0.1% BSA) at 37°C for 0, 5, 10, 20, 30, 60 or 120 minutes. Thrombin activation was stopped at the indicated time points by addition of 20 µl PPACK (30 µM final concentration). These activation mixtures are referred to as pTAFI activation mixtures and plasma activation mixtures, respectively. Alternatively, where indicated, plasmin activation was stopped by addition of 20 µl aprotinin (100 U/ml final concentration).

Twenty microliter of the substrate hippuryl-arginine (4 mM final concentration) was added and substrate conversion was allowed to proceed for 10 minutes at 22°C. Reactions were stopped by addition of 20 µl HCl 1 N followed by 20 µl NaOH 1 N and 25 µl sodium phosphate (1 M, pH=7.4). Subsequently, 6% cyanuric chloride, dissolved in 1,4-dioxane, was added and the mixtures were vortexed and centrifuged. One hundred microliter of supernatant was transferred into a 96-well microtiter plate and the absorbance at 405 nm was measured using an EL808 Ultra Microplate Reader (Bio-Tek instruments Inc). The activities were expressed as percentage of the maximum activity. To exclude the enzymatic activity of carboxypeptidase N in plasma, substrate conversion in plasma was also performed in the presence of PTCI (25 µg/ml final concentration) to measure carboxypeptidase N activity exclusively. Values obtained in the presence of PTCI were subtracted from total values (without PTCI) to calculate the specific TAFIa activity in plasma.

**Construction, selection and characterization of sandwich-type ELISAs for differential detection of TAFI fragments**
A panel of sandwich-type ELISAs was constructed as described previously. The constructed ELISAs were screened for their reactivity towards TAFI before and after activation and towards the recombinant AP. Three combinations, i.e., MA-T12D11/MA-T30E5-HRP, MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-T17D7-HRP, were ultimately selected and further characterized in detail. pTAFI activation mixtures (cfr chromogenic assay) and the recombinant AP were diluted to 1 µg/ml in PTA buffer (PBS pH=7.4 containing 0.002% Tween 80 and 1 g/l BSA), followed by serial two-fold dilutions up to 0.016 µg/ml, and applied in the ELISAs. Plasma activation mixtures (cfr chromogenic assay) were diluted to obtain a 1:10 dilution of plasma in PTAE buffer (PTA containing 5 mM EDTA), followed by serial two-fold dilutions up to 1:640, and applied in the ELISAs. Results were expressed as % response of the standard.

In the stage of development and characterization of the assays, either non-activated pooled human plasma (S₀) or activated pooled human plasma (S₁₀) were used as a standard (diluted to obtain a 1:40 dilution of plasma in PTAE buffer, followed by serial two-fold dilutions up to 1:2560). For preparation of S₁₀, plasma was incubated with thrombin (20 nM), thrombomodulin (5 nM), and CaCl₂ (17 mM) in Hapes buffer at 37°C for 10 minutes (i.e. maximum of TAFI activity generated as evidenced by the chromogenic assay). The reaction was stopped by addition of PPACK (30 µM final concentration).

For larger studies (see below) normal pooled human plasma was used as a calibrator and data were expressed relative to this normal plasma.

Time dependent evaluation of non-activated TAFI, the released activation peptide and TAFIa (antigen and activity) during clot lysis
Turbidity, TAFIa activity and antigen levels of TAFI and different TAFI fragments were measured during clot lysis. Pooled human plasma was incubated at 37°C with tPA (40 ng/ml in Hepes 20 mM, 0.1% Tween 20, pH=7.4), thrombomodulin (0, 0.5 or 5 nM) and CaCl₂ (12.5 mM final concentration) in Hepes buffer (Hepes 20 mM, pH=7.4) (1:2 final plasma dilution). These reaction mixtures were made in duplicate. One was used to evaluate the change in turbidity every 5 minutes at 405 nm (during a 120 minutes time course) with an EL808 Ultra Microplate Reader (Bio-Tek instruments Inc).

The duplicate reaction mixture was used to aliquot samples and stop the reaction at different time points (during a 120 minutes time course) by adding PPACK (30 µM final concentration) and aprotinin (100 U/ml final concentration). In these samples, TAFIa activity was determined with the described chromogenic assay and antigen levels of TAFI and different TAFI fragments were determined with the selected ELISAs. Samples were diluted to obtain a 1:12 dilution of plasma in PTAE buffer, followed by serial two-fold dilutions up to 1:768, and applied in the ELISA.

**Plasma elimination studies of the released activation peptide from TAFI and the catalytic domain from TAFI**

pTAFI (approximately 10 µM, 600 µg/ml) was incubated with thrombin (200 nM), thrombomodulin (50 nM), and CaCl₂ (50 mM) in 100 µl Hepes buffer for 5 minutes at 37°C. The reaction was stopped by addition of PPACK (300 µM final concentration). Approximately 30 µg of protein was injected into a lateral tail vein of female Balb/c mice. Blood samples of 28 µl were collected from the other tail vein at different time points with Hematocrit Tubes (Hirschmann Laborgerate®). Samples were diluted to obtain a 1:20 dilution in PTAE buffer, followed by serial two-fold dilutions up to 1:1280, and applied in the MA-
T12D11/MA-T18A8-HRP and MA-T30E5/MA-T17D7-HRP ELISA. The initial time point, taken 15-30 sec after injection, was considered to represent 100% of injected protein in the circulation.

**Study population and data collection**

The study population represents 300 subjects, aged 58–70 years, taking part in health surveys within the framework of the Sahlgrenska Academy Study of Ischemic Stroke (SAHLSIS). Subjects were randomly selected from participants in a population-based health survey in Göteborg or from the Swedish Population Register. Subjects without a history of atherothrombotic disease were invited and subjects showing signs of ischemic coronary artery disease on electrocardiogram (according to the Minnesota code 1982) were excluded. All investigations, including blood sampling, were performed between 8.00 and 10.30 AM after an overnight fast. Blood was collected in tubes containing 1/10 vol. of 0.13 M sodium citrate. The tubes were kept on ice and plasma was isolated within 2 h by centrifugation at 4°C and 2000 x g for 20 minutes. Plasma determination of t-PA antigen, t-PA activity, PAI-1 antigen and fibrinogen were performed as described. Plasma fibrinogen was measured by using an automated clot-rate assay (STA-R® analyzer, Diagnostica Stago) using the manufacturers reagents according to the instructions. Serum concentrations of cholesterol, high density lipoprotein (HDL) and triglycerides were determined by standard methods at the Department of Clinical Chemistry, Sahlgrenska University Hospital. Serum insulin levels were determined by a chemiluminescence assay on an IMMULITE 2000 analyzer (Diagnostic Products Corporation) using the manufacturers reagents according to the instructions. Information on risk factors was collected and defined as described previously. For technical and practical
reasons, all values of TAFI and TAFI fragments are expressed relative to pooled human plasma.

The study was approved by the Ethics Committee of Göteborg University, and the data handling procedures were approved by the National Computer Data Inspection Board. All participants gave their written informed consent.

**Statistical analysis**

Quantitative data were summarized by the mean and standard deviation (SD). Statistical analyses were performed with Graph Pad Prism 4.01 (Graph Pad Software, Inc., San Diego, USA).

For the SAHLSIS population study standard statistical methods were used. Non-normally distributed fibrinolytic and insulin variables are presented as medians and 25th and 75th percentiles. Differences between groups were evaluated by the non-parametric Mann-Whitney U test. Spearman’s r was calculated to assess the relationship between variables. Statistical significance was considered at $P<0.05$. 
References


Application of the developed assays in vitro and in vivo

Plasma samples of 13 healthy individuals were analysed in the different ELISAs, before and after 10 minutes activation with T/TM (cfr chromogenic assay) (see figure). Average values of intact TAFI antigen levels, released AP antigen levels and TAFIa antigen levels before activation were 107±45% (mean ± SD; median = 101%), 2.4±1.2% (mean ± SD; median = 2.3%) and 5.5±2.3% (mean ± SD; median = 5.6%), respectively. Average values of intact TAFI antigen levels, released AP antigen levels and TAFIa antigen levels after activation were 19±15% (mean ± SD; median = 16%), 96±35% (mean ± SD; median = 106%) and 107±49% (mean ± SD; median = 105%), respectively (see figure). A positive correlation was found between the decrease in TAFI antigen levels and the increase in released AP antigen levels (r=0.57, \(P=0.04\)) and between the increase in released AP antigen levels and the increase in TAFIa antigen levels (r=0.97, \(P<0.0001\)). The correlation between the decrease in intact TAFI antigen levels and the increase in TAFIa antigen levels was borderline significant (r=0.51, \(P=0.07\)).

Figure: Analysis of plasma samples of healthy individuals in the different ELISAs. Response (expressed in %) of intact TAFI (A), the released AP (B) and TAFIa (C) in healthy individuals before (●) and after (○) 10 minutes activation with T/TM.
Response of TAFI upon activation in the three selected ELISAs. TAFIa activity (○) generated upon activation of TAFI-Ala$^{147}$-Thr$^{325}$ (A) and TAFI-Ala$^{147}$-Ile$^{325}$ (B) between 0 and 120 minutes determined with a chromogenic assay. Response upon activation between 0 and 120 minutes in MA-T12D11/MA-T30E5-HRP (■), MA-T12D11/MA-T18A8-HRP (▲) and MA-T30E5/MA-T17D7 (▼) (mean ± SD, n≥3).
### Characteristics of subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (SD)</th>
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<tr>
<td>Age, years (SD)</td>
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<tr>
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<tr>
<td>Triglycerides, mM</td>
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<tr>
<td>HDL, mM</td>
<td>1.5 (0.4)</td>
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<tr>
<td>Insulin, µIU/mL</td>
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</tr>
<tr>
<td>Fibrinogen, g/L</td>
<td>3.1 (2.7-3.4)*</td>
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</tbody>
</table>

SBP denotes systolic blood pressure, DBP diastolic blood pressure, BMI body mass index, and HDL high density lipoprotein.

*Median (Interquartiles)
Quantitation of TAFI in plasma

Addition of pTAFI at a final concentration of 2, 5 and 10 µg/ml to TAFI-depleted plasma (cfr materials) revealed recoveries in MA-T12D11/MA-T30E5-HRP of 96±17%, 77±17%, and 94±4.6%, respectively.

Addition of pTAFI, 10 minutes activated with T/TM (cfr chromogenic assay), at a final concentration of 2, 5 and 10 µg/ml to TAFI-depleted plasma revealed recoveries in MA-T12D11/MA-T18A8-HRP of 72±12%, 79±15%, and 90±14%, respectively, and in MA-T30E5/MA-T17D7-HRP of 97±10%, 92±20%, and 77±11%, respectively. Addition of rAP at a final concentration of 0.5, 2, 5 and 10 µg/ml to TAFI-depleted plasma revealed recoveries in MA-T12D11/MA-T18A8-HRP of 92±31%, 62±4%, 103±17% and 83±16%, respectively.

The assay variability of the ELISAs was evaluated using 4 different plasma samples each assayed 4 times on 4 occasions. The intra-assay and inter-assay coefficients of variation for MA-T12D11/MA-T30E5-HRP were 6.2% and 8.3%, respectively, for MA-T12D11/MA-T18A8-HRP 3.1% and 7.3%, respectively and for MA-T30E5/MA-T17D7-HRP 3.3% and 6.4%, respectively.

The interdilution coefficients of variation were 8.2%, 5.1% and 7.3% for MA-T12D11/MA-T30E5-HRP, MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-T17D7-HRP, respectively. The detection limit of all three ELISAs was 1.6%. TAFI-depleted plasma revealed no detectable response in any ELISA.