Binding of Thrombin Activatable Fibrinolysis Inhibitor (TAFI) to Plasminogen May Play a Role in the Fibrinolytic Pathway

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Received August 30, 2008

Thrombin activatable fibrinolysis inhibitor (TAFI) or carboxypeptidase U are a carboxypeptidase B-like enzyme. TAFI modulates fibrinolysis by removing lysine and arginine residues from partially degraded fibrin, thereby inhibiting plasmin (Psn)-mediated fibrin degradation. Here, we investigated the binding and activation of TAFI in complex with plasminogen (Pgn) by thrombin (IIa) and thrombomodulin (TM). Bindings of TAFI, activated TAFI (TAFIa), and inactivated TAFIa to Pgn were demonstrated using Pgn-coated plate and anti-TAFI antibody conjugated with HRP in sandwich ELISA format. Next, the retained TAFIa catalytic activities in TAFIa/Pgn complex were monitored by using TAFI chromogenic substrate. In addition, IIa/TM could activate TAFI in the complex with Pgn, indicating the accessibility to its activation site at Arg-92 in the complex. Finally, the TAFI/Pgn complex was immuno-precipitated from pooled normal plasma with TAFI antibody conjugated magnetic beads. Pgn in the complex was detected by HRP conjugated anti-Pgn antibody. Indeed, TAFI/Pgn complex existed and was captured from plasma. Since higher concentration of Pgn existed in plasma than TAFI, most of TAFI isoforms would be bound to Pgn as complex, suggesting the TAFI/Pgn complex as the potential marker in cardiovascular, hemophiliac, or other hemorrhagic diseases.

Key Words : Thrombin activatable fibrinolysis inhibitor (TAFI), Carboxypeptidase U, Plasma, Plasminogen

Introduction

Thrombin activatable fibrinolysis inhibitor (TAFI),[1-5] also called plasma carboxypeptidase B, U, or R, is a carboxypeptidase B-like enzyme. TAFI is a 60 kDa glycoprotein zymogen, which can be converted to TAFIa by cleavage at Arg-92 with thrombin/thrombomodulin (IIa/TM) complex or by Psn.[1-5] TAFI in its active form, TAFIa, modulates fibrinolysis by removing lysine and arginine residues from partially degraded fibrin, thereby inhibiting plasmin (Psn)-mediated fibrin degradation.[3-9] C-terminal lysine residues on fibrin provides the binding moieties for both tissue plasminogen activator (tPA) and plasminogen (Pgn) through their lysine binding sites (LBS) in the kringle domains, forming a ternary complex between tPA, Pgn, and fibrin, which are critical anchors in the conversion from Pgn to Psn at the site of a thrombus by tPA.[10-18] Removal of lysine and arginine residues by TAFIa could interfere with the formation of Psn, indirectly inhibiting fibrinolysis (Figure 1).[19-22] TAFI exists in plasma along with an another basic carboxypeptidase called CPN, which also removes C-terminal Arg or Lys.[6] However, CPN does not regulate fibrinolysis nor interact with Pgn.[6] Hence, TAFI plays a significant balancing role between the coagulation and fibrinolysis cascades.[19-22] Furthermore, the inhibition of TAFIa could enhance fibrinolysis by increasing the rate of Psn binding to the partially degrade fibrin and amplification of Psn generation, which could provide a potential antithrombotic therapy.[23-26] While studies have indicated that TAFI could bind to Pgn, alpha-2-macroglobulin (A-2M), and pregnancy zone protein, the biological significance of TAFI in the complex with mentioned proteins in plasma has not been determined.[27] Psn also could inactivate TAFI by cleaving at additional sites at Arg-302, and Arg-330.[28] In this study, binding interactions of TAFI isoforms (TAFI, TAFIa and inactivated TAFIa) to Pgn were demonstrated using ELISA method and TAFI substrate. We investigated the capability of TAFI activation by IIa/TM in the complex. TAFI/Pgn complex was immuno-precipitated with anti-TAFI antibody, and detected with anti-Pgn antibody.

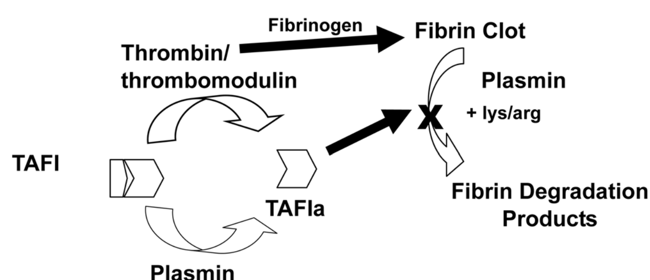

Figure 1. Schematic Diagram of the role of TAFI upon activation by thrombin and plasmin. Upon activation of thrombin/thrombomodulin, TAFIa removes lysine and arginine residues from partially degraded fibrin, thereby inhibiting plasmin (Psn)-mediated fibrin degradation.

Experimental Details

Fresh frozen citrated human plasma was obtained from the Kangwon University Medical School Hospital (Chuncheon, Korea). All plasmas were collected with Kangwon University's Internal Review Board and patient consent forms. The thrombin inhibitor D-Phe-Pro-Arg chloromethyl ketone

(PPAck) was purchased from Calbiochem (San Diego, USA). TAFI and anti-human TAFI (IgG2b) were from Hematological Associates (Essex Junction, VT). ActiChrome TAFI (#874) kit and anti-TAFI antibodies were from American Diagnostica (Greenwich, CT). Ready-made Phosphate Buffered Saline solution, Hepes buffer, Tris Buffered Saline solution (final 0.1% v/v), Glu-plasminogen (Glu-Pgn), tissue plasminogen activator (tPA), and 1-Stop 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase solution were purchased from Sigma. Superblock and Pico Chemiluminescent HRP Substrate from Pierce Inc. were used. Clear and black Nunc microtiter plates were used. Dynal Tosyl-activated M-280 magnetic beads were purchased from Invitrogen Inc. Anti-Pgn antibody conjugated with horseradish peroxidase (HRP) was purchased from abcam Inc.

Activation of TAFI. TAFI was activated with thrombin/thrombomodulin (IIa/TM) as previously reported.[3] Briefly, 50 ug of TAFI was incubated with 25 nM thrombin, 50 nM thrombomodulin and 5 mM $CaCl_2$ in 0.02 M Hepes and 0.15 M NaCl (pH 7.4) for 15 min at 37 °C. Afterwards, 30 nM of irreversible thrombin inhibitor, D-Phe-Pro-Arg Chloromethylketone, were added, and TAFIa was aliquoted for the storage at −80 °C until usage. Activation of TAFI in the presence of Pgn from 500, 250, 125, 62.5, 31.3, 15.6 and 0 ug/mL concentrations was carried out by following, TAFI (0.1 ug) was incubated with Pgn in 135 uL of Hepes buffer (0.02 M) with 5 mM $CaCl_2$ and 0.15 M NaCl at pH 7.4 for 30 min at 37 °C. TAFI/Pgn complex was activated as above with 15 uL of thrombin/thrombomodulin (IIa/TM, 100 nM thrombin, 200 nM thrombomodulin) at 37 °C for 15 min. TAFIa activities were measured with addition of TAFI substrate (20 uL), and the final optical density (OD) at 490 nm was measured after stopping the reaction with 50 uL of 2 N H_2SO_4 by SpectraMax Plus384 (Molecular Device Inc.) microtiter ELISA reader. Two controls, TAFI without activation and blank, were used.

Binding of TAFI and TAFIa to Glu-Pgn coated plate. Glu-Pgn coated-plate: Ninety-six well microtiter plate (Nunc, maxisorb) were incubated with 100 uL of Glu-Pgn (10 μg/mL), washed and blocked with Superblock as suggested by manufacturer, incubating the plate with 250 uL for 1 hr at room temperature. TAFI and TAFIa were incubated in the Pgn coated plates at 4 °C or at 37 °C in 20 mM TRIS buffer (pH 7.4) for 1 hr and washed four times with detergent containing Tris buffer. For the TAFI activation on the Pgn coated plate, 15 uL of Thrombin/thrombomodulin (100 nM thrombin, 200 nM thrombomodulin), 135 uL of Hepes buffer (0.02 M) with 5 mM $CaCl_2$ and 0.15 M NaCl at pH 7.4 for 15 min at 37 °C, and 20 uL of TAFI substrate were added into each wells. TAFIa activity was monitored at 420 nm for 1 hr on above microtiter ELISA reader, and final OD at 490 nm was also obtained. Inactivated TAFIa was obtained by incubating TAFIa at 37 °C for 90 min.

TAFI enzyme-linked immunosorbent assay (ELISA). TAFI and TAFIa (4.0, 3.0, 1.0, 0.5, 0.25, 0.13, 0.063 ug/mL) were incubated on the Pgn coated plates at 4 °C or at 37 °C in 20 mM TRIS buffer (pH 7.4) for 1 hr and washed four times with detergent containing Tris buffer. Anti-TAFI-HRP from American Diagnostica, Inc. (Greenwich, CT) was reconstituted with double distilled H_2O, and 100 uL were added to microtiter wells for 1 hour incubation at 37 °C. The wells were washed four times with detergent containing Tris buffer, and TMB substrate for HRP was added. After 20 minutes, the reaction was stopped with 50 uL of 2 N H_2SO_4, and the wells were read at 450 nm in SpectraMax Plus384 (Molecular Device Inc.) microtiter ELISA reader.

Conjugation of TAFI antibody on magnetic beads. Anti-human TAFI antibody was conjugated to Tosyl-activated M-280 magnetic beads by following the manufacturer's recommendation. Briefly, 200 uL of Tosyl-activated M-280 magnetic beads were incubated with 100 ug of TAFI antibody for 24 hrs in 0.1 M Phosphate buffer at pH 7.4, followed by blocking for 1 h with Superblock and washing. As negative control, magnetic beads with IgG2b isotype antibody were used. The magnetic beads with IgG2b isotype antibody were made as with anti TAFI antibody conjugation. The inactivated magnetic beads made by blocking the Tosyl group with Tris 1 M at pH 8.0 for for 24 hrs, and blocked for 1 h with Superblock.

Immuno-precipitation of Pgn with TAFI antibody conjugated magnetic beads. Two microliter of magnetic beads conjugated with anti-TAFI antibody, IgG2a isotype control or blocking agents were mixed with 100, 50, 25, 12.5, 6.25, 3.12, and 1.6 uL of plasma in total volume of 1 mL, and incubated for 1 hr at 37 °C. Afterwards, the beads were washed with detergent containing Tris buffer for three times. Anti-Pgn-HRP (1 mg/mL) from Abcam, Inc. was reconstituted with double distilled H_2O, and 100 uL of 0.4 ug/mL dilution were added for 1 hour at 37 °C. The beads were washed four times with detergent containing Tris buffer. Pico Chemiluminescent HRP Substrate (Pierce) was added and Chemiluminescent signal was detected with Perkin Elmer Victor 3 plate reader. Same set of experiments was carried out with the isotype magnetic beads as control.

Results and Discussion

Effect of Glu-plasminogen on the activity of TAFIa. IIa/TM activated TAFI (0.1 ug) in the presence of Pgn concentrations from 500, 250, 125, 62.5, 31.3, 15.6 and 0 ug/mL, and TAFIa activities were monitored after adding its substrate. When the initial Vmax and final optical density unit (OD) were compared, Vmax did not change in all Pgn concentrations with the average Vmax of 0.0615(deltaOD/delta min) ± 0.0027. The final OD were similar, 2.77 ± 0.12, in all Pgn concentrations. The average concentrations of Pgn and TAFI in normal plasma are 120 ug/mL and 4.5 ug/mL, respectively. Hence, all TAFI (0.1 ug) would bind to Pgn at 500, 250, 125 ug/mL, and exist as TAFI/Pgn complex in the experiment. Pgn at all concentrations did not show any interference with the TAFI activation by IIa/TM or preceding TAFIa activity. The results indicated that the TAFI activation site at Arg-92 was accessible to IIa/TM in the presence of Pgn. TAFI substrate could access to the catalytic site of

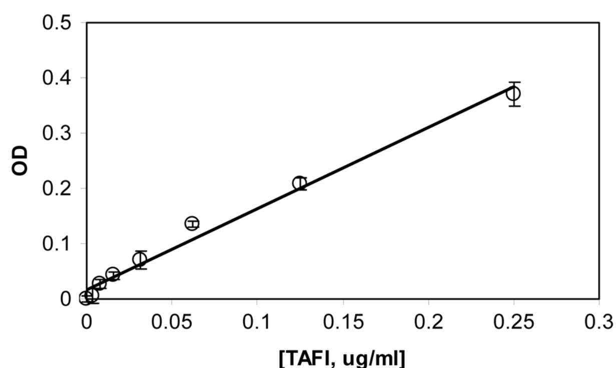

Figure 2. Detection of TAFI bound to the Pgn coated plate: peroxidase conjugated mouse monoclonal anti- TAFI antibody was used for the quantification of bound TAFI. TAFI was able to bind to Pgn with positive correlation. The lowest detection limit could be set at 0.008 ug/mL. Data points were linearly fitted, and the R^2 value was 0.986.

TAFI in high concentrations of Pgn.

Detection of TAFI bound to Glu-Pgn. The binding of TAFI to Glu-Pgn were quantitated by both ELISA and TAFIa enzymatic methods. Upon incubation of TAFI at concentrations, 0.250, 0.125, 0.063, 0.032, 0.016, 0.008, 0.004, and 0 ug/mL on Pgn coated plate for 1 hr at 37 °C and washes, the bound TAFI was measured by HRP conjugated anti-TAFI antibody (Figure 2). Indeed, TAFI was able to bind to Pgn with positive correlation. The lowest detection limit could be set at 0.008 ug/mL. In a duplicate experiment, instead of quantitating TAFI with antibody, the bound TAFI at concentrations, 4.0, 2.0, 1.0, 0.5, 0.25, 0.125, 0.063, and 0 ug/mL were activated with IIa/TM to TAFIa, and their activities were measured with addition of TAFI substrate (Figure 3). The linear relationship was also observed between the TAFI concentrations and activities for bound TAFI to Pgn. The lowest sensitivity of the TAFIa was at 0.063 ug/mL. Higher sensitivity of measuring TAFI was through HRP conjugated TAFI antibody, which would be due to the amplification from the conjugated HRP on the antibody. Observing TAFIa activity from TAFI/Pgn complex on the plate after activation with IIa/TM confirmed the open accessibility at the activation site Arg-92, and Pgn binding site did not overlap with the activation site or catalytic site.

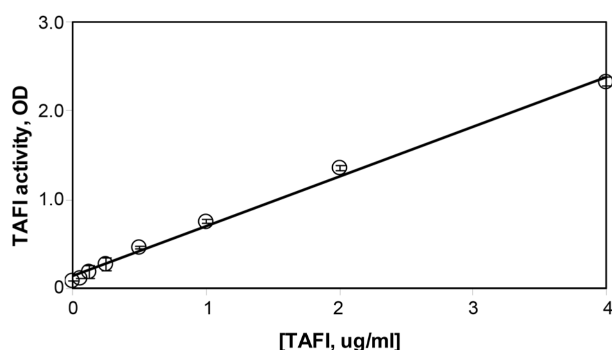

Figure 3. Quantification of the TAFI enzymatic activity from TAFI bound to the Pgn coated plate: Bound TAFI on Pgn plate was activated with IIa/TM, followed by the addition of TAFI substrate. The linear relationship was observed between the TAFI concentrations and activities for bound TAFI to Pgn. The lowest sensitivity of the TAFIa was at 0.063 ug/mL. Data points were linearly fitted, and the R^2 value was 0.994.

Binding of TAFIa to Glu-Pgn. TAFIa was activated by IIa/TM prior to the addition on Pgn coated plate. Binding of TAFIa was quantitated by the same methods as above, using HRP-conjugated TAFI antibody in ELISA format and TAFI substrate. The results from the ELISA and TAFI substrate were similar between them (Figure 4A, B). The lowest sensitivity was 0.063 ug/mL as before with TAFI. TAFIa was also able to bind to Pgn as with TAFI. Observation of TAFIa upon binding to Pgn supported the above results with TAFI, where the Pgn binding site would be located differently from the catalytic site of TAFIa. It was suggested that N-terminus before the IIa/TM cleavage site was responsible for the binding with Pgn based on reduced affinity of TAFIa than TAFI.[2] If only N-terminus of TAFI contained Pgn binding site, after IIa/TM activation and incubation on Pgn coated plate, TAFIa would be washed away and would not be detected. The detection of TAFIa activity indicated that TAFIa still contained Pgn binding site at its C-terminus region.

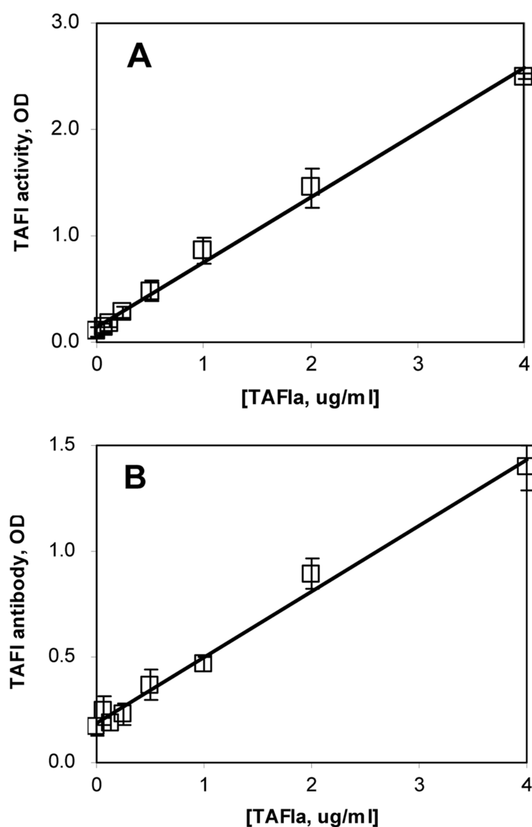

Figure 4. (A, B): Quantification of the bound TAFIa to the Pgn coated plate and its TAFI enzymatic activity. HRP conjugated anti-human TAFI (A) and TAFI substrate (B) were used for the quantification of bound TAFIa. The lowest sensitivity was 0.063 ug/mL as before with TAFI. TAFIa was also able to bind to Pgn as with TAFI. Data points were linearly fitted, and the R^2 values were 0.993 (A) and 0.989 (B), respectively.

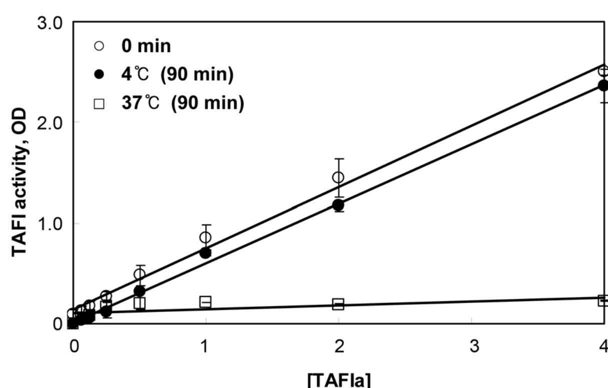

Figure 5. Quantification of the enzymatic activity from bound inactivated TAFI (open square, after 90 min. incubation at 37 °C) in comparison with incubated TAFIa at 0 min. (open circle) and 90 min. (closed circle) incubations at 4 °C prior to binding with Pgn coated plate. The lowest sensitivity was 0.063 ug/mL as before with TAFI. TAFIa was also able to bind to Pgn as with TAFI. Data points were linearly fitted, and the R^2 values were 0.993, 0.997, and 0.414 for 0 min, 4 °C (90 min), and 37 °C (90 min), respectively.

Binding of inactivated TAFIa with Pgn. TAFIa catalytic activity was sensitivity to the temperature.[1-3] The half-life of TAFIa at physiological temperature 37 °C was reported as ~10 min, whereas greater than 30 min at 0 °C.[1-3] TAFIa could be inactivated by incubating for 90 min at 37 °C. Inactivated TAFIa was applied to Pgn coated plate in comparison with TAFIa stored at 0 °C (Figure 5). Inactivated TAFIa revealed its diminished catalytic activity. TAFIa incubated at 4 °C sustained its activity in comparison with the control TAFIa (unfrozen just before the experiment).

HRP conjugated TAFI antibody revealed that the inactivated TAFIa with diminished activity along with TAFIa was still able to bind to Pgn (not shown). The mechanism of the TAFI's short half-life at 37 °C was suggested as structural changes caused by conformational instability at the catalytic site. Interestingly, a mutation at Thr-325 to Ile was able to increase its half-life by 100% to 20 min.[7] All TAFI isomers, TAFI, TAFIa, inactivated TAFIa, were able to bind to Pgn. Hence, TAFI could interact with Pgn through both N- and C-termini.

Detection of TAFI/Pgn complex in plasma. In an attempt of capturing TAFI/Pgn complex in plasma, anti-TAFI antibody was used as capturing antibody for several reasons, instead of anti-Pgn antibody. TAFI existed in lower concentrations (~4.5 ug/mL or 75 nM) than other active components of the haemostatic system, especially against Pgn (~120 ug/mL or 1.3 uM) in plasma. Higher concentrations of Pgn in the plasma in comparison with TAFI would allow Pgn to interact with other peptides and proteins in blood. TAFI/Pgn complex would exist only as a fraction in comparison with other complex with Pgn, and only small quantity of the complex would be captured by the anti-Pgn magnetic beads, requiring larger quantity of the magnetic beads to capture significant quantity of TAFI. Because Pgn could bind to partially degraded fibrinogen and fibrin with high affinity, which existed at higher concentrations in plasma than TAFI. On the other hand, TAFI was reported to bind with Pgn or Alpha2-macroglobulin in plasma,[27] hence, using anti-TAFI-antibody would yield higher possibility of capturing and concentrating the complex. Therefore, anti-TAFI antibody conjugated magnetic beads (Anti-TAFI-MB) was used to immunoprecipitate TAFI/Pgn complex in pooled normal plasma and to detect with anti-Pgn antibody conjugated with HRP. Detection of Pgn indicated the presence of the complex with TAFI and the complex in plasma could be captured by Anti-TAFI-MB. The isotype magnetic beads were used as control, where did not show any significant binding towards the complex. Therefore, wherever Pgn was presented, TAFI in complex with Pgn could be ready to play their role of removing C-terminal Lys residues upon IIa/TM activation.

Lysine binding kringe domains of Pgn are responsible for many interactions with blood proteins, like fibrin, fibrinogen, TAFI, tPA, uPA, and antiplasmin-2. In TAFI, four lysine residues were located at 42, 43, 44, and 59 in the N-terminus of TAFI before the activation site at 92, and 17 lysine residues at 124, 133, 140, 144, 149, 211, 212, 218, 240, 268, 282, 304, 306, 324, 357, 380, and 392 in the C-terminus region.[1-3] Decreased affinity upon TAFI activation from zymogen suggested the N-terminus region as potential region for the binding with Pgn.[2] And kringle 1 of Pgn might be responsible for the binding interaction with TAFI, based on the reduced affinity after a2-antiplasmin and 6-amino-*n*-hexanoic acid (e-amino caproic acid, eACA) treatments.[27] Since the exact Pgn binding site and the structure of TAFI were not known, we speculated the potential Lys binding sites in TAFI by comparing the structure of human procarboxypeptidase B as template.[29] Among them, Lys residues at 42, 43, 44, and 59 in the N-terminus, and 149, 212, 240,

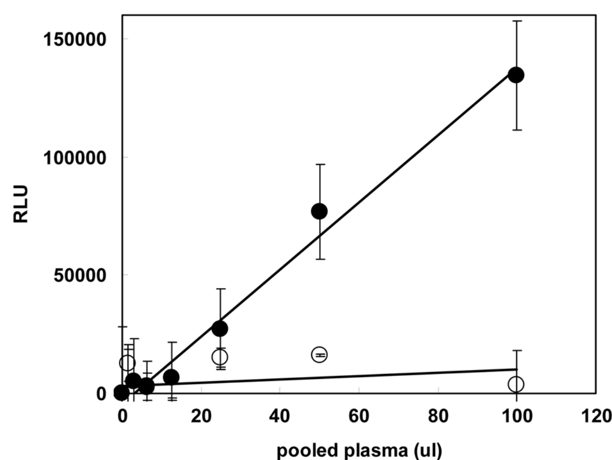

Figure 6. Quantification of the bound Pgn in complex with TAFI: Anti-TAFI antibody (closed circle) was used to immuno-precipitate the TAFI/Pgn complex in plasma, and anti-Pgn antibody conjugated with HRP was used to quantify Pgn in series of plasma concentrations from 100, 50, 25, 13, 6, 3 and 0 uL. The isotype magnetic beads (open circle) were used as control, where did not show any significant binding towards the complex. Data points were linearly fitted, and the R^2 values were 0.986 and 0.070 for Anti-TAFI antibody and isotype magnetic beads, rexpectively.

304, 306, and 324 (residue numbering of TAFI) in the C-terminus seemed to be located on the surface, exposed more to the solvent, and could be the potential binding sites. Since conserved residues near catalysis site were Glu-363 and Arg-217, Arg-235, Tyr-341, and Asn-234 for the substrate-binding, and His-181, Glu-184, and His-310 for the zinc-binding in the catalytic domain of TAFIa,[31] lysine residue at 218 may not able to provide Pgn anchoring site. From the structural analysis, Lys residues 133, 140, 144, 268, 282, 357, 380, and 392 were located more towards to the inner part of the structure. Moreover, since the mutation at Thr-325 to Ile increased TAFI stability slightly,[30] binding of Pgn to the lysine residues at 304, 306, and 324 might provide small stability by binding to TAFIa, hence, increased its lifetime of TAFI. In addition, if Pgn binding site would be located only at the N-terminus, TAFIa would be displaced from Pgn coated plate, diminishing TAFIa activity. Our results indicated other potential Pgn binding sites in the C-terminus region, supporting that TAFI isoforms, TAFI, TAFIa, and TAFai, could exist in complex with Pgn.

Recently, TAFI has been implicated in hemorrhagic fevers,[33] Crimean-Congo[34] and dengue hemorrhagic fevers,[35] in addition to other cardiovascular diseases, atherothrombosis (angina pectoris),[32] hypertension hemophilia,[36,37] vWD,[38] sepsis,[30] DIC,[33] and DVT.[40,41] Decreased TAFI antigen and activity levels were observed in all patients with Crimean-Congo or dengue hemorrhagic fevers, which were similar to observing decreased levels of TAFI activity in bacterial sepsis, hemophilia or vWD patients.[33-39] In all cases, decreased antigen or functional TAFI levels would contribute to the severity of bleeding complications in bleeding disorders due to the impaired capacity of the coagulation system to protect the fibrin clot from fibrinolysis. Other possibilities of observing lower levels of TAFI activity could be due to liver dysfunction during active viral infection.[42] But other proteins levels did not change, suggesting other mechanisms responsible for the decreased levels of TAFI in these hemorrhagic diseases. In all hyperfibrinolysis cases, Psn could be implicated in decreasing the level of TAFI by digestion. Since they would exist as complex, Psn could efficiently cleave and inactivate TAFI.

TAFI may not be the best specific maker for a particular cardiovascular disease. On the other hand, TAFI could be a sensitive maker for the general cardiovascular events, indicating the imbalance in the coagulation and fibrinolysis. Using an ELISA, TAFI substrate, and activation of TAFI and Pgn in the complex, we demonstrated the presence of TAFI/Pgn complex in the pooled normal plasma. It would be interesting to measure the levels of TAFI/Pgn complex in the various cardiovascular diseases, such as hemophilia, vWD, sepsis, DIC, DVT, and other cardiac diseases.

Conclusion

This study demonstrated that TAFI isomers, TAFI, TAFIa and inactivated TAFIa, could bind to Pgn. TAFI/Pgn complex was detected in the pooled normal plasma. The complexation between TAFI and Pgn did not interfere with TAFI activation by IIa/TM or its catalytic activity, suggesting different binding site for Pgn. Upon activation of TAFI in complex with Pgn by IIa/TM, the complex could work as a multifunctional unit for their respective roles in the TAFI-dependent fibrinolysis pathway.

Acknowledgments. This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, KRF-2007-314-E00157), Kyung-won Research Starting Fund, and GRRC program of Gyeonggi province.

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