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ORIGINAL ARTICLE

Evaluation of thrombomodulin/thrombin activatable fibrinolysis inhibitor function in plasma using tissue-type plasminogen activator-induced plasma clot lysis time

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Abstract

Background: Thrombin activatable fibrinolysis inhibitor (TAFI) is one of the most important physiological fibrinolysis inhibitors. Its inhibitory efficacy under physiological conditions remains uncertain.

Objectives: Elucidate the role of soluble thrombomodulin (sTM)/TAFI axis in the regulation of fibrinlysis.

Methods: Since thrombin is required to generate activated TAFI (TAFIa) that targets the C-terminal lysine of partially digested fibrin, a clot lysis assay is suitable for evaluating its function. Using tissue-type plasminogen activator-induced plasma clot lysis time (tPA-PCLT) together with TAFIa inhibitor and recombinant sTM (rsTM), we evaluated the specific function of TM/TAFI in the plasma milieu.

Results: tPA-PCLT values were significantly shortened by the TAFIa inhibitor. rsTM supplementation prolonged tPA-PCLT, which was shortened by the TAFIa inhibitor to a time similar to that obtained without rsTM and with the TAFIa inhibitor. Plasma obtained from patients treated with rsTM showed prolonged tPA-PCLT, which was shortened by the TAFIa inhibitor but not further prolonged by rsTM. However, no significant correlation was observed between tPA-PCLT and parameters of TM/TAFI system in the plasma.

Conclusion: The role of the TM/TAFI system in regulating fibrinolysis was successfully evaluated using TAFIa inhibitor and rsTM. Trace amounts of soluble TM in normal plasma appeared sufficient to activate TAFI and inhibit fibrinolysis. Further, a therapeutic dose of rsTM appeared sufficient to activate TAFI and regulate fibrinolysis in the plasma milieu.

KEYWORDS
clot lysis assay, fibrinolysis, TAFI, thrombomodulin, tPA

Essentials

- Thrombomodulin (TM)/thrombin activatable fibrinolysis inhibitor function was successfully evaluated using tissue-type plasminogen activator-induced plasma clot lysis time (tPA-PCLT).
- Soluble TM (sTM) in normal plasma appeared to effectively activate thrombin activatable fibrinolysis inhibitor and prolong tPA-PCLT.
- Supplementation of recombinant sTM appeared to further prolong tPA-PCLT.
- Recombinant sTM administration to disseminated intravascular coagulation patients prolonged tPA-PCLT; the effect of which lasted at least 24 hours.

1 | INTRODUCTION

Fibrinolysis removes unnecessary thrombi generated in the vasculature. Its native machinery takes place when fibrin is formed, where the assembly of tissue-type plasminogen activator (tPA) and Glu1-plasminogen (Glu1-plg) on the fibrin surface is a prerequisite [1–3]. The effective activation of Glu1-plg on the surface of fibrin is explained by a template mechanism and a conformational change of Glu1-plg to an easily activatable looser form after binding to the C-terminal lysine residues of partially digested fibrin through its lysine-binding site in Kringle 5 [4]. The importance of this mechanism is clearly demonstrated by the fact that dissociation of this binding by tranexamic acid, a lysine analog, inhibits effective fibrinolysis by disturbing the trimolecular complex formation. The generated plasmin then cleaves fibrin at the peptide bonds at the C-terminus of either lysine or arginine, which amplifies Glu1-plg accumulation at the newly generated C-terminal lysine residues on fibrin [1,5]. This effective amplification of fibrinolysis has been demonstrated in real-time imaging studies [6,7]. There is also a physiological mechanism targeting this machinery, which is mediated by a thrombin activatable fibrinolysis inhibitor (TAFI), whose active form (TAFIa) cleaves C-terminal lysine residues as a carboxypeptidase and inhibits fibrinolysis [7,8].

Thrombomodulin (TM)-bound thrombin [9] and plasmin [10] effectively activate TAFI. Since TAFIa has a very short half-life [8], the loci and phase of TAFI activation are important to fully express its specific activity under physiological and pathological conditions. TM exists on the surface of normal vascular endothelial cells (VECs), and thrombin is generated at the site where the vascular surface is injured and coagulation factors are activated. Therefore, the border between injured and normal VECs is the anticipated locus where stable hemostatic thrombi are needed and TAFI is likely activated. Platelets also contain TM [11]. They might provide their surface as loci for TAFI activation since the platelets' surface is the place for activating vitamin K–dependent coagulation factors and thrombin generation.

In addition to these specific loci, TAFI can be effectively activated in other intravascular locations where thrombin is generated, since plasma contains a trace amount of soluble TM (sTM) [12]. The amounts of sTM in the plasma are known to increase under various pathological conditions including sepsis, disseminated intravascular coagulation (DIC), and cardiovascular diseases [13]. Furthermore, under certain conditions including sepsis-associated DIC, recombinant sTM (rsTM) is frequently used in several countries including Japan [14]. However, the mechanism by which sTM contributes to TAFI activation remains unknown.

In the present study, we employed tPA-induced plasma clot lysis assay (tPA-PCLT) together with TAFIa inhibitors and rsTM and analyzed how the TM/TAFI system regulates plasma clot lysis using normal plasma obtained from healthy volunteers. We also applied this method to patients' plasma obtained from septic DIC patients who were treated with rsTM as well as from an abnormal TM patient whose TM lacked thrombin-binding site (TM-Nagasaki) and analyzed how sTM contributes to TM/TAFI dependent modulation of fibrinolysis in plasma.

2 | METHODS

2.1 | Proteins and reagents

We obtained the materials from each of the following sources: RecombiPlasTin 2G human recombinant tissue factor (TF; Instrumentation Laboratory), human recombinant tPA (TD2061; TOYOBO Co, Ltd), human rsTM (TM alpha, molecular weight = 64,000 Da; Asahi Kasei Pharma Corporation), TAFIa inhibitor (DS45251085, a DS-1040 analog, WO2013039202; Daiichi Sankyo Company Limited), human thrombin (Tanabe-Mitsubishi Pharma), and fluorogenic substrate of thrombin (Z-G-G-R-AMC; Technothrombin TGA SUB, Technoclone GmbH).

2.2 | Blood collection and preparation

Blood samples from healthy adult subjects (N = 11) and patients were collected into vacuum blood collection tubes containing 0.1 volumes of 3.2% trisodium citrate. Platelet-poor plasma was obtained by

centrifugation at 2000 × *g* for 10 minutes at 4 °C and was kept at −80 °C until use.

The septic DIC patients (*N* = 6) were diagnosed with DIC score ≥ 4 based on the Japanese Association for Acute Medicine's acute-stage DIC diagnostic criteria [15] and were treated by rsTM in intensive care unit in the hospital of Hamamatsu University School of Medicine. rsTM was administered at a 380-U/kg/d dose (numbers 2, 5, and 6). The dosage was reduced to 130 U/kg/d when the patient's kidney function was impaired (numbers 1 and 3) and to 200 U/kg/d when the platelet count was low (31,000/mm^3; number 4). Plasma samples were obtained at different time points: before and at 1, 6, and 24 hours after rsTM treatment. These patients were treated by rsTM for 1 to 7 days according to their DIC status.

A patient having congenital abnormal TM, a homozygous substitution of glycine by aspartate at amino acid residue 412 (Gly412Asp) in the thrombin-binding domain (TM-Nagasaki) [16], was also involved in this study. Due to lack of activation of both protein C and TAFI, the patient showed thrombosis and recurrent subcutaneous hemorrhage, which recovered well after rsTM administration together with improvements in abnormal laboratory tests including elevated D-dimer. We obtained one patient with congenial abnormal TM 1 week after rsTM treatment and before the next rsTM administration (regarded as "before rsTM treatment") and another sample immediately after rsTM administration (regarded as "after rsTM treatment").

The study was conducted following the principles of the Declaration of Helsinki. It was approved by the Ethics Committee of Hamamatsu University School of Medicine (reference numbers are 16-286 for healthy subjects and 17-237 and 15-114 for patients). All the participants provided written informed consent.

2.3 | Euglobulin clot lysis time

The euglobulin fraction was obtained by isoelectric precipitation of the acidified diluted plasma (20 times dilution at pH 5.2). After 1-hour incubation at 4 °C, the precipitate was obtained by centrifugation at 2000 × *g* for 10 minutes at 4 °C and was reconstituted to an original plasma volume with 0.1 M Tris/HCl buffer, pH 7.4. Euglobulin clot lysis time (ECLT) was assayed using a 96-well microtiter plate reader (Multiskan FC spectrophotometer, Thermo Fisher Scientific) as described previously [17,18], both in the absence and presence of calcium chloride [19–21]. Lysis time was defined as the time required for halfway decrease during clot lysis.

2.4 | tPA-PCLT and rsTM supplemented tPA-PCLT

tPA-PCLT was assayed similarly to the ECLT with a turbidimetric method [22]. A volume of 75 μL of plasma fraction supplemented with 1.0 nM tPA in 20 mM HEPES buffer (pH 7.4) containing 150 mM NaCl and 0.01% Tween 80 was mixed with calcium chloride (final concentration 10 mM) together with either a constant concentration of human thrombin (1 U/mL, tPA-PCLT [Th]) or TF (final 3000 times dilution, TF concentration was less than 0.1 pg/mL; tPA-PCLT [TF]) in total 200 μL to initiate clot formation. rsTM and TAFIa inhibitors (final concentration 5 nM: 0.32 μg/mL and 1 μM, respectively) were also supplemented when necessary. After covering the surface of the clot (final volume 200 μL) with soluble paraffin, the turbidity of each well was continuously monitored by an automatic microtiter plate reader at 37 °C.

2.5 | Thrombin generation assay

In tPA-PCLT (TF), a thrombin generation assay was conducted as previously reported [23] with a slight modification by using a fluorogenic substrate of Z-G-G-R-AMC, and lag time, time to peak, peak height, and area under the curve were calculated [22,24].

2.6 | Enzyme-linked immunosorbent assay of TAFI, TAFIa, TAFI inactive derivative, sTM, and plasminogen activator inhibitor 1

Plasma concentrations of total TAFI, activated TAFI and its inactive derivative (TAFIa/ai), sTM, and plasminogen activator inhibitor 1 (PAI-1) were assayed using the following commercially available enzyme-linked immunosorbent assay (ELISA) kits: total TAFI: IMUCLONE Total TAFI ELISA (BioMedica Diagnostics Inc); TAFIa/ai: ASSERACHROM TAFIa/TAFIai (Diagnostica Stago); sTM (kit): Quantikine ELISA Human Thrombomodulin/BDCA-3 (R&D Systems); and PAI-1: Human PAI-1 ELISA Kit (catalog number EP1100-1, Assaypro). Functionally active PAI-1 was also assayed by enzyme immunoassay [18].

2.7 | Statistical analysis

Correlation coefficients between the 2 groups were evaluated using Spearman's rank correlation methods. When the absolute values of the obtained Spearman's rank correlation coefficient (rs) were larger than their limit value (rs = 0.618 for 2-sided for 11 samples), the correlation was considered significant (*P* = .05). The significance of the differences in each parameter was evaluated using the Wilcoxon signed-rank test. The critical values for 2-tailed tests were obtained from the Wilcoxon signed-rank table, and *P* values less than .05 were considered significant.

3 | RESULTS

3.1 | tPA-PCLT and its modification by TAFIa inhibitor

tPA-PCLT initiated by thrombin supplementation (tPA-PCLT [Th]) showed a wide range of values among individual samples, although

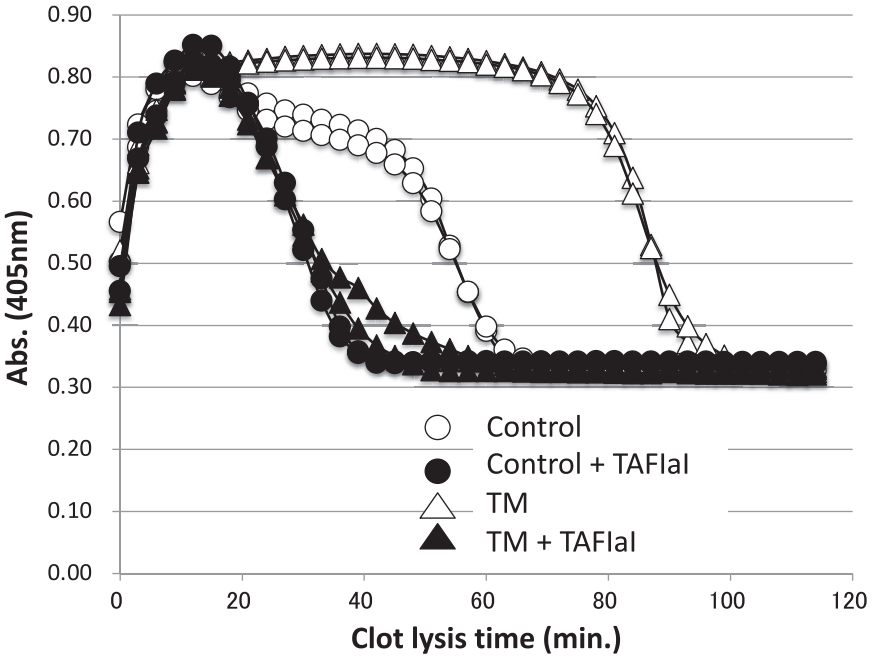

FIGURE 1 The representative clot lysis curves of tissue-type plasminogen activator-induced plasma clot lysis time (tPA-PCLT.) The raw data of tPA-PCLT human thrombin (Th) conducted in duplicate are shown. Open circles: tPA-PCLT (Th); closed circles: tPA-PCLT (Th) + activated thrombin activatable fibrinolysis inhibitor (TAFIa) inhibitor, open triangles: tPA-PCLT (Th) + recombinant soluble thrombomodulin (rsTM); closed triangles: tPA-PCLT (Th) + rsTM + TAFIa inhibitor. Abs, absorbance; TAFIai, inactive derivative of thrombin activatable fibrinolysis inhibitor.

duplicate measurements of each sample revealed good reproducibility (Figure 1). tPA-PCLT (Th) was significantly shortened by the TAFIa inhibitor (Figure 2), suggesting that a certain amount of TAFI was activated either in the plasma or during the assay and prolonged clot lysis. As was previously reported by others [25] and our group [7], a trace amount of sTM in the plasma (Table 1) seemed to be responsible for TAFI activation under the conditions employed above.

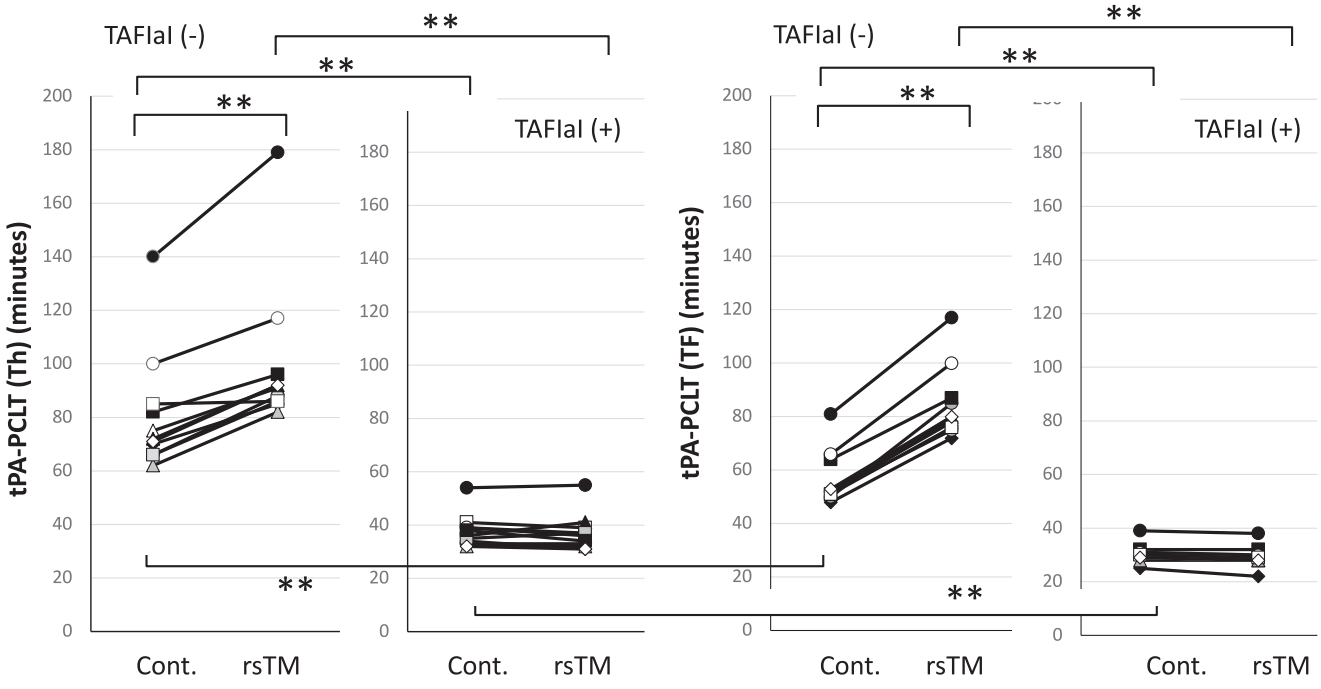

FIGURE 2 Tissue-type plasminogen activator-induced plasma clot lysis time (tPA-PCLT) with human thrombin (Th) in normal volunteers. tPA-PCLT (Th) with and without recombinant soluble thrombomodulin (rsTM) in the absence and presence of activated thrombin activatable fibrinolysis inhibitor (TAFIa) inhibitor in normal volunteers' plasma (left 2 figures). tPA-PCLT with human recombinant tissue factor (TF) with and without rsTM in the absence and presence of TAFIa inhibitor in normal volunteers' plasma (right 2 figures). The significance of the differences between the 2 groups was evaluated using Wilcoxon signed-rank test ($N = 11$). $^{**}P < .01$. Cont., control; TAFIai, inactive derivative of thrombin activatable fibrinolysis inhibitor.

TABLE 1 Plasma concentrations of fibrinolysis-related factors in normal volunteers.

	PAI-I (ng/mL)			sTM	Total TAFI	TAFIa/ai
Entry no.	Total	Complex	Free	(ng/mL)	(%)	(ng/mL)
No. 1	65.4	12.6	52.8	3.24	112	18.6
No. 2	9.0	4.1	4.9	4.33	125	16.8
No. 3	5.4	3.4	2.0	3.21	120	12.2
No. 4	5.1	3.4	1.6	4.15	99	10.6
No. 5	17.8	7.7	10.1	3.56	109	10.4
No. 6	4.2	3.0	1.2	3.29	119	10.4
No. 7	93.7	14.0	79.7	3.11	117	13.1
No. 8	8.2	8.7	0.0	3.27	121	14.3
No. 9	11.0	3.9	7.1	3.44	113	11.1
No. 10	5.1	5.1	0.0	3.24	119	10.6
No. 11	11.0	5.4	5.6	3.19	111	14.3

PAI-I, plasminogen activator inhibitor 1; sTM, soluble thrombomodulin; TAFI, thrombin activatable fibrinolysis inhibitor; TAFIa/ai, activated or the inactive derivative of thrombin activatable fibrinolysis inhibitor.

tPA-PCLT initiated by TF supplementation together with $CaCl_2$ (tPA-PCLT [TF]) was significantly shorter than tPA-PCLT (Th) ($P < .01$; Figure 2) but showed a positive correlation with tPA-PCLT (Th) (rs = 0.682; $P < .05$; Supplementary Table S1). tPA-PCLT (TF) was also shortened by the TAFIa inhibitor, which was also shorter than that of tPA-PCLT (Th) supplemented with the TAFIa inhibitor ($P < .01$; Figure 2), and these 2 parameters showed a good correlation (rs = 0.893; $P < .05$; Supplementary Table S1).

3.2 | rsTM supplemented tPA-PCLT

A supplementation of rsTM significantly prolonged tPA-PCLT (Th), which was shortened by the TAFIa inhibitor, to values like tPA-PCLT (Th) obtained without rsTM and with the TAFIa inhibitor (Figures 1 and 2). This suggests that the prolongation was mediated by TAFIa, likely by thrombin bound to rsTM. tPA-PCLT (TF) was similarly prolonged by rsTM to tPA-PCLT (Th) but was still shorter than tPA-PCLT (Th) with rsTM ($P < .01$; Figure 2). The tPA-PCLT (TF) with rsTM was also shortened by the TAFIa inhibitor. tPA-PCLT (Th) supplemented with rsTM showed a good correlation with tPA-PCLT (TF) supplemented with rsTM (rs = 0.84; $P < .05$). Similarly, tPA-PCLT (Th) with rsTM and the TAFIa inhibitor showed a good correlation with tPA-PCLT (TF) with rsTM and the TAFIa inhibitor (rs = 0.847; $P < .05$; Supplementary Table S1).

This suggests that only a part of the total TAFI in plasma was activated in the control tPA-PCLT by a trace amount of endogenous sTM, and supplementation with rsTM successfully contributed to further activation of TAFI and prolongation of tPA-PCLT.

3.3 | Contribution of the plasma levels of TAFI/TM-related factors to tPA-PCLT

Total TAFI, TAFIa/ai, and sTM plasma concentrations were assayed, and their contributions to tPA-PCLT (Th) were analyzed. Plasma levels of these proteins were not significantly different among healthy individuals (Table 1). Neither total TAFI nor sTM levels in the plasma were significantly correlated with tPA-PCLT (Th), tPA-PCLT (Th) with rsTM, and delta tPA-PCLT (Th) obtained by rsTM supplementation. They also did not show significant correlation with the number obtained by subtracting tPA-PCLT (Th) with rsTM and the TAFIa inhibitor from tPA-PCLT (Th) with rsTM (Supplementary Table S1). The thrombin generation was measured in tPA-PCLT (TF). However, no parameter related to thrombin generation showed a meaningful correlation with tPA-PCLT (TF) with rsTM or the number obtained by subtracting tPA-PCLT (TF) with rsTM and the TAFIa inhibitor from tPA-PCLT (TF) with rsTM (Supplementary Table S2).

3.4 | Correlations of tPA-PCLT (Th) and other clot lysis assays

Using the same sample, we conducted other clot lysis assays and analyzed their correlations with tPA-PCLT (Th) (Supplementary Tables S1 and S2). tPA-PCLT (Th) correlated well with both tPA-PCLT (Th) with the TAFIa inhibitor and tPA-PCLT (Th) with rsTM. Further, both tPA-PCLT (Th) and tPA-PCLT (TF) showed positive correlations with both ECLT Ca(−) and ECLT Ca(+).

3.5 | Correlations between tPA-PCLT (Th) and PAI-1 as well as other parameters of fibrinolysis in plasma

To analyze how the factors in the plasma contributed to tPA-PCLT, we analyzed the correlations between tPA-PCLT and plasma concentrations of the regulators of fibrinolysis (Supplementary Tables S1 and S2). tPA-PCLT (TF) positively correlated with total PAI-1 levels (Supplementary Table S2). This result was surprising because large amounts of tPA were used to overcome PAI-1 activity and initiate plasma clot lysis in the assay, and thus the contribution of PAI-1 was expected to be small. The facts that tPA-PCLT (TF) showed a strong correlation with plasma PAI-1 levels coincide with the facts that tPA-PCLT (TF) showed a strong correlation with ECLT, which is basically regulated by plasma PAI-1 levels [17,18].

3.6 | tPA-PCLT of septic DIC patients' plasma

Plasma samples obtained from DIC patients before and after rsTM treatment were subjected to the tPA-PCLT (Th) assay. Plasma levels of sTM, total TAFI, and TAFIa/ai are shown in Table 2. Changes in

TABLE 2 Plasma concentrations of fibrinolysis-related factors in septic disseminated intravascular coagulation patients.

Entry no.	sTM (ng/mL)	Total TAFI (%)	TAFIa/ai (ng/mL)
No. 1	12.90	64	55.7
No. 2	5.07	114	65.3
No. 3	2.19	107	239.7
No. 4	9.20	55	42.3
No. 5	4.69	107	73.8
No. 6	2.59	115	78.8

sTM, soluble thrombomodulin; TAFI, thrombin activatable fibrinolysis inhibitor; TAFIa/ai, activated or the inactive derivative of thrombin activatable fibrinolysis inhibitor.

plasma concentration of sTM after rsTM administration are shown in Figure 3A. The patients' plasma showed a wider range of tPA-PCLT (Th) levels than those of normal volunteers (Figure 3B). As in normal volunteers, those before treatment showed prolonged tPA-PCLT (Th) with rsTM supplementation. However, samples obtained after treatment (1, 6, and 24 hours) did not show an apparent prolongation by rsTM, and the tPA-PCLT (Th) obtained in the presence and absence of rsTM were similar. The TAFIa inhibitor was successfully shortened to a similar value in all the cases.

3.7 | tPA-PCLT of a patient's plasma of TM-Nagasaki

We also analyzed plasma obtained from TM-Nagasaki patient. The patient's plasma before rsTM treatment showed shorter tPA-PCLT compared with those of normal volunteers, which was not further shortened by inactive derivative of TAFI (TAFIai) (Figure 4). However, plasma obtained after rsTM administration showed prolonged tPA-PCLT, which was shortened by TAFIai to a time similar to that of before rsTM treatment. These results suggest that TAFI is not activated in plasma of TM-Nagasaki and that administered rsTM

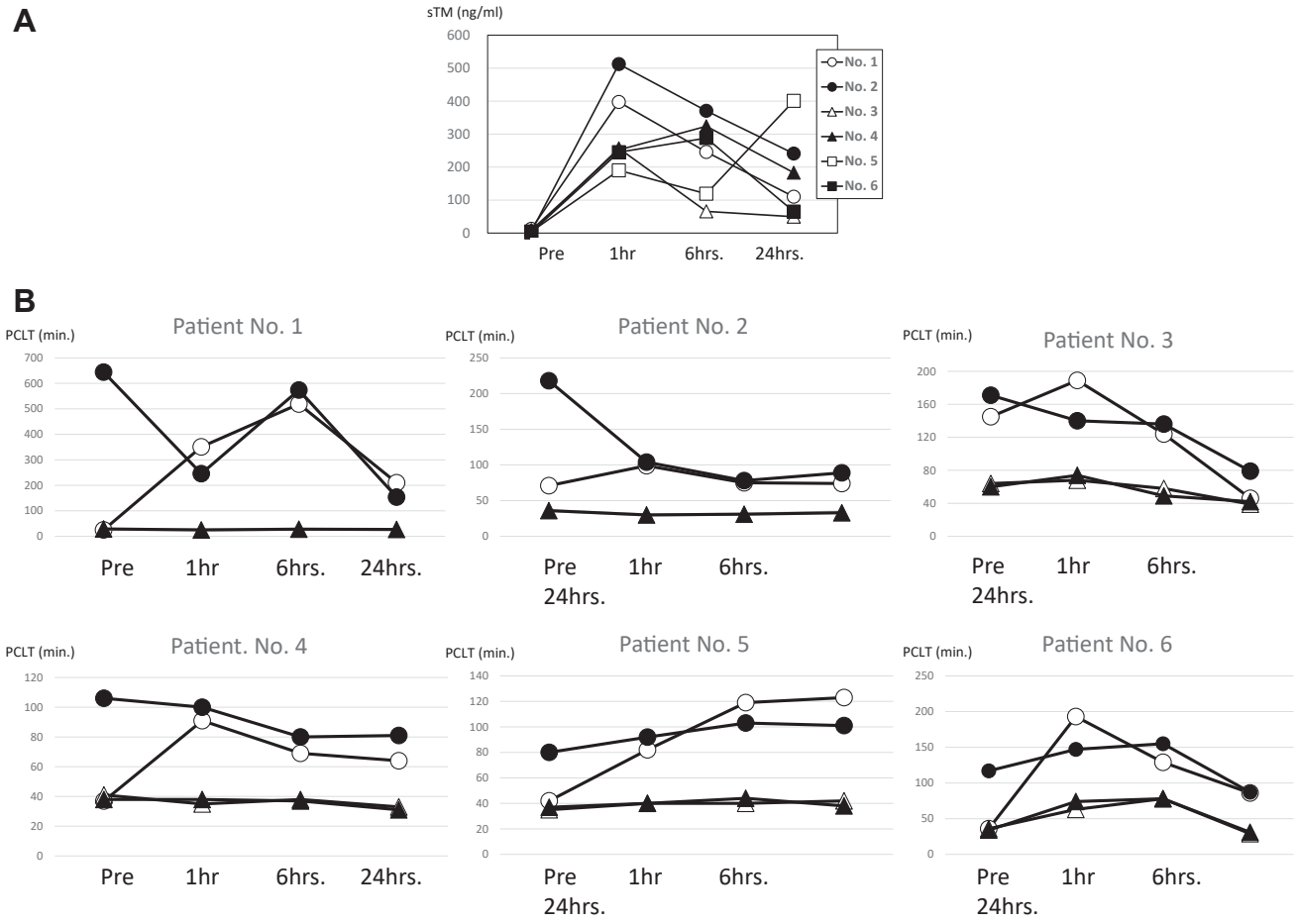

FIGURE 3 Changes in soluble thrombomodulin (sTM) and tissue-type plasminogen activator-induced plasma clot lysis time (tPA-PCLT) with human thrombin (Th) in septic disseminated intravascular coagulation patients' plasma before and after sTM treatment. Plasma samples were obtained at different times before (Pre) and at 1, 6, and 24 hours after recombinant sTM (rsTM) treatment. (A) Plasma sTM levels are shown. (B) tPA-PCLT (Th) in the presence and absence of rsTM and activated thrombin activatable fibrinolysis inhibitor (TAFIa) inhibitors of patients' samples. Data are presented as the average tPA-PCLT time for duplicate data. Open circle: tPA-PCLT (Th); closed circle: tPA-PCLT (Th) + rsTM; open triangle: tPA-PCLT (Th) + TAFIa inhibitor; closed triangle: tPA-PCLT (Th) + rsTM + TAFIa inhibitor.

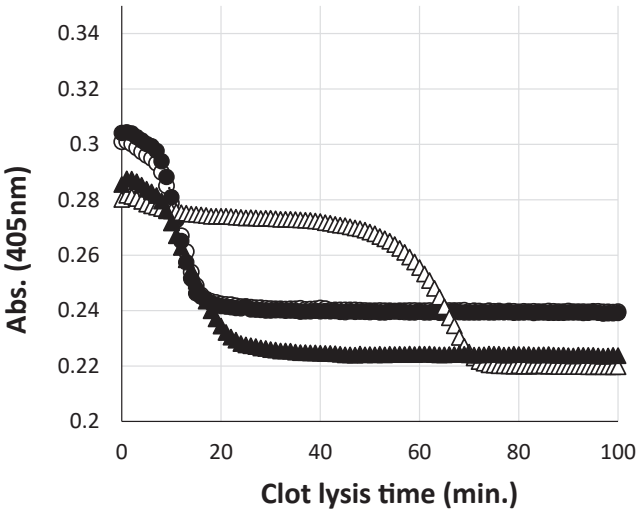

FIGURE 4 The clot lysis curves of tissue-type plasminogen activator-induced plasma clot lysis time (tPA-PCLT) with human thrombin in TM-Nagasaki. The patient's plasma of TM-Nagasaki was obtained before and immediately after recombinant soluble thrombomodulin administration and was subjected to tPA-PCLT either in the presence or absence of an activated thrombin activatable fibrinolysis inhibitor (TAFIa) inhibitor. tPA-PCLT with human thrombin before treatment without (open circle) and with TAFIa inhibitor (closed circle), and after treatment without (open triangle) and with TAFIa inhibitor (closed triangle). Abs, absorbance; TM-Nagasaki, patient with abnormal thrombomodulin whose thrombomodulin lacks a thrombin-binding site.

successfully functioned to activate TAFI. These also suggest that TAFI activation in plasma is mainly mediated by TM-bound thrombin but by neither free thrombin nor plasmin.

4 | DISCUSSION

In the present study, we confirmed that a certain amount of TAFI can be activated in the plasma milieu in an endogenous sTM-dependent manner by demonstrating significant shortening of tPA-PCLT by TAFIa inhibitor. The fact that supplemented rsTM significantly prolonged tPA-PCLT suggests that rsTM given as therapeutics likely further modifies fibrinolysis in *in vivo* situations, as was also shown in the patients' plasma.

Fibrinolysis is primarily regulated by 3 distinct regulators of PAI-1, TAFI, and alpha2-antiplasmin. To evaluate either the total activity of fibrinolysis or the potential to exhibit fibrinolytic activity in plasma, global fibrinolytic activity assays such as diluted plasma clot lysis time, ECLT, and tPA-PCLT have been developed [26]. However, these regulators inhibit their target molecules or systems at different time points and in different places *in vivo* [3], and thus evaluating each factor's role in a single assay method seems difficult. In the present study, using tPA-PCLT together with a specific inhibitor of TAFIa and rsTM, a modifier of the TM/TAFI system, we successfully evaluated the contribution of the TM/TAFI system to fibrinolysis in the plasma milieu.

TAFIa, a carboxypeptidase that cleaves C-terminal lysine residues of peptides, inhibits the binding and activation of plasminogen on fibrin or cell surfaces [8,27]. Since the gene-deficient animals do not develop an apparent phenotype when invasive challenges are not applied [28,29], their physiological relevance in the regulation of fibrinolysis remains uncertain. Recent findings of TM-Nagasaki [16], a genetic variant of TM that lacks TAFI activation properties and shows a bleeding tendency, suggest physiological relevance of TAFI in stabilizing hemostatic thrombi. To accurately evaluate the function of TM/TAFI in plasma, a clot lysis assay in the presence of both TM and TAFI is required. Our previous and present findings suggest that trace amounts of sTM in normal plasma efficiently prolong tPA-PCLT via TAFI activation [7]. Thus, the value obtained by subtracting tPA-PCLT with the TAFIa inhibitor from tPA-PCLT indicated endogenous plasma TM/TAFI activity, which likely develops without additional TM. Lack of the shortening by TAFIa inhibitor in the plasma obtained from TM-Nagasaki patients clearly suggests an essential function of TM-bound thrombin for TAFI activation in plasma. Though both free thrombin and plasmin [10] are suggested to activate TAFI, their role in the plasma milieu appeared much less than that of sTM-bound thrombin.

However, neither tPA-PCLT (Th) nor tPA-PCLT (TF) showed meaningful correlations with the plasma levels of TM/TAFI-related factors including TAFI, sTM, and TAFIa/ai (Supplementary Tables S1 and S2). The values obtained by subtracting tPA-PCLT (Th) supplemented with the TAFIa inhibitor from tPA-PCLT (Th), as well as that of tPA-PCLT (TF) supplemented with the TAFIa inhibitor from tPA-PCLT (TF), also did not show meaningful correlations with these values. We calculated the amount of thrombin generated using an established method [23]. Contrary to our expectations, it did not show meaningful correlations with tPA-PCLT (TF), in which thrombin generated during the assay likely participated in the activation of TAFI. Only a small difference in the plasma concentrations of these factors among healthy volunteers could explain these findings. However, rsTM supplementation successfully prolonged both tPA-PCLT (Th) and tPA-PCLT (TF) levels, which were reversed by the TAFIa inhibitor. These facts suggest that TM present *in vivo*, probably either as sTM in the plasma or as membrane-bound TM on VECs in the vessels, efficiently generates TAFIa and suppresses fibrinolysis together with thrombin.

In addition, plasma obtained from patients treated with rsTM showed TAFIa inhibitor-sensitive prolongation of tPA-PCLT, which was not further prolonged even after rsTM supplementation (Figure 3B), suggesting that sTM existing in plasma efficiently suppresses fibrinolysis. rsTM has been clinically used as an anticoagulant for patients with DIC owing to its cofactor activity on thrombin-catalyzed activation of protein C [30]. However, our results suggest that the plasma sTM levels in these patients are sufficient to activate TAFI and inhibit fibrinolysis. The fact that the concentration of sTM required for the activation of TAFI is lower than that required for protein C in the absence of the endothelial protein C receptor [31,32] supports these findings. Thus, rsTM treatment likely suppresses fibrinolysis when thrombin is generated. In septic DIC, fibrinolysis suppression is a critical feature that develops thrombogenesis and multiple organ failures [33]. Therefore, the enhancement of TAFI

activation capacity by rsTM treatment was speculated to deteriorate their clinical features. If we consider evident beneficial effects without serious adverse effects [34,35], rsTM-dependent TAFI activation might contribute rather to stabilize microthrombi and mitigate inflammation to become beneficial to septic DIC whose underlying pathophysiology is multifactorial [33]. Further analyses are required to understand how rsTM induces beneficial effects on patients with septic DIC and others by modifying both their anticoagulant and antifibrinolytic activities using specific laboratory tests including the one introduced in the present study.

The tPA-PCLT generated by both thrombin and TF showed positive correlations with ECLT both in the presence and absence of Ca^{2+} (Supplementary Tables S1 and S2). tPA-PCLT (TF) also showed positive correlations with PAI-1. Although a large amount of tPA is supplemented in tPA-PCLT to overcome PAI-1 in the plasma and express sufficient tPA activity, the balance between tPA and PAI-1 in the plasma seems to be responsible for determining the fibrinolytic activity to be expressed after clot formation. To evaluate the involvement of a particular regulatory system in tPA-PCLT, the use of a specific inhibitor of the system, such as a TAFIa inhibitor, is essential. For ECLT, we proved that supplementation of $CaCl_2$ significantly shortened ECLT by quenching the PAI-1 activity. Thrombin generated in the euglobulin fraction through the activation of the coagulation cascade by the addition of $CaCl_2$ was shown to neutralize PAI-1 activity through both high molecular weight complex formation and subsequent cleavage of PAI-1 [20]. The absence of shortening of ECLT by $CaCl_2$ in patients with lethal bleeding is a good indication for studying the PAI-1 gene to identify their distinct PAI-1 deficiency [36,37].

In a global fibrinolytic assay, a specific technique is required to evaluate the function of a specific regulator. In the present study, we used a TAFIa inhibitor and rsTM, an important regulator of the TM/TAFI system, to evaluate the function of TM/TAFI in tPA-PCLT. We demonstrated that trace amounts of endogenous sTM in plasma were sufficient to activate TAFI and inhibit fibrinolysis and that rsTM, either supplemented into the tPA-PCLT assay system or intravenously administered to patients as therapeutics, further suppressed fibrinolysis. We believe that these assays will contribute to evaluating the function of the TM/TAFI system under different pathological conditions and identifying abnormalities in this system. As is well known, however, TM also functions on the surface of VECs and modulates coagulation, fibrinolysis, and inflammation together with other functional molecules, including endothelial protein C receptor and proteoglycans. Though the present study clearly demonstrates sTM functions in plasma in the absence of VECs, possible collaborative functions with other molecules on cell surfaces need to be evaluated to understand the whole function of TM *in vivo*.

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ETHICS STATEMENT

The study was conducted following the principles of the Declaration of Helsinki. It was approved by the Ethics Committee of Hamamatsu University School of Medicine (reference numbers are 16-286 for healthy subjects and 17-237 and 15-114 for patients). All the participants provided written informed consent.

AUTHOR CONTRIBUTIONS

T.U. designed the study, performed the experiments, analyzed and interpreted the data, and wrote the paper. Yuko Suzuki designed the study, analyzed and interpreted the data, and wrote the paper. Y. Sano performed the experiments, analyzed the data, and discussed the results. Yuji Suzuki and M.D. collected the septic disseminated intravascular coagulation patient samples and discussed the results. M.O. collected the patient's sample of TM-Nagasaki and discussed the results. H.S., N.H., and N.M. discussed the results. All the authors read and approved the manuscript.

RELATIONSHIP DISCLOSURE

The activated thrombin activatable fibrinolysis inhibitor of the DS-1040 analog was provided by Daiichi Sankyo Co, Ltd. The authors declare no competing financial interests.

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SUPPLEMENTARY MATERIAL

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