Visualization of Domain- and Concentration-Dependent Impact of Thrombomodulin on Differential Regulation of Coagulation and Fibrinolysis

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Abstract

Background Thrombomodulin (TM) functions as a dual modulator—anticoagulant and antifibrinolytic potential—by the thrombin-dependent activation of protein C and thrombin-activatable fibrinolysis inhibitor (TAFI). Activated TAFI cleaves the C-terminal lysine of partially degraded fibrin and inhibits both plasminogen binding and its activation on the fibrin surface. We have reported previously that activated platelets initiate fibrin network formation and trigger fibrinolysis after the accumulation of tissue-type plasminogen activator and plasminogen.

Objective To analyze the effects of domain-deletion variants of TM on coagulation and fibrinolysis at different concentrations.

Methods Domain-deletion variants of TM, such as D123 (all extracellular regions), E3456 (minimum domains for thrombin-dependent activation of protein C and TAFI), and E456 (minimum domains for that of protein C but not TAFI), were used at 0.25 to 125 nM for turbidimetric assay to determine the clotting time and clot lysis time and to visualize fibrin network formation and lysis in platelet-containing plasma.

Results and Conclusions A low concentration of either D123 or E3456, but not of E456, prolonged clot lysis time, and delayed the accumulation of fluorescence-labeled plasminogen at the activated platelets/dense fibrin area due to effective TAFI activation. Conversely, only the highest concentrations of all three TM variants delayed the clotting time, though fibrin network formation in the vicinity of activated platelets was almost intact. TAFI activation might be affected by attenuation in thrombin activity after the clot formation phase. These findings suggest that the spatiotemporal balance between the anticoagulant and antifibrinolytic potential of TM is controlled in domain- and concentration-dependent manners.

Keywords
- fibrinolysis
- thrombomodulin
- anticoagulant
- platelets
- fluorescence

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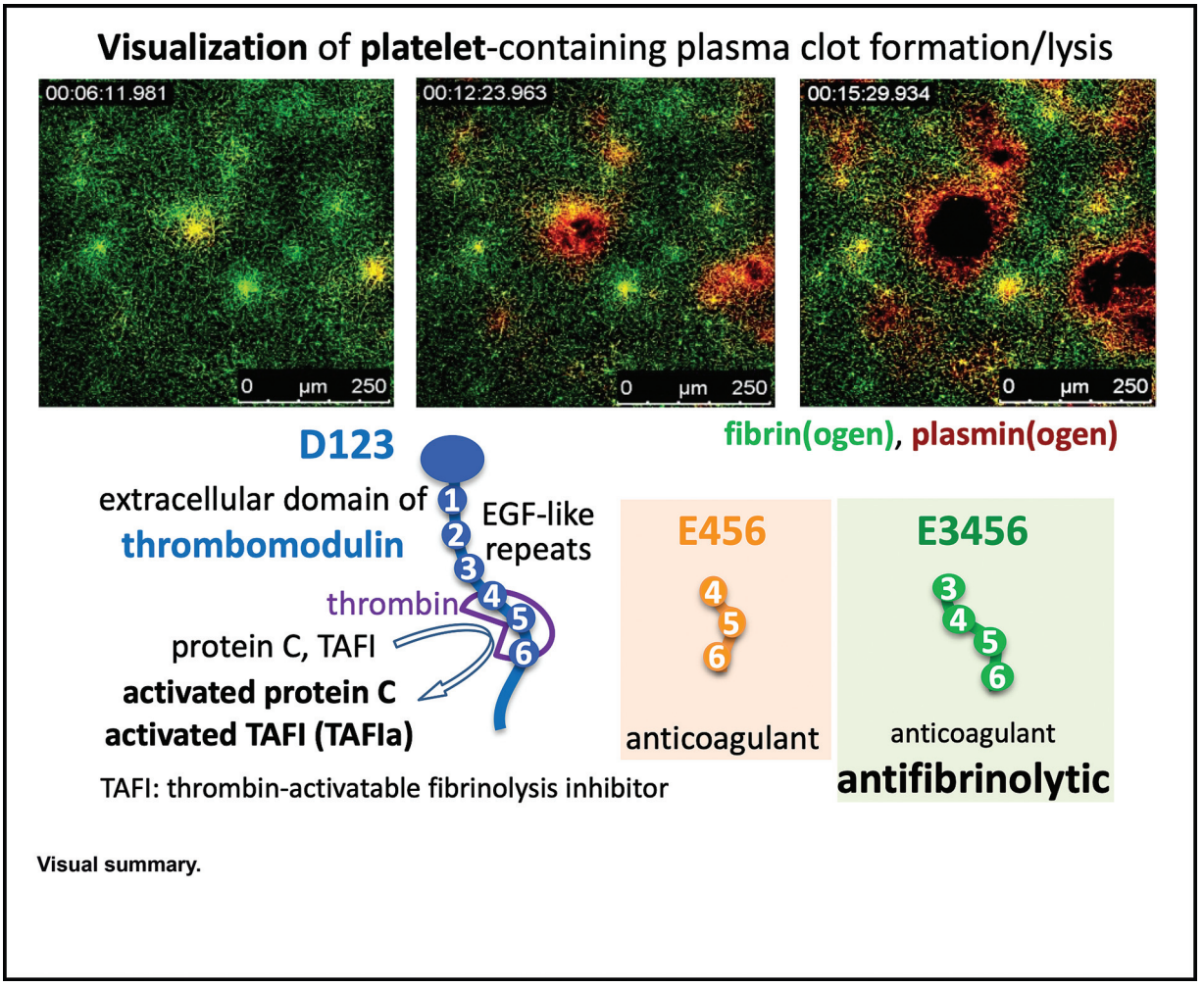

Visual summary.

Introduction

Nearly 40 years after the discovery of thrombomodulin (TM), several studies have revealed its involvement in the regulation of coagulation, innate immunity, inflammation, and cell trafficking, as well as therapeutic use.[1,2] TM, primarily expressed on the vascular endothelium, is a single-stranded integral membrane glycoprotein and was initially identified as a membrane-bound cofactor for thrombin-catalyzed activation of protein C.[3,4] Activated protein C is known to proteolytically cleave activated coagulation factors V and VIII and function as a potent anticoagulant. Thus, thrombin-dependent procoagulant activity is converted to anticoagulant activity when TM is available.

TM-bound thrombin also strongly activates thrombin-activatable fibrinolysis inhibitor (TAFI).[5] Activated TAFI (TAFIa), a metallocarboxypeptidase, fundamentally removes C-terminal lysine or arginine from peptides and proteins. The C-terminal lysine on partially degraded fibrin, to which the lysine-binding sites of plasminogen bind, plays essential roles in the native machinery of effective plasmin generation and fibrinolysis.[6–8] Although TAFIa easily exhibits its inhibitory effect on fibrinolysis where TM is expressed, and thrombin is generated, no physiological inhibitor of TAFI has been reported. Only the thermal instability of TAFIa is considered to terminate its activity.[9,10] Although the correlation between elevated TAFI/TAFIa levels and the risk of ischemic stroke or cardiovascular disease has been demonstrated,[11] low TAFI levels due to gene polymorphisms resulted in an increase in the risk of thrombotic diseases, which is in contrast to our expectation; thus, the physiological relevance of the TM–TAFI system remains controversial.[8]

TM is structurally composed of five domains: three extracellular domains, a transmembrane region, and a short cytoplasmic tail. The extracellular region consists of N-terminal domains homologous to the family of C-type lectins (lectin-like domain), six tandemly repeated epidermal growth factor-like (EGF-like) domains, and a serine–threonine-rich domain.[12] The fourth, fifth, and sixth EGF-like structures are the minimum domains required for protein C activation potential,[13] whereas additional regions of the C-loop in the third EGF-like structure are required for TAFI activation potential.[14,15] Protein C and TAFI activation depend on different TM structures; however, these substrates competitively bind to TM-bound thrombin in solution,[14] and the activation rates of both protein C and TAFI seem to be equal in the presence of saturating thrombin–TM complex.[5] Conversely, the thrombin-dependent activation of TAFI is

enhanced at low TM concentrations, which is canceled with increasing TM concentrations depending on a functional protein C pathway.[16] Wu et al demonstrated the activation of protein C and TAFI on vascular endothelial cells and concluded that both substrates did not compete for the thrombin-dependent activation by cell-surface-expressed TM.[17] Interestingly, how these two physiologically important events are either independently or cooperatively regulated in the vasculature remains to be elucidated.

Our previous studies, using real-time imaging analyses of human platelet-containing plasma, clarified that both endogenous[18] and supplemented[19] recombinant human-soluble TM (rhsTM; Recomodulin), which are complexed with thrombin, effectively activated TAFI, resulting in delayed clot dissolution, although fibrin network structure formation seemed unaffected. Such real-time imaging techniques have great advantages in visualizing different clot formation and dissolution processes—platelet activation, fibrin network formation initiated from activated platelet surface, plasminogen accumulation with tissue-type plasminogen activator (tPA) on the activated platelet surface, and fibrin network dissolution expanding to the periphery.

In this study, we analyzed the effects of different concentrations of recombinant TM (rTM) variants on both coagulation and fibrinolysis. The domain-deletion variants of rTM used herein include the following: D123 consisting of a lectin-like domain (D1), six EGF-like domains (D2), and a serine–threonine-rich domain (D3); E3456 consisting of a third to sixth EGF-like structure; and E456 consisting of a fourth to sixth EGF-like structure. Results obtained from both turbidimetric assay and real-time imaging of fibrin formation and lysis in the platelet-containing plasma demonstrated the existence of differential regulation of coagulation and fibrinolysis by thrombin-bound TM through the domain- and concentration-dependent activation of TAFI and protein C including in the vicinity of the activated platelets where fibrin generation is initiated.

Materials and Methods

The concentrations of the reagents and the platelet count indicated here are the final concentrations.

Proteins and Reagents

The following reagents were purchased: Alexa Fluor fluorescent dyes (Life Technologies Corporation, Eugene, Oregon, United States), Dade Innovin human recombinant tissue factor (TF; Siemens, Germany), human fibrinogen (Enzyme Research Laboratories, South Bend, Indiana, United States), and type B gelatin (Sigma-Aldrich Inc., St. Louis, Missouri, United States).

TAFIa inhibitor (biosimilar compound of DS-1040),[20] human recombinant tPA (TD2061), and rTM variants (D123, E3456, and E456)[21] were kindly provided by Daiichi Sankyo Company Limited (Tokyo, Japan), Toyobo Co., Ltd. (Osaka, Japan), and Asahi Kasei Pharma (Tokyo, Japan), respectively. Glu1-plasminogen was purified from freshly frozen human plasma by affinity chromatography on sepharose lysine.[22]

Blood Collection and Preparation

Blood samples from healthy participants were collected in tubes containing 0.1 volumes of 3.2% trisodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation at 250 × g for 10 minutes at 22°C, and the platelet concentration was determined using a whole-blood cell counter, pocH-80i (Sysmex Corporation, Kobe, Japan). Platelet-free plasma was prepared by centrifugation at 1,800 × g for 10 minutes at 4°C, followed by subsequent centrifugation at 3,000 × g for 10 minutes at 4°C. Following this, the platelets were counted, and the plasma was confirmed to be free of platelets. PRP was diluted with platelet-free plasma to obtain plasma containing 4.0×10^4 platelets/μL. For experiments, plasma samples were diluted to a final concentration of 50% with 10 mM HEPES buffer, pH 7.4 (HBS; 140 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$). All samples were used within 6 hours after blood collection. The study was conducted following the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Hamamatsu University School of Medicine (No. 16–286). All participants provided written informed consent.

Turbidimetric Assay for Clotting and Clot Lysis Times

To determine the clotting and clot lysis times, platelet-containing plasma (4.0×10^4 platelets/μL) was mixed with various concentrations (0.25, 0.5, 1, 5, 25, and 125 nM) of D123, E456, E3456, and 1.5 nM tPA in a 96-well microplate. As necessary, 5 μM TAFIa inhibitor was added. After adding 10 mM $CaCl_2$ with Dade Innovin (dilution, 1:6,000), the measurement of absorbance at 405 nm was immediately started at 37°C, with the absorbance being measured every minute (Multiskan FC; Thermo Fisher Scientific, Waltham, Massachusetts, United States). Clotting time was calculated as the time of the half-maximal increase in turbidity during clot formation. Clot lysis time was defined as the time interval between the half-maximal increase in turbidity during clot formation and the half-maximal decrease during clot lysis.

Enzyme-Linked Immunosorbent Assay for TM in Washed Platelets

To prepare washed platelets, PRP supplemented with 0.5 μM prostacyclin was centrifuged at 700 × g for 15 minutes at 22°C. The platelets were resuspended in HBS supplemented with 0.5 μM prostacyclin, then centrifuged again at 1,100 × g for 10 minutes at 22°C. The washing step was repeated, and the platelet pellet was lysed using cell lysis reagent (CelLytic-M; Sigma-Aldrich). The TM concentration in washed platelets was determined using Quantikine ELISA Human TM/BDCA-3 (R&D Systems, Minnesota, United States).

Real-Time Imaging Using a Confocal Laser Scanning Microscopy

Real-time imaging analysis was performed using a fluorescence microscope, TCS SP8 (Leica Microsystems GmbH, Wetzlar, Germany), equipped with a 20× (NA 0.8) objective lens and a stage-top incubator (Tokai Hit Co., Ltd., Shizuoka, Japan) to maintain the temperature of the samples at 37°C.

All experiments were conducted in a 0.1% gelatin-coated, 35-mm glass-based dish (AGC Inc., Tokyo, Japan). Platelet-containing plasma (4.0×10^4 platelets/μL) was supplemented with 2.0 nM tPA, Alexa Fluor 488-labeled fibrinogen, and Alexa Fluor 568-labeled plasminogen and incubated for 3 minutes in a heating chamber on a stage at 37°C. Coagulation was initiated by adding 10 mM $CaCl_2$ combined with Dade Innovin (dilution, 1:6,000). Immediately after that, a focal plane approximately 10 μm above the bottom of the dish in a randomly selected platelet field was chosen, and the image capture was started. The observation field was maintained during the entire duration of the experiment. Three focal planes, scanning at 1-μm steps each, were captured, and a single image was created by stacking the three planes.

Analysis of Real-Time Imaging Data

To estimate the efficacy of TAFI activity, the time from the appearance of fibrin fiber structures to the achievement of maximal labeled plasminogen fluorescence intensity at a dense fibrin network area (circular region of interest with a diameter of 30 pixels) was measured and defined as the plasminogen accumulation time. Fibrin formation in the vicinity of activated platelets was evaluated as a percentage of delta increase in the Alexa Fluor 488-labeled fibrinogen fluorescence intensity of the maximum increase in the intensity in the aforementioned area 1 to 5 minutes after the triggering of coagulation.

Statistical Analysis

All data were statistically analyzed using Statcel 3, an add-in software for Excel (OMS Publishing Inc., Saitama, Japan). For multiple comparisons, a Shirley–Williams test (►**Fig. 1**) or a Steel–Dwass test (►**Fig. 2**) was used to compare all nonparametric pairs. For between-group comparisons, the Student's *t*-test or the Mann–Whitney U-test was used.

Results

The Effects of rTM Variants on the Plasma Clot Formation and Lysis

We analyzed the effects of rTM variants on plasma clot formation and lysis. D123, an rTM variant with the complete extracellular domain of TM, showed a significant anticoagulant effect only when added at the highest concentration of 125 nM (►**Fig. 1A**). Other variants—E3456, which has the minimum required domain for the thrombin-dependent activation of protein C alone, and E456, which has the minimum required domain for activating thrombin-dependent protein C alone but not TAFI—significantly prolonged the clotting time at the highest concentration of 125 nM

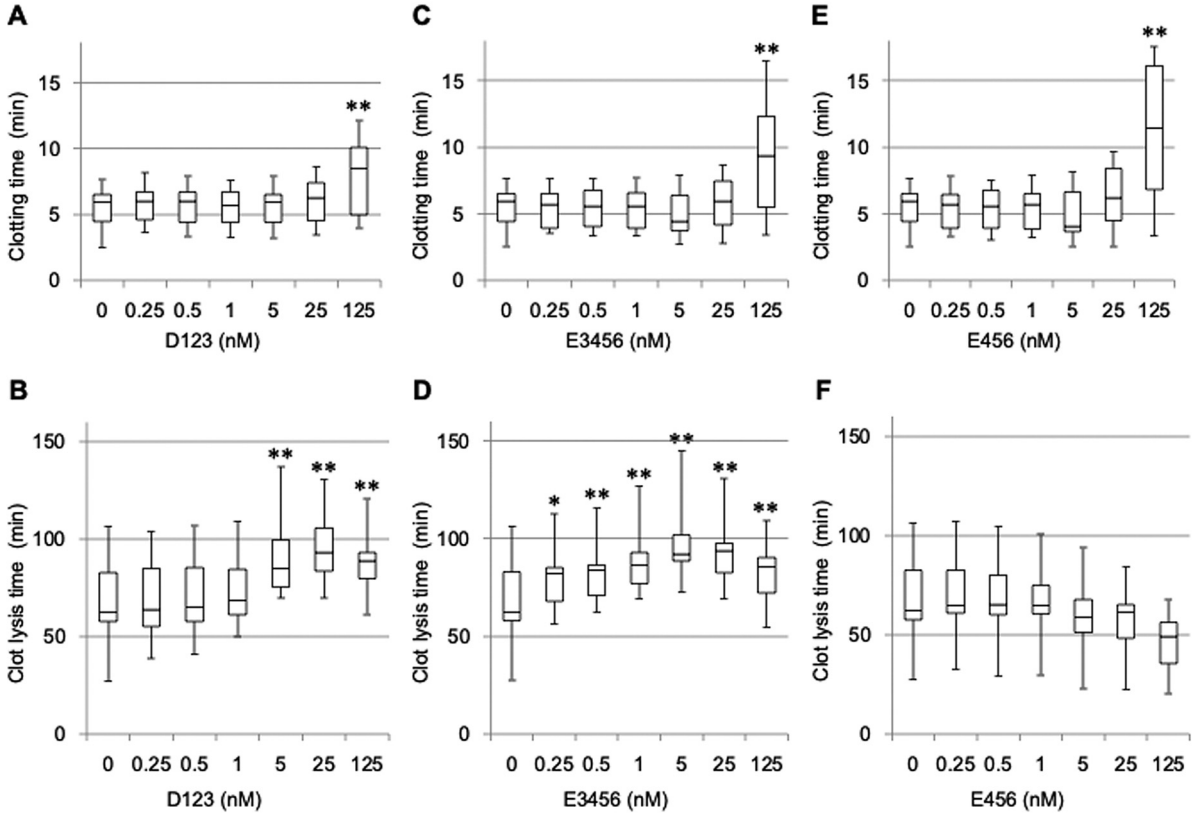

Fig. 1 The effects of recombinant thrombomodulin (rTM) variants on the platelet-containing plasma clotting time and clot lysis time determined by the turbidimetric assay. Diluted thromboplastin (tissue factor)-triggered clotting time (**A, C, E**) and tissue-type plasminogen activator-initiated clot lysis time (**B, D, E**) of the platelet-containing plasma (4.0×10^4 platelets/μL) in the presence of D123 (A, B), E3456 (C, D), and E456 (**E, F**) are shown. For all conditions, >15 samples in total, unevenly from 6 healthy individuals were used. Data are shown as the median and interquartile range, and the increasing trends were analyzed using the Shirley–Williams multiple comparison tests ($^*p < 0.05$, $^{**}p < 0.01$).

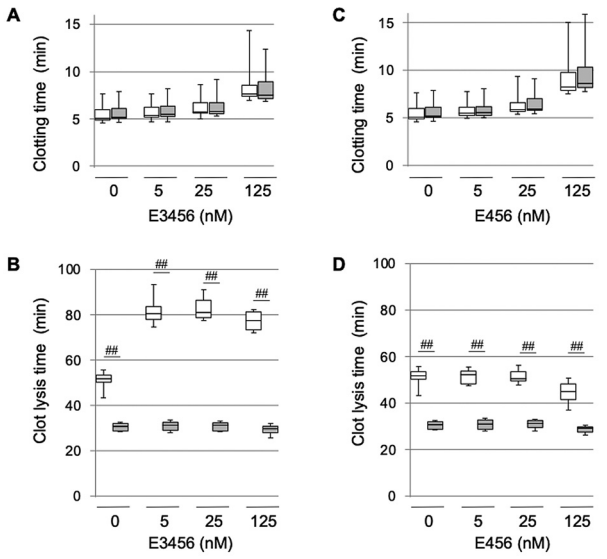

Fig. 2 Reduction of TM-associated prolongation of clot lysis time by TAFIa inhibitor. Platelet-containing plasma (4.0×10^4 platelets/μL) clotting time (**A, C**) and clot lysis time (**B, D**) in the absence (open bars) or presence of 5 μM TAFIa inhibitor (closed bars) are shown. (A) and (B) represent E3456, and (C) and (D) represent E456. For all conditions, >8 samples in total, unevenly from 3 healthy individuals were used. Data are shown as median and interquartile ranges and were analyzed using the Mann–Whitney *U*-test ($^{\#\#}p < 0.01$). TAFIa, activated thrombin-activatable fibrinolysis inhibitor; TM, thrombomodulin.

(►**Fig. 1C, E**); thus, their effects were slightly greater than those of D123. These turbidimetric analyses suggested that a high concentration of TM domain was required for preventing fibrin clot formation, through an inhibitory effect of TM on the procoagulant activity of thrombin and/or TM-bound thrombin catalyzing protein C.

The clot lysis time was prolonged by both D123 and E3456 at lower concentrations than those required to prolong the clotting time, which was more apparent in E3456 (►**Fig. 1B, D**). At the highest concentration of 125 nM, however, prolongation of clot lysis time was limited. Thrombin-bound E456, which cannot activate TAFI, did not prolong the clot lysis time but rather shortened it at high concentrations, albeit not significantly (►**Fig. 1F**). TAFIa inhibitor strongly shortened the clot lysis time both in the absence and presence of rTMs at all the concentrations employed (►**Fig. 2B, D**). The differences in the clot lysis times are considered TAFIa activity-dependent, and both endogenous TM and supplemented rTM were responsible for the development of thrombin-dependent TAFIa activity. It was reasonable that the TAFIa inhibitor did not affect the clotting time (►**Fig. 2A, C**)

Activated Platelet-Dependent TAFI Activation in the Presence of E456

The clot lysis time for the platelet-containing plasma was longer than that of the platelet-free plasma both in the absence and presence of supplemented rTM, which was shortened by the TAFIa inhibitor to similar values (►**Fig. 2B, D** and ►**Supplementary Fig. S1**). These results support our recent findings that activated platelets inhibited fibrinolysis by activating TAFI, which is dependent on platelet-derived TM and/or soluble TM in the plasma.[18] Although the amounts of TM were very low in the platelets (15.3 ± 2.27 pg in the single well of 8.0×10^6 platelets [$n = 3$], mean ± standard deviation) and in plasma (0.86 ± 0.17 ng/200 μL: single well volume,[18] mean ± standard deviation), they appeared to have successfully activated TAFI together with thrombin. In the presence of high E456 concentrations, the TAFIa inhibitor significantly shortened the clot lysis time only in the platelet-containing plasma (►**Fig. 2D**) but not in the platelet-free plasma (►**Supplementary Fig. S1**). Therefore, TAFI seemed to be effectively activated by endogenous TM-bound thrombin in the presence of platelets even when the activation of the coagulation cascade was suppressed by the high concentration of E456.

Visualization of Platelet-Oriented Coagulation and Fibrinolysis

Our previous real-time imaging in vitro studies[18,19,23] demonstrated a series of events leading to clot lysis after the accumulation of a large amount of plasminogen along with the fibrin network, which was displayed as a montage in control (►**Fig. 3**). A dense fibrin network formed on and around the activated platelet is also the place where both tPA and plasminogen accumulation and fibrinolysis are initiated.[19] The effects of TM variants on clot formation and the lysis process in the platelet-containing plasma are shown in ►**Fig. 4**. All TM variants showed localized dense clot formation in the vicinity of activated platelets within 3 minutes after the initiation of the coagulation reaction with diluted thromboplastin and Ca^{2+} (►**Fig. 4B–D**; left panels). Plasminogen already accumulated at a dense fibrin network at 10 minutes in the control (►**Fig. 4A**) and E456-treated plasma (►**Fig. 4C**), which increased the amount and triggered the initiation of fibrinolysis and its propagation to the periphery. Plasminogen accumulation was significantly delayed at two different concentrations by D123 (►**Fig. 4B**) and E3456 (►**Fig. 4D**).

Estimation of TAFI Activation in the Vicinity of Activated Platelets

The fluorescence intensity of labeled plasminogen represents the amounts of C-terminal lysine residues on fibrin fibers. TAFIa, a carboxypeptidase, cleaves C-terminal lysine and thereby decreases its amount. Suppression of the fluorescence intensity in the labeled plasminogen on the fibrin network and activated platelets is considered to show the effect of TAFI activation. The average fluorescence intensity of labeled fibrinogen (►**Fig. 5**, closed circles) and plasminogen (►**Fig. 5**, open circles) in a single dense fibrin region, just in the vicinity of activated platelet, is shown as a representative result. The plasminogen accumulation time, defined as the time from the appearance of the fibrin network to the peak of plasminogen fluorescence intensity, was strongly delayed by D123 and E3456 but not by E456. Furthermore, the plasminogen accumulation time was more significantly delayed by 5 nM E3456 than by 5 nM D123 or 125 nM E3456. Delayed plasminogen accumulation

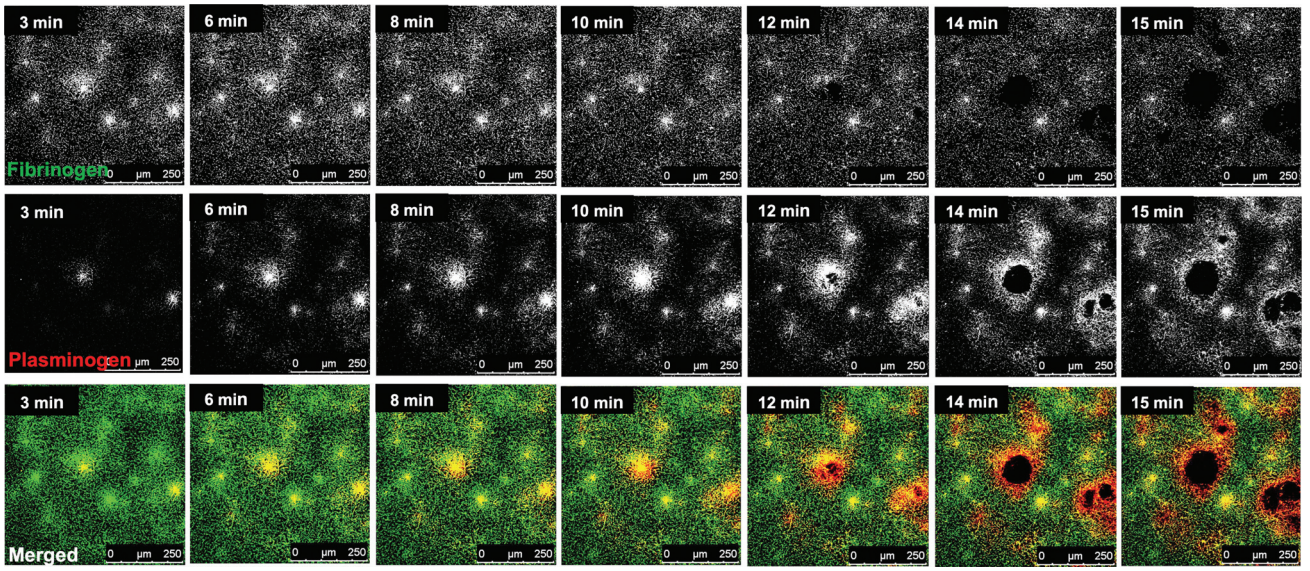

Fig. 3 Visualization of fibrin network formation and lysis by confocal laser scanning microscopy. A montage of an Alexa Fluor 488-labeled fibrinogen (top) panel, an Alexa Fluor 568-labeled plasminogen (middle) panel, and an overlaid (bottom; green: fibrinogen, red: plasminogen) panel is shown. Coagulation and fibrinolysis were initiated by the addition of tissue factor and 2 nM tPA to recalcified platelet-containing plasma (4.0×10^4 platelets/μL) in the absence of rTM. The indicated time refers to the time after the initiation of image capture. Scale: 250 μm. rTM, recombinant thrombomodulin; tPA, tissue-type plasminogen activator.

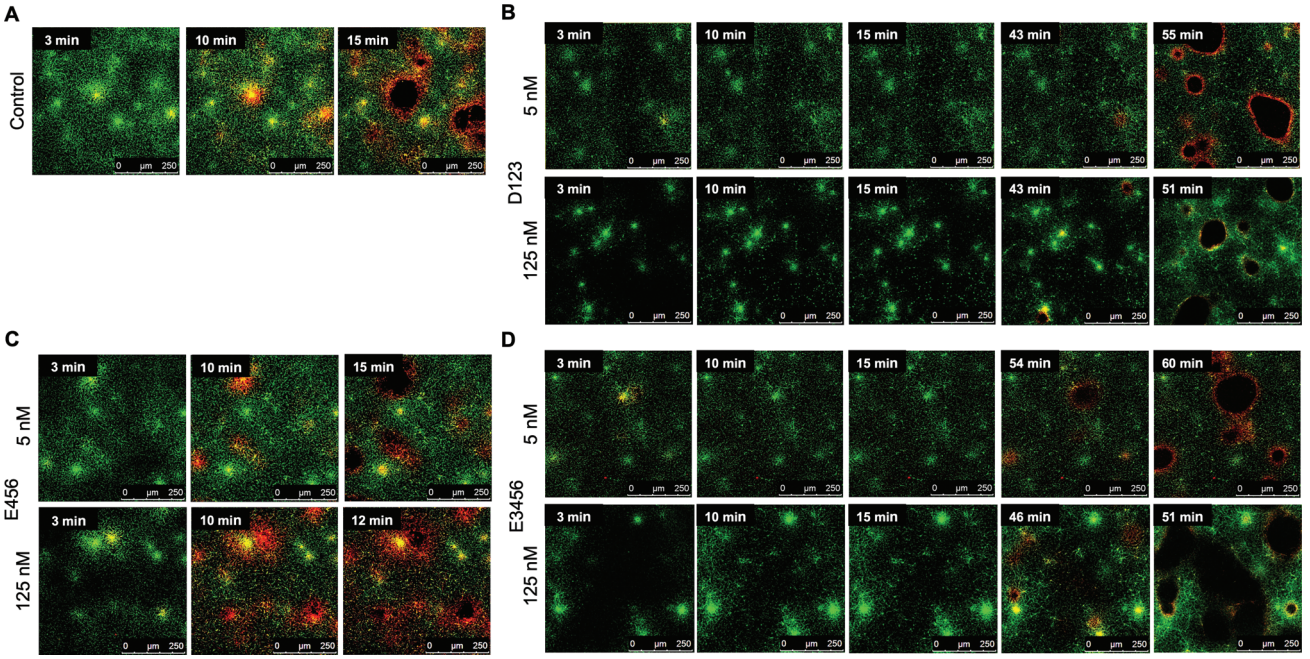

Fig. 4 rTM-modified fibrin network formation, plasminogen accumulation, and lysis. Representative sequential overlaid images of Alexa Fluor 488-labeled fibrinogen (green) and Alexa Fluor 568-labeled plasminogen (red) are displayed at the time indicated after starting the video capture. All conditions showed similar tissue factor-triggered fibrin formation initiated by activated platelets from the initiation of image capture. In control (**A**) and E456 (**C**), plasminogen also accumulated at the dense fibrin network formed at the site of activated platelets where fibrinolysis was initiated and propagated at the periphery with the addition of 2 nM tPA. In the presence of either D123 (**B**) or E3456 (**D**), plasminogen accumulation and fibrinolysis initiation were significantly delayed. Scale: 250 μm. rTM, recombinant thrombomodulin; tPA, tissue-type plasminogen activator.

by E3456 completely disappeared, and early fibrinolysis was also recovered using a TAFIa inhibitor (►**Fig. 6**). These results suggested that the thrombin-dependent activation of TAFI is differently regulated in TM domain- and concentration-dependent manners.

The Effect of TMs on Fibrin Network Formation in the Vicinity of Activated Platelets

The anticoagulant activity promoted by inhibition of thrombin procoagulant and/or actibated protein C was detected only at the highest concentration of TM in the turbidimetric

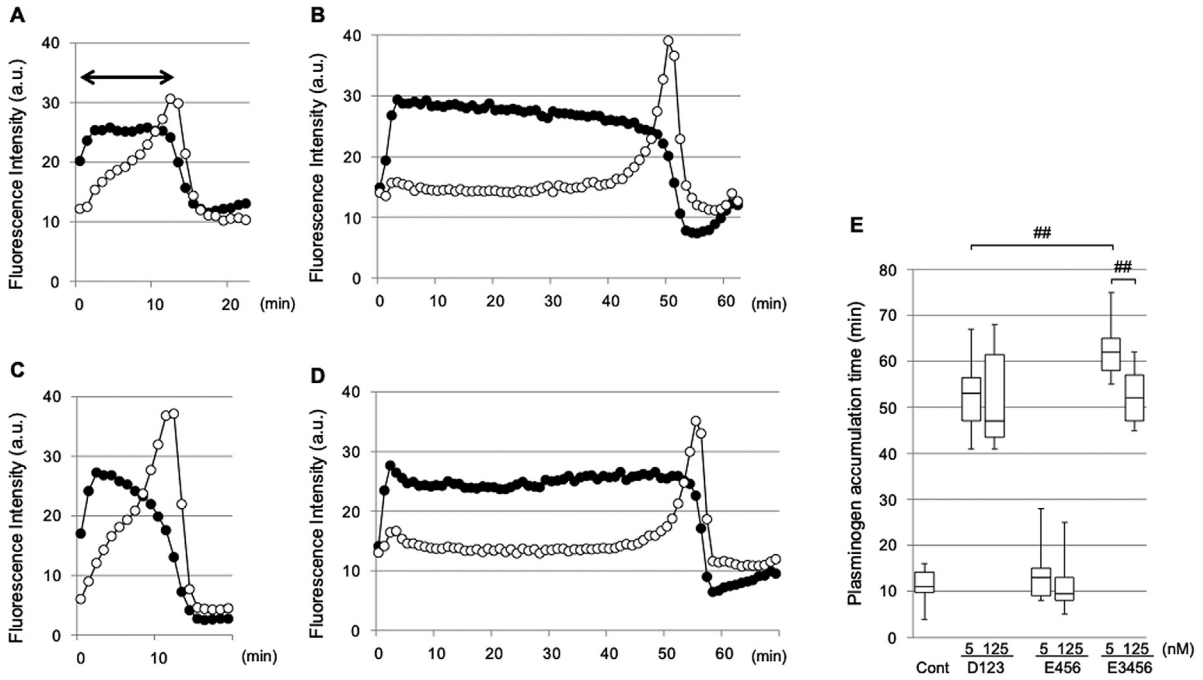

Fig. 5 Estimation of TAFI activation by attenuation of labeled plasminogen accumulation. The average fluorescence intensities of labeled fibrinogen (closed circles) and labeled plasminogen (open circles) within a 30-pixel diameter circle of dense fibrin regions under the conditions in ►**Fig. 3** are traced and shown in (**A**) control: without TM, (**B**) 5 nM D123, (**C**) 5 nM E456, and (**D**) 5 nM E3456 in arbitrary unit (a.u.). The double-headed arrow represents the time from the appearance of the fibrin network formation to the maximum intensity of the labeled plasminogen. This time was defined as plasminogen accumulation time. (**E**) Values of plasminogen accumulation time (median and interquartile range) in 3 to 5 regions selected from >3 experiments under each condition are shown, and a significant difference was analyzed using Student's *t*-test ($^{\#\#}p < 0.01$). TAFI, thrombin-activatable fibrinolysis inhibitor; TM, thrombomodulin.

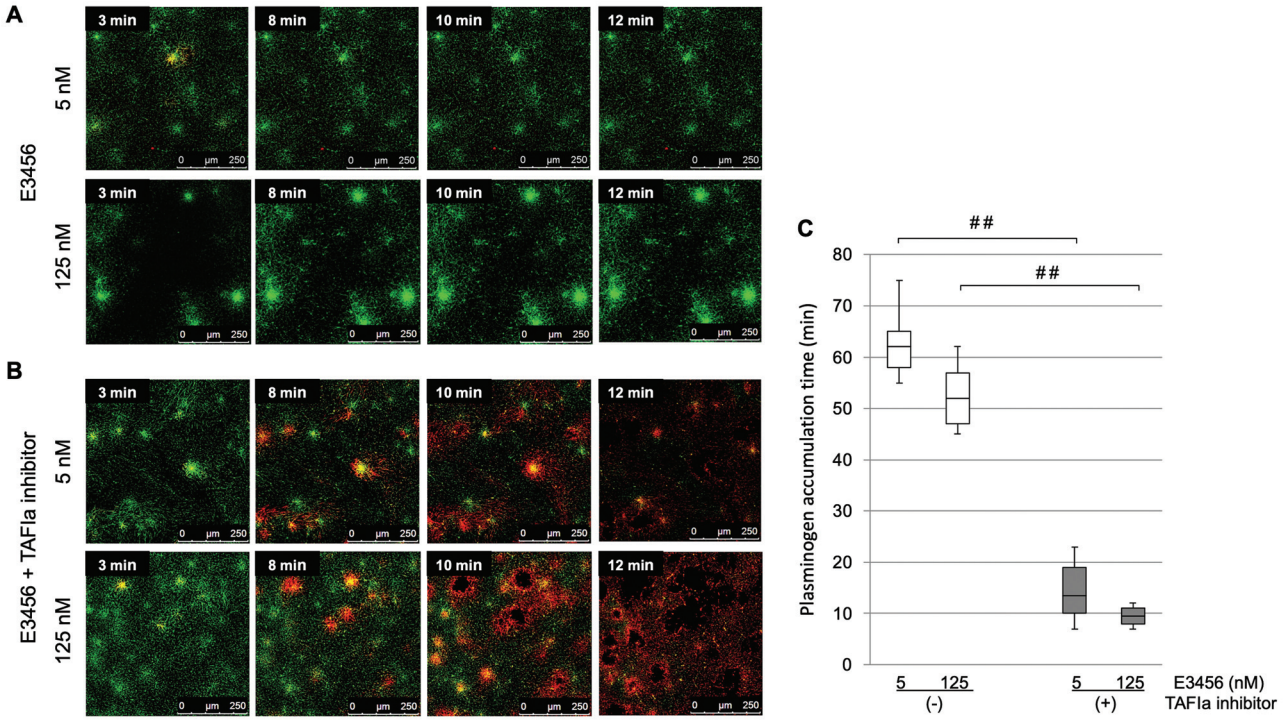

Fig. 6 TM-dependent activation of TAFI was strongly blocked by the TAFIa inhibitor. Representative sequential overlaid images of Alexa Fluor 488-labeled fibrinogen (green) and Alexa Fluor 568-labeled plasminogen (red) at early time points (up to 12 minutes) in the presence of different concentrations (5 nM and 125 nM) of E3456 are shown in (**A**) and their modifications by TAFIa are shown in (**B**). Scale: 250 µm. (**C**) Values of plasminogen accumulation times in 3 to 5 regions selected from >3 experiments under each condition are shown as median and interquartile range and were analyzed using Student's *t*-test ($^{\#\#}p < 0.01$). aTAFI, activated thrombin-activatable fibrinolysis inhibitor; TM, thrombomodulin.

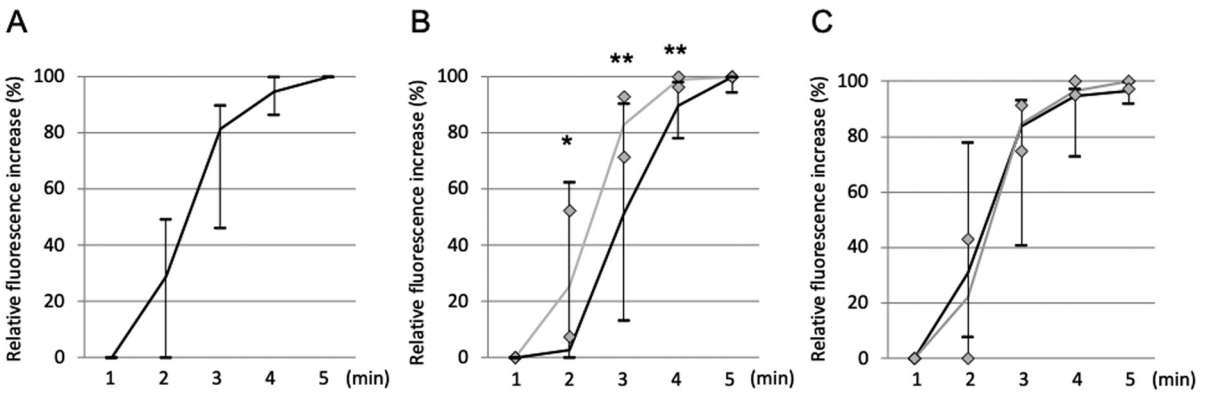

Fig. 7 The difference in anticoagulant effect between low and high concentrations of TMs at an early phase of fibrin network formation. The fluorescence intensity of Alexa Fluor 488-labeled fibrinogen in the same region of interest as in ►**Fig. 5** was measured and represented as a percentage from the initial intensity to the maximum intensity. The median and interquartile ranges from 3 to 5 regions selected from >3 experiments at the time point after triggering the coagulation cascade are shown in (**A**) control, (**B**) E456, and (**C**) E3456. The gray lines and the diamonds of interquartile ranges indicate 5 nM, and the black lines and the minus symbols of interquartile ranges indicate 125 nM of TMs (B, C), respectively. A significant difference in the value between 5 and 125 nM was analyzed using the Mann–Whitney *U*-test ($^{*}p < 0.05$, $^{**}p < 0.01$). TM, thrombomodulin.

assay (►**Fig. 1**). We quantified this anticoagulant effect using visual images of dense fibrin network formation around the activated platelets. The fluorescence increases due to fibrin (ogen) deposition at an earlier phase (1–5 minutes) after being triggered by the coagulation cascade were slightly suppressed by E456 at a concentration of 125 nM (►**Fig. 7B**; black line) compared with that at 5 nM (►**Fig. 7B**; gray line). In the case of E3456, however, there was no difference in the fibrin deposition increase (►**Fig. 7C**). Even when a large amount of E3456 was supplemented into platelet-containing plasma, a certain amount of thrombin enough to satisfactorily convert fibrinogen to fibrin was generated, at least in the vicinity of activated platelets. The amount of thrombin was not enough, however, to activate TAFI in the presence of 125 nM of E3456, due to activated protein C-dependent attenuation of thrombin generation, as was suggested by the significantly shorter plasminogen accumulation time compared with that obtained at 5 nM (►**Fig. 5E**). Therefore, anticoagulant and antifibrinolytic potentials of TM appeared to be controlled in domain- and concentration-dependent manners.

Discussion

This study demonstrated how distinct TM domains affect coagulation and fibrinolysis at different concentrations in two methods: turbidimetric assay of platelet-containing plasma clot formation and lysis and direct visualization of the activated platelet-enhanced dense fibrin network formation and plasminogen accumulation. First, all variants containing E3456 (minimum domains to activate both protein C and TAFI together with thrombin) prolonged the fibrin clot lysis time through TAFI activation at low concentrations; however, those were limited in high concentrations. Second, all three rTM variants containing E456 (domains to activate protein C only together with thrombin) delayed fibrin formation at a higher concentration than those required to activate TAFI through the E3456 domain. Third, the E456 domain-dependent conversion of procoagulant activity of thrombin to anticoagulant activity resulted in the delay in net fibrin formation and in the suppression of both thrombin-bound TM-dependent TAFI activation and the associated resistance to fibrinolysis, though the former was less affected in the vicinity of activated platelets at least in the very early phase. Lastly, the lectin domain appeared to suppress the TM-bound thrombin activity to activate TAFI through the E3456 domain at low concentrations.

Addition of either D123 (complete extracellular domain) or E3456 to the platelet-containing plasma prolonged the clot lysis time (►**Fig. 1B, D**) and delayed the accumulation of fluorescence-labeled plasminogen at the dense fibrin area (►**Figs. 4B, D** and **5B, D, E**). These appeared to be caused by effective TAFI activation because both completely disappeared in the presence of TAFIa inhibitor. Although such effects by E3456 were concentration-dependent, the highest concentration of E3456 (125 nM; approximately four times higher than the pharmacological concentration used for rhsTM treatment) showed rather limited prolongation of the clot lysis time compared with low concentrations. Further, labeled plasminogen accumulation in the region centered at the dense fibrin area occurred significantly faster in the presence of 125 nM E3456 than in the presence of 5 nM E3456 (►**Fig. 5E**). Inhibition of the coagulation cascade through thrombin activity and/or protein C activation by E3456 at this concentration, especially after fibrin clot formation, seems to have impaired TAFI activation.

In comparison, the anticoagulant effects of TM evaluated based on clotting time in our experimental setting were shown only at the highest concentration of either D123, E3456, or E456 (►**Fig. 1A, C, E**). This is consistent with the

finding that the clotting time was prolonged in the presence of 30 nM of rhsTM in the clot waveform analysis.[24] Although the thrombin generation assay showed the inhibitory effect of rhsTM at a rather lower concentration of 5 nM,[25] results obtained from imaging analysis of fibrin network formation in the vicinity of activated platelets revealed only a limited effect of TMs even at the highest concentration. Although thrombin has a broad range of diverse substrates, including both pro- and anticoagulant functions,[26] fibrin network formation initiated from the conversion of fibrinogen to fibrin seems intact, at least in the vicinity of activated platelets. Therefore, the existence of such a gradient of thrombin activity seems to be also involved in the balance between pro- and anticoagulant function in a spatiotemporal manner.

The anticoagulant effect of high TM concentration observed herein seemed to have decreased TAFI activation, demonstrated by the decreased prolongation of the clot lysis time by both D123 and E3456 at high concentrations. Decreased TAFI activation at high TM concentrations was similarly demonstrated by Mosnier et al, who concluded that it was dependent on the functional protein C pathway.[16] Faster clot lysis at higher TM concentrations than at lower concentrations was also obtained using a recently developed automated, global coagulation technique, i.e., simultaneous clot–fibrinolysis waveform analysis.[24] These are based on the well-established concept that de novo thrombin generation triggered by the coagulation cascade in the plasma, particularly mediated by the activation of factor XI (intrinsic) feedback loop, resulted in the activation of TAFI and fibrinolytic resistance.[27,28] Other reports also focused on the relationship between TAFI and protein C activation by TM/thrombin. Competitive binding of TAFI and protein C to 1 nM TM in the presence of 5 nM thrombin[14] and an equal activation of protein C and TAFI by TM/thrombin[5] were shown in early studies performed in purified conditions. Although it has been evident that the thrombin-dependent activation of these two molecules by endothelial cell-expressed TM is noncompetitive and their activation kinetics are comparable,[17] how augmented thrombin generation on the surface of activated platelets affects these activation kinetics on the endothelial cells has not been determined. Therefore, de novo thrombin generation on the activated platelet surface may provide conditions different from those in purified systems in terms of the amount and/or velocity of thrombin generation required to activate TAFI and protein C.

Our results showed that the inhibitory effect of 5 nM D123 on fibrinolysis, which was detected in both the clot lysis and plasminogen accumulation times, was slightly but significantly lesser than that of E3456. The lectin-like domain of D123 is homologous to the C-type lectin family, a group of Ca^{2+}-dependent carbohydrate-binding proteins.[29] This domain is particularly important for the anti-inflammatory and cytoprotective effects of TM, which was first demonstrated in transgenic mice lacking the D1 region.[1,30] However, it plays no role in anticoagulant activity.[29] Under our experimental conditions, the clot was formed by the endogenously generated thrombin due to TF supplementation; thus, D123 and E3456 showed different results in fibrinolysis. The lectin-like domain in TM is known to bind to glycans in TAFI and to hinder TAFI binding to the thrombin–TM complex.[31,32] Therefore, E3456 lacking a lectin-like domain could simply activate the TAFI more effectively as the glycan-dependent attenuation was lacking. Regarding clotting time, compared with D123, it tended to extend in both E3456 and E456, albeit not significantly, suggesting that the interaction of the lectin-like domain with protein C affects the anticoagulant activity of protein C, which is reported to increase two to three times by the elimination of glycosylation sites in the heavy chain of protein C.[33]

Although in vitro plasma assay has successfully demonstrated increased protein C activity induced by thrombin combined with supplemented rTM, a recent study[34] demonstrated that the circulating activated protein C levels in patients with sepsis treated with rhsTM did not show any significant increase in plasma-activated protein C activity. However, coagulation activity was slightly but significantly decreased. A systematic review also did not find any evidence of the relationship between activated protein C in the plasma and coagulation suppression in acute coagulopathy in patients with trauma-induced shock.[35] Such discrepancy might be due to the local effect of protein C activation in vivo, which does not affect the circulating plasma level.

Similarly, the regulation of TAFI activation by TM/thrombin in vivo also crucially plays an essential role in the stabilization of hemostatic thrombi, and thus, at least two factors seem relevant in the regulation. One is thrombin activity, which seems to be sufficiently generated at the injury site. Thrombin generation is disrupted in patients with coagulopathy, particularly in patients with hemophilia[24,36,37] and prothrombotic patients undergoing anticoagulant therapy,[38] both of which are known to result in poor clot stability and premature clot lysis. The experiments using plasma from hemophilic patients and ex vivo/in vivo analysis in hemophilic dogs showed improved clot strength by soluble TM at very low concentration,[39] as a result of effective TAFI activation, although thrombin generation was insufficient. The other is the existence of sufficient amounts of functional TM. The main symptom in recently reported TM variants is bleeding.[40–42] In the case of TM-Nagasaki, a single amino acid substitution of glycine by aspartate at 412 inhibits its thrombin binding and results in recurrent subcutaneous hemorrhage.[40] The border of the vascular injury site surrounded by normal vascular endothelial cells could be the candidate place where both thrombin activity and TM are expected to exist at sufficient amounts to efficiently activate TAFI. Further analysis to determine the spatiotemporal regulation of TAFI activation on TM-expressing vascular endothelial cells, as well as in vivo real-time imaging techniques performed by our group[43–45] and others,[46–48] seems to contribute to the understanding of when and where to activate TAFI and protein C.

What Is Known about This Topic?

- Thrombomodulin (TM)-bound thrombin activates thrombin-activatable fibrinolysis inhibitor (TAFI) and protein C.

What Does This Paper Add?

- Low-concentration TM was enough to express its antifibrinolytic potential and strongly attenuated plasminogen accumulation in the vicinity of activated platelets as well.
- High-concentration TM was required to express its anticoagulatory potential according to the turbidimetric assay using platelet-containing plasmas.
- Fibrin formation in the vicinity of activated platelets was not interfered even with high-concentration TM supplementation, though faster plasminogen accumulation was observed due to less TAFI activation by the attenuation of thrombin generation.

Authorship and Conflict-of-Interest Statements

L. Mochizuki performed the experiments, analyzed the data, and wrote the manuscript; Y. Suzuki designed the study, analyzed and interpreted the data, and wrote the manuscript; H. Sano, N. Honkura, and K. Masumoto discussed the results; T. Urano conceptualized the study and wrote the manuscript. All authors read and approved the manuscript.

All authors declare no competing financial interests.

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