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THROMBIN-ACTIVATABLE FIBRINOLYSIS INHIBITOR (TAFI) DEFICIENT MICE

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1. ABSTRACTS

In order to examine the physiological role of thrombin-activatable fibrinolysis inhibitor (TAFI), we generated homozygous TAFI deficient mice by targeted gene disruption. Intercrossing of heterozygous TAFI mice showed that TAFI mice were born in the expected Mendelian ratio, indicating that transmission of the mutant TAFI allele did not lead to embryonic lethality. TAFI deficient mice developed normally and reached adulthood. No physical abnormalities were observed. They were fertile and pregnancies were carried to full term. Hematological analysis of TAFI deficient mice did not show any major differences compared with their wild type littermates, including plasma fibrinogen level, PT and aPTT. Prolongation of lysis time upon activation of TAFI was observed only with plasma from wild type and heterozygous mice in an *in vitro* clot lysis assay. TAFI deficiency did not lead to increased bleeding as determined by blood loss following tail transection. *In vivo*, TAFI deficiency did not influence occlusion time in either an arterial or a venous thrombosis model. The effects of TAFI deficiency were also investigated in thrombin-induced pulmonary thromboembolism, Factor X coagulant protein-induced thrombosis and endotoxin-induced disseminated intravascular coagulation models. In these models, TAFI deficiency did not improve the morbidity or mortality. Based on the kaolin-induced writhing test, TAFI did not play a major role in bradykinin degradation under normal conditions. These studies demonstrate that TAFI deficiency is compatible with murine life.

2. INTRODUCTION

2.1. THE FIBRINOLYTIC SYSTEM

The fibrinolytic system removes fibrin clots from the circulation in order to maintain blood vessel patency, and it mediates the activation of matrix metalloproteases, which degrade extracellular matrix proteins (1-8). Thus abnormalities in the fibrinolytic system can lead to pathological conditions ranging from thrombosis and hemorrhage to atherosclerosis and tumor metastasis. The molecular components of the fibrinolytic system have been extensively characterized, and consist of plasminogen, plasminogen activators, matrix metalloproteases and their

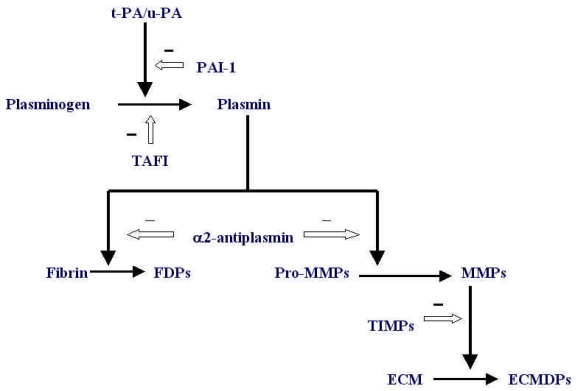

Figure 1. Schematic presentation of the fibrinolytic system. t-PA; tissue type plasminogen activator, u-PA; urokinase-type plasminogen activator, PAI-1; plasminogen activator inhibitor-1, TAFI; thrombin-activatable fibrinolysis inhibitor, FDPs; fibrin degradation products, Pro-MMPs; pro-matrix metalloproteinases; MMPs; matrix metalloproteinases, TIMPs; tissue inhibitors of matrix metalloproteinases, ECM; extracellular matrix, ECMDPs; extracellular matrix degradation products.

various inhibitors which interact in a tightly regulated manner as shown in Figure 1 (9, 10). The first step in fibrinolysis is generation of a limited amount of plasmin, an active serine protease, from Glu-plasminogen by a plasminogen activator. There are two physiological plasminogen activators; tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Based on knockout animal studies, t-PA plays the more important role in thrombolysis in plasma while u-PA exerts its main functions in tissues (11, 12). When both t-PA and Glu-plasminogen bind to fibrin, the rate of conversion of plasminogen to plasmin by t-PA is increased dramatically (13). Thus, on the clot surface, plasmin initiates clot lysis by proteolytic cleavage of internal lysine residues of Aa-chain of fibrin.

Fibrinolysis is accelerated by several mechanisms. One of the major feedback mechanisms involves newly exposed C-terminal lysine residues of Aa-chain of fibrin following its partial degradation by plasmin. Since both Glu-plasminogen and t-PA have high affinities for these newly exposed C-terminal lysine residues, this leads to increased binding of the substrate and its activator to fibrin, leading to amplified production of plasmin on clot surface (14, 15). The fibrinolytic system is tightly regulated at the level of plasmin by a2-antiplasmin, plasminogen activators by plasminogen activator inhibitor-1 (PAI-1), and matrix metalloproteases by their inhibitors (TIMPs). These inhibitors bind directly to the active sites of their cognate enzymes and prevent excess fibrinolysis and the matrix degradation. Recently, another protein that exhibits carboxypeptidase B-like activity has been shown to modulate fibrinolysis/thrombolysis. This protein, named thrombin-activatable fibrinolysis inhibitor (TAFI), inhibits the amplification of plasmin production by removing the C-terminal lysine residues from the partially degraded fibrin, thereby slowing down fibrinolysis/thrombolysis.

2.2. Thrombin-activatable fibrinolysis inhibitor (TAFI)

2.2.1. Structure of TAFI

Thrombin-activatable fibrinolysis inhibitor (EC 3.4.17.20), also known as plasma procarboxypeptidase B, procarboxypeptidase U ("U" for unstable) or arginine procarboxypeptidase, is a 60kDa glycoprotein that circulates in plasma at around 75 nM (16-23, for recent review, see 24-27). Originally it was described as a novel arginine carboxypeptidase generated in serum following coagulation (22, 23). The protein was purified from human plasma on a plasminogen-Sepharose affinity column, and its cDNA was isolated from a liver cDNA library (16). The protein consists of a 22-amino acid-long signal peptide, a 92-amino acid-long activation peptide and a 309-amino acid catalytic domain, which shows 50% identity with pancreatic carboxypeptidase A and B (CPA and CPB, for review on carboxypeptidases, see 28, 29). The overall structure of TAFI is very similar to that of pancreatic CPA and CPB. The presence of aspartic acid at position 256 of the catalytic domain suggests that it is a basic carboxypeptidase. There are 4 potential N-linked glycosylation sites in the activation peptide. Amino acid sequences of TAFI from mouse and rat have also been elucidated and they show 80 % homology with that of human TAFI (30-32). Amino acid sequence of TAFI from rabbit also shows a similar homology (our unpublished data).

2.2.2. Enzymatic action of TAFI

As is the case with pancreatic CPA and CPB, TAFI can be activated in vitro by high concentrations of trypsin, thrombin, or plasmin via cleavage at Arg92 (16-18, 33). Activated TAFI, denoted as TAFIa, is a zinc metalloprotease that hydrolyzes various synthetic and natural peptides with C-terminal arginines and lysines, with a preference for arginine. Its activity can be inhibited either by a synthetic compound such as guanidinoethylmercaptosuccinic acid (GEMSA) or a naturally occurring small protein, carboxypeptidase inhibitor from potato (CPI).

Unlike pancreatic CPA and CPB, activated TAFI is very unstable since it undergoes conformational changes that result in thermal instability. This thermal instability in turn facilitates the proteolytic cleavage of TAFIa at Arg302 by thrombin resulting in the loss of a substrate-binding site (33-35). The stability of TAFIa is enhanced when its catalytic site is occupied with inhibitors such as GEMSA and aminohexanoic acid.

The proposed physiological activator of TAFI is a thrombin/thrombomodulin complex. Thrombomodulin enhances the efficiency of thrombin-induced activation of TAFI by a factor of 1250, almost exclusively through its effect on *k*cat (36, 37). Furthermore, thrombomodulin protects activated TAFI by blocking the cleavage of Arg302 by free thrombin. There has also been a report on the activation of TAFI by plasmin, especially in the presence of heparin and other glycosaminoglycans (38).

2.2.3. TAFI and fibrinolysis *in vitro*

While TAFIa has been shown to cleave arginine and lysine residues from various peptides with C-terminal basic residues *in vitro*, the physiological substrate of TAFIa

is likely fibrin that has been partially degraded by plasmin. Activated TAFI prolongs the lysis time of clots formed in the presence of Glu-plasminogen up to three-fold in a clot lysis assay using purified protein components (18, 36, 37). This effect is dose-dependent with a half-maximal effect obtained at a TAFI concentration of 1 nM (36). Since that is well below the concentration of circulating TAFI, sufficient active enzyme can be generated in plasma to modulate fibrinolysis. In a plasma clot lysis assay, activation of TAFI by the thrombin/thrombomodulin complex results in a retardation of t-PA-induced lysis (39-43). This prolongation was abolished when activation of TAFI was inhibited with either monoclonal anti-TAFI antibody or anti-thrombomodulin antibody. The known inhibitors of TAFIa, CPI and GEMSA also blocked the inhibitory effect of TAFIa on clot lysis. In a whole blood clot lysis assay, similar results of TAFIa were observed on clot lysis (44, 45). In these assays, carboxypeptidase N (EC 3.4.17.3), the second basic carboxypeptidase present in blood which is not inhibited by CPI, had no effect on clot lysis time (44). Taken together these experiments suggest that TAFI plays a role in the regulation of fibrinolysis *in vivo*.

2.2.4. TAFI in experimental animals

The *in vivo* effect of activated TAFI has been reported in a number of animal models using CPI. Minnema et al. (46) demonstrated that incorporation of CPI or anti-Factor XI antibody in the thrombus at the time of its formation resulted in a two-fold increase in the rate of endogenous fibrinolysis compared with the control in a rabbit jugular vein thrombolysis model. There was no synergism between the effect of anti-Factor XI antibody and CPI, which is consistent with the concept that they work through the same mechanism, namely that Factor XI is involved in the amplification of thrombin generation which leads to activation of TAFI. Using a rabbit arterial thrombosis model, Klement et al. (47) showed that systemic administration of CPI with t-PA resulted in shortening of reperfusion time and longer duration of patency of the occluded vessel compared with t-PA only. Co-administration of CPI strongly inhibited thrombus growth. Similar effects of TAFI on t-PA-induced thrombolysis were also reported in a rabbit arterio-venous shunt model and in a rabbit jugular vein thrombolysis model (48, 49). Furthermore, prevention of venous thrombosis in the presence of TAFI inhibitor was observed in both rabbit and rat model (48, 50). These *in vivo* studies provide further evidence that TAFI is involved in the physiological regulation of fibrinolysis/thrombolysis.

2.2.5. TAFI in humans

The correlation between fibrinolytic potential and plasma level of TAFI antigen has been documented in the general population (51). In normal healthy subjects, plasma TAFI antigen level varies between 45 % and 150 % of the mean value (51-54). There have been some reports on the influence of age on the plasma TAFI antigen level (52-54). The presence of polymorphisms in the TAFI gene has been observed and it is known that some of the polymorphisms are responsible for variations in the plasma TAFI antigen level (55-59). To date, there has been no report of an individual deficient in plasma TAFI. The gene encoding human TAFI has been mapped to chromosome 13q14.11 (60, 61). No known hemostatic disease is currently associated with this region. However, one study indicated an increased TAFI level as a mild risk factor for venous thrombosis (53). On the other hand, surprisingly, there has been a recent report indicating lower levels of TAFI as a risk factor for coronary heart diseases (62). Such conflicting reports reflect the current state of our understanding of pathophysiological role(s) of TAFI in various diseases.

3. TAFI deficient mice

3.1. Rationale for generation of TAFI deficient mice

Mouse models deficient in each component of the fibrinolytic system have been generated to examine the role of the fibrinolytic system in development, thrombosis, fibrinolysis, reproduction, wound repair and cell migration (11, 12, 63-67). Unlike deficiency in coagulation factors such as tissue factor or Factor V, that cause embryonic lethality (68, 69), deficiency in the components of the fibrinolytic system results in animals that develop normally with no obvious phenotypic abnormalities and are fertile. However, plasminogen deficient mice suffer spontaneous thrombosis and impaired wound healing, which is alleviated by a concomitant deficiency in the fibrinogen gene (65, 66, 70). Furthermore, plasminogen activator deficient mice have impaired lysis of microthrombi in the lung, especially in response to endotoxin challenge (64), while mice deficient in either PAI-1 or a2-antiplasmin have increased thrombolysis and are more resistant to endotoxin-induced thrombosis (63, 67).

While accumulating evidence indicates a role of TAFI in the regulation of fibrinolysis/thrombolysis, all the studies have been performed with non-selective inhibitors of TAFIa. These inhibitors are known to inhibit other carboxypeptidases that are localized in different compartments of the body and exert various functions (28, 29). In order to investigate a role of TAFI in the regulation of endogenous fibrinolysis/thrombolysis in the light of the current conflicting clinical data, we generated homozygous TAFI deficient mice by targeted gene disruption (71). Such animal models can also be utilized to determine if TAFI plays a role in other plasmin-mediated processes and in the turnover of biologically active peptides possessing C-terminal basic residues. During the preparation of this review, Wagenaar et al. independently reported generation of TAFI deficient mice and observed a lack of abnormal phenotypes in their TAFI deficient mice, consistent with our result (72). The following sections cover the generation and characterization of our TAFI deficient mice.

3.2. Characterization of TAFI deficient mice

3.2.1. Generation of TAFI deficient mice

The full-length murine TAFI cDNA was isolated from a mouse liver cDNA library (lambda ZAP, Stratagene, La Jolla, CA) using a partial human TAFI cDNA as probe. The murine TAFI cDNA was used to screen a P1 genomic library prepared from the 129/Sv mouse (Genome Systems, St. Louis). A 9.2-kb SacI DNA fragment containing exons 6 to 9 and a 6.4-kb SpeI DNA fragment containing exon 10

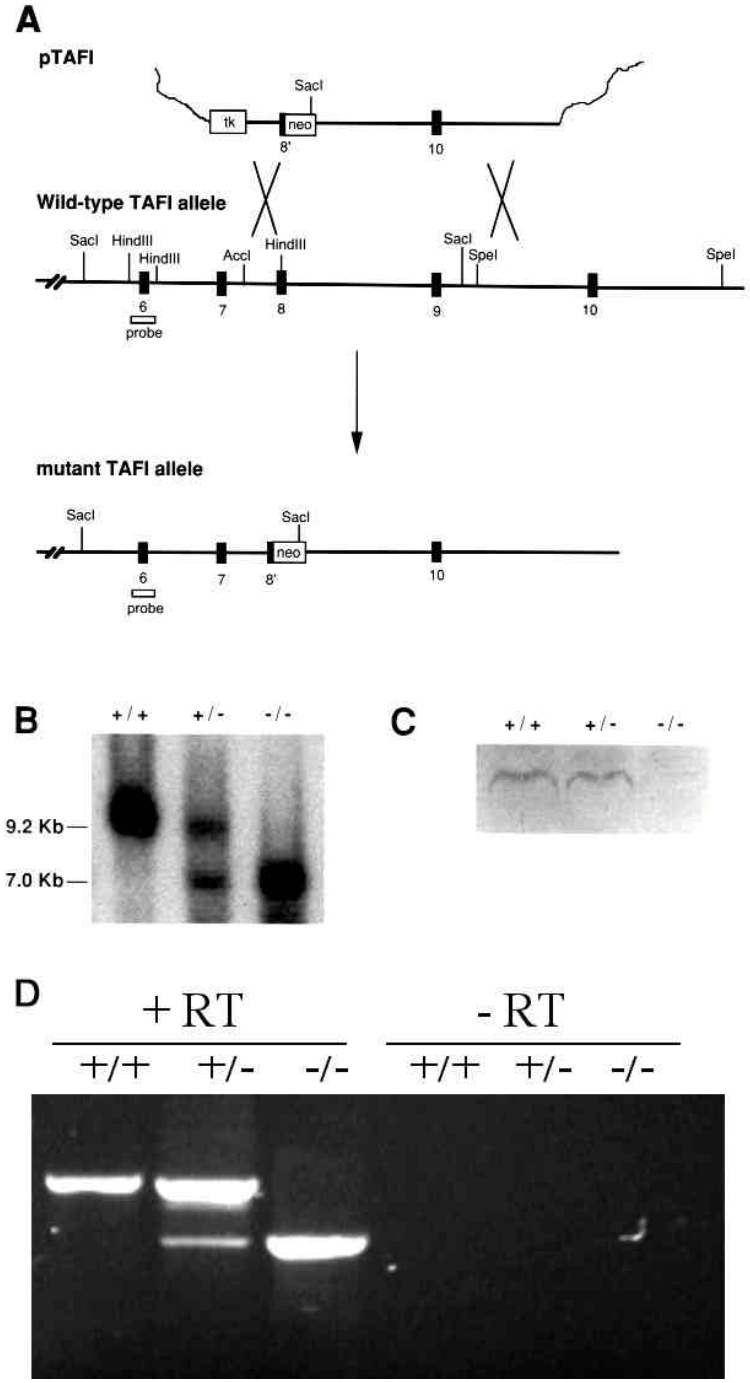

Figure 2. TAFI gene-disruption in mice. (A) TAFI replacement construct and partial restriction map of the endogenous locus. Exon 8 and 9 encode some of the zinc and substrate binding sites of TAFI. Neo, neomycin phosphotransferase gene; tk, thymidine kinase gene; probe, TAFI-specific probe used in Southern blot analysis. (B) Southern blot analysis of TAFI gene disruption. The probe indicated in (A) was used to detect 9.2-kb DNA fragment in the wild type allele and 7.0-kb DNA fragment in the mutant allele following Sac1 digestion of genomic DNA. (C) Western blot analysis of TAFI from plasma of $TAFI^{+/+}$, $TAFI^{+/-}$ and $TAFI^{-/-}$ mice. Antibody raised against TAFI peptide (CGIHAREWISPAF) hybridized to a band with a molecular weight similar to that of murine albumin in the wild type and heterozygous mice. It was absent in the homozygous TAFI deficient mice. (D) RT-PCR analysis of liver mRNA isolated from $TAFI^{+/+}$, $TAFI^{+/-}$ and $TAFI^{-/-}$ mice. TAFI PCR products obtained with primers, one annealing to the 5' end of TAFI cDNA (5'-CAAGTCACTGTTGGGATGAAGC -3') and the second one annealing to the 3' end of TAFI cDNA (5'-ATTAACTGTTCCTGATGACATGCC -3') are shown in the left panel. No bands are detected in the absence of reverse transcriptase (right panel).

Table 1. Plasma level of TAFI antigen and carboxypeptidase activity

Features	$TAFI^{+/+}$	$TAFI^{+/-}$	$TAFI^{-/-}$
TAFI antigen (nM, equivalent)	0.62 ± 0.23 (5)	0.34 ± 0.12 (6)	0.07 ± 0.03 (7)
TAFIa activity (mOD/min)	0.88 ± 0.33 (5)	0.52 ± 0.29 (6)	0.1 ± 0.15 (7)
CPN activity (mOD/min)	3.72 ± 1.18 (5)	3.63 ± 0.67 (6)	4.7 ± 1.06 (7)

TAFI antigen level in plasma was measured using an ELISA assay kit (Enzyme Research Laboratories, South Bend, IN) with purified human TAFI as a standard. TAFI activity in plasma was determined using 0.4 mM FA-Ala-Arg (Bachem Biosciences Inc., Bubendorf, Switzerland) as substrate following its activation with thrombin (40 nM), thrombomodulin (50 nM) and $CaCl_2$ (17 mM). CPN activity was determined similarly without addition of thrombin, thrombomodulin and $CaCl_2$. mOD is defined as milli-optical density. The number of mice used in each group is indicated in parentheses.

of the isolated murine TAFI gene were subcloned into pBluescript II KS (Stratagene) producing plasmid A and B, respectively. For the construction of the targeting vector, a HSV-thymidine kinase (tk) cassette was placed at the KpnI site within the multiple cloning site of pBluescript II KS using blunt end ligation. A 0.9-kb AccI-HindIII TAFI DNA fragment spanning a portion of intron 7 and half of exon 8 from plasmid A was subcloned into pET-21b (Stratagene) at its multiple cloning site. A XhoI-HindIII fragment containing the TAFI DNA was removed from the resulting pET-21b plasmid and was inserted between XhoI and HindIII sites directly after the tk cassette in the targeting vector. A SalI fragment containing a 1.5 kb PGK-neomycin phosphotransferase (neo) cassette was inserted into plasmid B directly upstream of the 6.4-kb TAFI DNA. Construction of the targeting vector was then completed by inserting a 7.9-kb XhoI-NotI DNA fragment from plasmid B containing the neo cassette and exon 10 of TAFI gene, between SalI and NotI sites of the targeting vector (Figure 2A). The targeting vector was linearized with NotI and introduced by electroporation into 129/Sv-derived R1 embryonic stem cells (gift from A. Nagy, University of Toronto, Ontario, Canada), and stable transfectants were selected as described previously (73). Of 420 clones, two contained the expected allele as determined by Southern blot analysis (Figure 2B). These two independent embryonic stem cell clones were subsequently injected into C57BL/6 blastocysts as described (73), and the resulting male chimera were bred to C57BL/6 females to generate F1 offspring. Heterozygous TAFI deficient mice were crossed to produce $TAFI^{+/+}$, $TAFI^{+/-}$ and $TAFI^{-/-}$ F2 littermates.

Successful targeting of the TAFI gene resulted in the replacement of a portion of exon 8 and 9 with the neo cassette, thereby removing the DNA segment encoding for residues critical for substrate and zinc binding which are essential for catalytic activity (Figure 2A). The expected structure of the targeted TAFI locus was confirmed by Southern blot analysis. Thus, after SacI digestion of genomic DNA, the probe that encompasses exon 6 recognized a band of approximately 9.2-kb in the wild type allele and a band of about 7.0-kb in the disrupted allele (Figure 2B). Germline transmission of the TAFI-null allele was achieved in a number of chimeric mice derived from two independent embryonic stem cell clones. Mice generated from both embryonic stem cell clones were characterized independently and were shown to be phenotypically indistinguishable. Consequently, most of the work described here was performed with one line.

The targeted disruption of the TAFI gene resulted in the truncation of the TAFI transcripts in homozygous TAFI null mice as shown by reverse transcriptase-polymerase chain reaction (RT-PCR) of mRNA isolated from liver (Figure 2D, left panel). The liver was the main tissue in which TAFI mRNA was detected by Northern blot analysis in mouse (30, 31). Using primers that correspond to the 5' and 3' end of TAFI cDNA, the RT-PCR of liver mRNA isolated from TAFI deficient mice produced a fragment of about 1000-bp compared with a fragment of about 1,350-bp with liver mRNA from wild type littermates. The RT-PCR amplified both fragments when mRNA from heterozygous mice was tested. In the absence of reverse transcriptase, no products were obtained (Figure 2D, right panel).

To determine if the truncated TAFI transcript in TAFI deficient mice leads to the presence of circulating truncated TAFI protein, Western blot analysis of plasma samples from different genotypes was performed. Polyclonal antibodies raised against a synthetic peptide CGIHAREWISPAF in the mature TAFI were used. Since this region of TAFI is encoded by exon 5, which is upstream of the disrupted region, if a truncated form of TAFI protein existed in circulation, it should be detected using these antibodies. The antibodies bound to a band present only in plasma samples from wild type and heterozygous TAFI mice (Figure 2C). The position of the band indicated that its molecular weight was similar to that of albumin, expected size for full-length mouse TAFI. There was no detectable truncated TAFI present in the plasma from TAFI deficient mice. The lack of TAFI protein in the plasma from TAFI deficient mice was also demonstrated using a commercial ELISA kit for detection of human TAFI and purified human TAFI as standard. The plasma level of TAFI in heterozygous mice was found to be about half of that detected in wild type mice (Table 1). Only background values were found with plasma samples from TAFI deficient mice, which were similar to those obtained with samples of TAFI-deficient human plasma.

3.2.2. Viability and fertility of TAFI deficient mice

DNA analysis of 259 progeny derived from heterozygous TAFI mice intercrosses showed that TAFI mice were born in the expected Mendelian ration of 1: 2: 1 (Table 2). This indicated that TAFI deficiency did not lead to embryonic lethality. TAFI deficient mice developed normally and reached adulthood. No physical abnormalities were observed in these mice up to 24 months of age. Mating of homozygous males with homozygous

Table 2. F2 offspring of TAFI $^{+/-}$ matings

Genotype	TAFI$^{+/+}$	TAFI$^{+/-}$	TAFI$^{-/-}$
Number of pups	64	127	68
% of total	25 %	49 %	26 %

Genotype of offspring from intercrossing of heterozygous TAFI mice was determined by PCR. Genomic DNA prepared from either tail or ear biopsies was used as templates with primers derived from 1) the upstream portion of exon 8 (5'-AGAAAGGTGCGTCAAGTTCCTCC-3'), 2) a portion of exon 8 deleted in the mutant allele (5'-GTTCTTCGTGGTCCTTGCTTTTG-3') and 3) the neo cassette (5'-TTCCTGACTAGGGGAGGAGTAGAAG-3'). The PCR product derived from the wild type allele is 190-bp and that from the mutant allele is 390-bp.

Table 3. Hematological and hemostasis analysis

Features	TAFI$^{+/+}$	TAFI$^{-/-}$
Hemoglobin (gram/%)	14.7 ± 1.4 (15)	14.5 ± 1.2 (15)
Platelet count (x 10^9/L)	1013 ± 176 (15)	1053 ± 288 (15)
Fibrinogen (%)	100 ± 35 (15)	96 ± 26 (15)
Alkaline phosphatase (IU/L)	85.4 ± 22 (5)	87.5 ± 20 (4)
Blood urea nitrogen (mg/dL)	25.8 ± 4.7 (5)	24.3 ± 5.8 (4)
Creatine (mg/dL)	0.44 ± 0.06 (5)	0.5 ± 0.08 (4)
PT (s)	10.9 ± 0.41 (6)	11.3 ± 0.38 (6)
aPTT (s)	19.3 ± 0.97 (6)	20.0 ± 0.83 (6)

For blood chemistry analysis, serum was obtained from the clotted whole blood and analyzed by IDEXX Veterinary Services Inc (West Sacramento, CA). Fibrinogen level was measured according to the method described by Macart (89). Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were performed with citrated platelet-poor-plasma using an Electra 900C coagulometer (Beckman Coulter, Brea, CA). The number of mice used in each group is indicated in parentheses.

females produced viable offspring of normal litter size indicating that TAFI deficient mice were fertile and pregnancies were carried to full term. Hematological analysis of TAFI deficient mice did not show any major differences in blood cell counts, plasma fibrinogen level, PT and aPTT, compared with those of wild type littermates (Table 3). There was no abnormality in liver and kidney functions as shown by blood chemistry analysis.

TAFI deficient mice did not suffer from any apparent untoward effects of tail transection. In order to study the effect of TAFI deficiency on bleeding more carefully, blood loss was determined during 30 minutes following tail transection of a 1-mm segment. The mice used in the study had intact tails prior to the amputation because routine genotyping was performed with ear biopsies instead of tail clips. There was no statistically significant difference in the amount of blood loss between TAFI deficient mice and the control mice (Figure 3, left panel). It has been shown previously that the 129/Sv mice have significantly longer bleeding times than the C57BL/6 mice, indicating that the genetic background can influence hemostasis (74). In this study, we compared offspring of littermates, rather than comparing among the same littermates. However, the present results were similar to those obtained from our previous study in which tail bleeding time in TAFI deficient mice was compared with their littermates and no differences were found (74). The effect of TAFI deficiency on bleeding was also studied in the presence of an antithrombotic agent that promoted exaggerated bleeding. In the presence of Lovenox, a low molecular weight heparin, there were wide individual differences in the extent of bleeding. However, the range of blood loss in TAFI deficient mice was similar to that in the wild type indicating that TAFI had little or no influence on coagulation (Figure 3, right panel). These findings are consistent with the results of aPTT and PT in TAFI deficient mice, which were identical to control values (Table 3).

3.2.3. Carboxypeptidase activity in TAFI deficient mice

To demonstrate a role of activated TAFI in fibrinolysis, the effect of TAFI deficiency was tested in a plasma clot lysis assay. Coagulation and fibrinolysis were initiated in mouse platelet-poor plasma with thrombin and t-PA, respectively. In the presence of thrombomodulin, which enhances the activation of TAFI, clot lysis time was prolonged at least two-fold in plasma from wild type mice (Figure 4, left panel). This prolongation was likely due to activation of TAFI as the inhibitor of carboxypeptidase from potato reversed it (data not shown). A similar extent of prolongation was observed in plasma from heterozygous mice, indicating that while their plasma level of TAFI is about half of that in the wild type (Table 1), enough TAFI is activated to perturb fibrinolysis. There was no prolongation of clot lysis time in plasma from TAFI deficient mice upon the addition of thrombomodulin (Figure 4, right panel). On the other hand, an addition of purified human TAFI to the same plasma resulted in a dose-dependent prolongation of clot lysis time as seen with plasma from the wild type and heterozygous mice (data not shown). Therefore, activated TAFI is responsible for the retardation of fibrinolysis in the wild type and heterozygous mice. These results are in agreement with potential TAFIa activity measured directly in these plasma samples with a small molecule substrate, FA-Ala-Arg (Furylacryloyl-alanyl-arginine), following activation with the thrombin/thrombomodulin complex (Table 1). Furthermore, it was evident that the activity of the second basic carboxypeptidase in plasma, carboxypeptidase N, remained relatively constant in the three groups of mice and that it did not influence fibrinolysis to any significant extent.

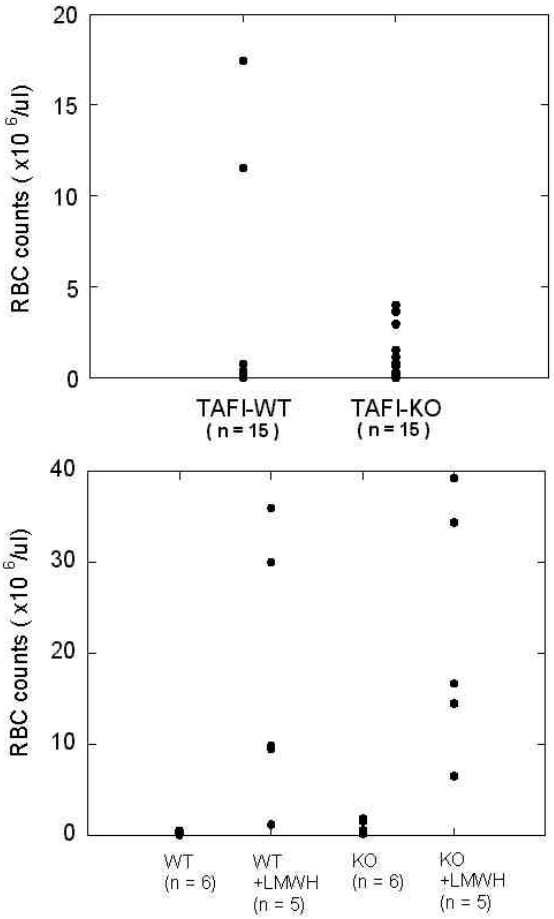

Figure 3. Tail bleeding. Wild type mice and TAFI deficient mice were anaesthetized with an intraperitoneal injection of pentobarbital (62.5 mg/kg body weight) and a 1-mm segment was amputated from the tip of the tail using a device. The tail was immediately immersed in saline pre-warmed to 37 º C. The amount of red blood cells released from the tail wound into saline during 30 minutes following amputation was determined with a Baker 9120+CP Cell Counter (ABX Diagnostics, Inc., Irvine, CA) and shown in the top panel. For tail bleeding in the presence of low molecular weight heparin, mice were injected with Lovenox (3 mg/kg body weight) via tail vein 2 minutes before tail transection and the amount of blood loss was measured similarly (bottom panel). The number of animals used in each group is indicated in the parentheses.

3.2.4. Effects of TAFI deficiency in thrombosis models

To investigate the effect of TAFI deficiency on the development of arterial thrombosis, TAFI deficient mice and their littermates were subjected to a photochemical injury in the presence of Rose bengal in the carotid artery and blood flow was monitored continuously as described previously (75). The time required to form an occlusive thrombus, defined as no detectable flow for 1 minute or longer, was recorded. Mean time was 59.2 ± 5.2 minutes (n=15) in TAFI deficient mice compared with 59.1 ± 6.3 minutes in the wild type littermates (n=14), indicating that TAFI deficiency did not influence the rate of acute thrombus formation in this arterial thrombosis model. The effect of TAFI deficiency was also tested on venous thrombosis. Mean time for occlusion of a jugular vein in TAFI deficient mice was 55.8 ± 43.8 minutes (n = 4) and 81.8 ± 41.4 minutes (n = 6) in the wild type, indicating the lack of impact of TAFI deficiency on venous thrombosis. In these models, initial formation of an occlusive thrombus depends on the balance between coagulation and fibrinolysis. While TAFI deficiency did not have a significant influence on the rate of acute thrombus formation, it is possible that enhanced endogenous fibrinolysis due to the absence of activated TAFI may facilitate spontaneous lysis of the residual thrombus. To test this possibility, some animals were allowed to recover, and the size of the residual thrombus was measured 24 hours later. We did not observe any statistically significant difference in the cross-sectional area of the residual venous thrombus between two groups. Such results imply that TAFI does not greatly influence the rate of spontaneous reperfusion either. In a similar arterial thrombosis model, PAI-1 has been shown to affect both occlusion and spontaneous reperfusion rate (76, 77). One of the possible reasons that TAFI deficiency does not influence the rate of thrombosis is that PAI-1 released from the vessel wall in response to a photochemical injury may well mask the more subtle effects of TAFI.

The potential involvement of TAFI in endogenous fibrinolysis under pathological conditions was examined in thrombin-induced acute thromboembolism, Factor X coagulant protein (XCP)-induced subacute thrombosis and endotoxin-induced disseminated intravascular coagulation. In the acute thromboembolism model, intravenous injection of human thrombin into the tail vein of conscious wild type mice over 2 to 3 seconds resulted in death within 10 minutes in a dose-dependent manner (Table 4). In similar models reported previously, intravenous injection of human thrombin into mice caused quick death (78, 79). Fibrin deposition was detected in both large and small vessels in the lung. The authors demonstrated that thrombin-induced feedback activation of clotting factors, and subsequent additional formation of thrombin, contributed to organ failure (79). Furthermore, it was proposed that thrombin-induced activation of Factor XIII and/or TAFI, which render newly formed fibrin more resistant to plasmin degradation, as additional pathological mechanism. In our study, survival rate determined at 10 minutes after thrombin injection was similar among TAFI deficient mice compared with the wild type. To investigate subtle effects of TAFI deficiency in response to thrombin injection, tissue fibrin deposition was measured using ^{125}I-labled human fibrinogen in separate experiments. There was, however, no significant accumulation of radioactivity in the lung and kidney of the control mice at any time points up to 6 hours after thrombin injection (our unpublished data). Thus in these experiments in mice, an intravenous injection of thrombin did not lead to substantial fibrin deposition in kidney or lung, preventing us from studying the effects of TAFI deficiency by this methodology.

Table 4. Survival rate in thrombin-induced acute pulmonary thromboembolism

Thrombin dose (NIH U/kg body weight, intravenous injection)	Survival rate in TAFI$^{+/+}$	Survival rate in TAFI$^{-/-}$
1,600	87 % (n = 8)	62 % (n = 8)
2,400	78 % (n = 9)	64 % (n = 11)
3,200	60 % (n = 15)	50 % (n = 12)

Conscious mice were injected with human thrombin over 2 to 3 seconds into the tail vein at various doses. The survival rate at 10 minutes after thrombin administration was taken as an index of the severity of pulmonary thromboembolism. The number of mice used in each group is indicated in the parentheses.

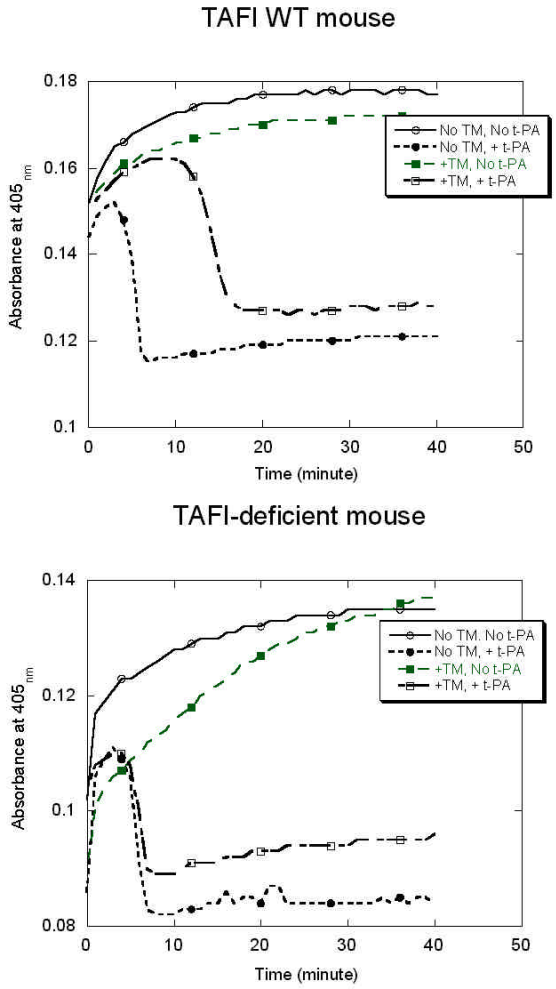

Figure 4. Plasma clot lysis profile. Clot formation and lysis were initiated in recalcified (Ca^{2+}, 17 mM) mouse citrated plasma with human thrombin (from American Diagnostica Inc., Greenwich, CT, at a final concentration of 2.5 NIH unit/mL) and t-PA (from Genentech inc., South San Francisco, CA, at a final concentration of 0.017 μg/mL), respectively. Human recombinant soluble thrombomodulin (TM) was added at a final concentration of 50 nM to enhance activation of endogenous TAFI. Absorbance at 405 nm is plotted against incubation time. A typical clot lysis profile obtained in the absence of TM and t-PA (solid line with open circle), in the absence of TM but presence of t-PA (dotted line with closed circle), in the presence of TM but absence of t-PA (dashed line with closed square) and in the presence of both TM and t-PA (dashed line with open square) is shown. The top panel is for the plasma from the wild type mice and the bottom panel is for the plasma from TAFI deficient mice.

While a high dose of thrombin activates TAFI *in vitro*, it is not known if TAFI is activated following thrombin injection in this *in vivo* acute pulmonary thromboembolism model. Thus, TAFI deficient and the wild type mice were challenged with endotoxin in a chronic model. Sato et al. demonstrated previously that LPS injection increased activation of TAFI as well as induction of hepatic TAFI mRNA in mice (31). Following LPS injection (either 30 mg/kg body weight or 40 mg/kg body weight), survival rates were similar between TAFI deficient and the wild type mice (Figure 5). Because of reports on transient fibrin deposition in the kidney of mice injected with LPS (80), in separate experiments animals were injected with either a sublethal dose (2 mg/kg body weight) or a high dose (30 mg/kg body weight) of LPS. Three hours later, kidneys were harvested and were stained for fibrin using rabbit anti-fibrinogen/fibrin antiserum (kindly provided by J. L. Degen, University of Cincinnati, Ohio, USA). While there was some specific fibrin staining within the glomeruli of both the wild type and TAFI deficient mice following LPS injection, there was no significant difference in the extent of staining between the two groups (determined by a pathologist who was blinded with respect to the genotype of the samples). Therefore, TAFI does not appear to exert a major effect on renal fibrin deposition. On the other hand, an enhanced endogenous fibrinolytic system in PAI-1 deficient mice or in a_2-antiplasmin deficient mice resulted in reduced tissue fibrin deposition in response to non-lethal dose of endotoxin (63, 67). Recently it has been reported that inhibition of TAFI activity reduced tissue fibrin deposition in rat DIC models (81, 82). The differences in the results of these studies in rat using inhibitors of TAFI activity and of the current study using TAFI deficient mice may be attributable to the difference in the extent of TAFI involvement in the pathogenesis of LPS-induced fibrin deposition in different species. Another explanation is that the inhibitors used to block TAFI activity may also inhibit another basic carboxypeptidase that is critical for development of fibrin deposition in response to LPS injection.

In a factor X coagulant protein (XCP)-challenged subacute thrombosis model, mice were injected intraperitoneally with XCP (150 μg/kg body weight) from Russell viper venom and survival rate, platelet counts and plasma fibrinogen level were determined. Survival rates at 16 hours after injection of XCP were similar in both wild type and TAFI deficient mice. Furthermore, significant decreases in plasma fibrinogen level and platelet counts were observed 2 hours and 16 hours after the stimulus, and the extent of decreases were similar in two groups (Table 5). These results confirm the conclusion from the other models of systemic thrombotic challenge, that no

Table 5. Survival rate, fibrinogen level and platelet counts following XCP challenge

Features	$TAFI^{+/+}$ (n = 15)	$TAFI^{-/-}$ (n = 15)
Survival rate (%)		
at 2 h	93	87
at 16 h	53	60
Plasma fibrinogen level (100 %)		
at 0 h	100 ± 35	96 ± 26
at 2 h	52 ± 23	56 ± 16
at 16 h	83 ± 34	106 ± 56
Platelet count (x 10^9/L)		
at 0 h	1013 ± 176	1053 ± 288
at 2 h	328 ± 252	416 ± 239
at 16 h	319 ± 285	353 ± 142

Mice were injected intraperitoneally with 150 µg/kg body weight Factor X coagulant protein from Russell Viper venom. The survival rate, platelet counts and fibrinogen level were determined at 2 and 16 hours later. The number of mice used in each group is indicated in the parentheses.

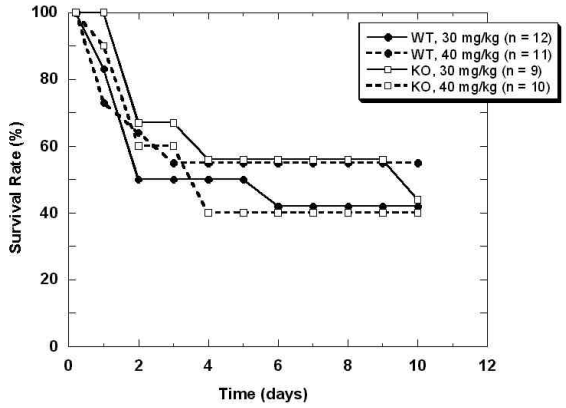

Figure 5. Effect of endotoxin on the survival of the wild type and TAFI deficient mice. Mice were injected intraperitoneally with endotoxin dissolved in saline (Escherichia coli lipopolysaccharide, serotype 0111:B4, from Sigma, St. Louis, MO) at a dose of either 30 mg/kg or 40 mg/kg body weight. The mice were observed daily for signs of endotoxemia daily such as lethargy, watery eyes and scruffy fur, and survival time was recorded. Survival curves for wild type mice injected with 30 mg/kg body weight (solid line with closed circle), with 40 mg/kg (dotted line with closed circle), TAFI deficient mice injected with 30 mg/ kg (solid line with open square) and with 40 mg/kg (dotted line with open square) are shown. The number of animals used in each group is indicated in the parentheses.

differences in response of TAFI deficient and wild type mice could be detected.

3.2.5. Kaolin-induced writhing response in TAFI deficient mice

Since *in vitro* TAFI has been shown to cleave C-terminal arginine residue from bradykinin (33, 83, 84), the effect of TAFI deficiency on bradykinin degradation was studied using a kaolin-induced writhing model (85). In this model, kaolin induced bradykinin release via activation of Factor XII, which in turn induced a writhing response. Previously, a nonselective inhibitor of basic carboxypeptidase had been shown to potentiate the writhing response. The number of writhes during the 15 minutes following intraperitoneal injection of kaolin (2.5 mg/mouse) was 1.29 ± 1.78 (n = 14) in wild type compared with 1.21± 1.85 (n = 14) in TAFI deficient mice. Thus, these results implied that TAFI does not play a major role in bradykinin metabolism under normal conditions.

4. PERSPECTIVE

Our study demonstrates that mice with a targeted mutation in the TAFI gene that leads to TAFI deficiency are born without any abnormalities and develop normally to reach adulthood. They are fertile and produce viable offspring, which demonstrates that TAFI deficiency is compatible with embryogenesis and growth. Hematological and hemostatic analyses reveal no major differences between TAFI deficient mice and the wild type including tail bleeding. Previously it has been shown that lack of activated TAFI resulted in premature lysis induced by exogenous t-PA of clots formed from plasma deficient in coagulation factor VIII, IX, X or XI (86). Such data implicated a physiological role for TAFI in stabilization of newly formed fibrin clots. The study with TAFI deficient mice shows that loss of TAFI gene does not lead to grossly impaired hemostasis. While lack of TAFI activity results in the loss of thrombomodulin-dependent prolongation of clot lysis *in vitro*, TAFI deficiency does not influence the rate of an occlusive thrombus formation in arteries and veins *in vivo*. Furthermore, TAFI deficiency does not improve survival rate compared with the wild type in thrombin-induced thromboembolism, XCP-induced thrombosis and endotoxin-induced disseminated intravascular coagulation, demonstrating that responses to acute challenges tested so far are not compromised in TAFI deficient mice.

It is noteworthy that Biemond et al. recently reported the lack of an effect of TAFI deficiency on pulmonary fibrinolysis of ^{125}I-labeled microthrombi (87). On the other hand, Swaisgood et al. reported that TAFI deficiency resulted in an increased pulmonary fibrinolysis when TAFI mice were in the background of heterozygosity for plasminogen gene (88). Furthermore, in these mice, leukocyte migration into the peritoneal cavity in response to thioglycollate injection was increased significantly.

Their results indicate a definitive role of TAFI in fibrinolysis and cell migration *in vivo* and that the influence of TAFI becomes critical under certain pathological conditions such as reduced level of circulating plasminogen.

The current study shows that the absence of TAFI activity in TAFI deficient mice is not compensated for by an increase in carboxypeptidase N since the plasma level of carboxypeptidase N activity remains relatively constant. Furthermore, it is confirmed that carboxypeptidase N dose not participate in the regulation of fibrinolysis. Both carboxypeptidase N and TAFIa can cleave the C-terminal arginine from bradykinin *in vitro*. Based on the results of the kaolin-induced writhing test with TAFI deficient mice, it is unlikely that TAFI is involved in the metabolism of bradykinin under normal conditions *in vivo*. However, there are a number of other biologically active peptides possessing C-terminal basic residues such as anaphylatoxins that might be other physiological substrates of activated TAFI (84). TAFI deficient mice will be suitable models for studying potential roles of TAFI in processing of other biologically active peptides.

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