


# Pharmacological Differentiation of Thrombomodulin Alfa and Activated Protein C on Coagulation and Fibrinolysis In Vitro

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## Abstract

Although thrombomodulin alfa (TM alfa), recombinant human soluble thrombomodulin, exerts antithrombogenic effects through activated protein C (APC), clinical trials suggested that TM alfa has a lower bleeding risk than does recombinant human APC. To address the mechanism explaining this difference, effects of TM alfa and APC on thrombogenic, coagulation, and fibrinolytic processes were compared *in vitro*. TM alfa and APC inhibited generation of thrombogenic markers, thrombin, and prothrombin fragment F1+2 and prolonged coagulation parameters, activated clotting time (ACT), and activated partial thromboplastin time (APTT). Concentrations of TM alfa effective for thrombin and F1+2 generation inhibition were comparable to those of APC. However, effects of TM alfa on ACT and APTT were clearly weaker than those of APC. TM alfa significantly prolonged clot lysis time (CLT) and decreased LY30, a parameter of degree of fibrinolysis in thromboelastography, whereas APC significantly shortened CLT and increased LY30. These results suggested that while the antithrombogenic effects of TM alfa were similar to those of APC, its anticoagulant effects were lower. In addition, effects of TM alfa were antifibrinolytic, while those of APC were profibrinolytic.

## Keywords

thrombomodulin, activated protein C, thrombosis, coagulation, fibrinolysis

## Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection.<sup>1</sup> Activation of coagulation, which represents the cross-talk between hemostasis and inflammation, is believed to be involved in the pathogenesis of sepsis. Proinflammatory cytokines can activate the coagulation system and downregulate physiological anticoagulant mechanisms. In normal circumstances, coagulation is controlled by 3 physiological anticoagulant pathways, antithrombin, activated protein C (APC), and tissue factor (TF) pathway inhibitor. In sepsis, all 3 pathways are dysregulated. Significant downregulation of thrombomodulin (TM) by inflammatory cytokines results in impaired conversion of protein C to APC. Restoration of normal levels of APC or TM in sepsis is considered to be a rational approach to treatment.<sup>2,3</sup>

APC proteolytically degrades coagulation factors Va and VIIIa, thereby producing antithrombogenic effect. In addition, APC has a profibrinolytic effect, thus inducing clot lysis.<sup>4</sup> A

phase 3 study of recombinant human APC (rhAPC, drotrecogin alfa, Xigris, Eli Lilly) showed that rhAPC decreased mortality in patients with severe sepsis.<sup>5</sup> However, in some clinical situations, rhAPC increased the risk of severe bleeding.<sup>6</sup> In the most recently reported large clinical trial, rhAPC, compared with

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placebo, did not significantly reduce mortality at 28 or 90 days, but significantly increased the rate of nonserious bleeding events in patients with septic shock.<sup>7</sup> Subsequently, the manufacturer of rhAPC decided to withdraw the product from the market.<sup>8</sup>

Currently, a promising intervention for sepsis is recombinant soluble TM, composed of 498 amino acids and all extracellular domains of TM (TM alfa, also known as ART-123).<sup>2</sup> TM alfa, like natural TM, has antithrombogenic effects through protein C activation.<sup>9,10</sup> TM alfa has antifibrinolytic effects, through activation of thrombin activatable fibrinolysis inhibitor (TAFI).<sup>11</sup> TM alfa can enhance the generation of both antithrombotic APC and antifibrinolytic activated TAFI (TAFIa), demonstrating its dual effects on regulation of thrombosis and fibrinolysis. Clinical trials of TM alfa in Japan in patients with disseminated intravascular coagulation (DIC) demonstrated that the DIC resolution rate was higher, and the incidence of bleeding-related adverse events was lower in the TM alfa group, compared with the heparin group.<sup>12,13</sup> The global phase 2b study for sepsis and suspected DIC demonstrated pharmacologic effects and suggestive evidence for efficacy of TM alfa.<sup>14</sup> Based on these results, TM alfa is considered to be a safe intervention in critically ill patients with coagulopathy caused by sepsis, even in settings where some patients are given prophylactic doses of heparin. No difference in the incidence of bleeding complications between TM alfa- and placebo-treated patients was observed.<sup>14</sup> A global phase 3 study of TM alfa for severe sepsis with coagulopathy is ongoing (ClinicalTrials.gov number, NCT01598831).

Although the global phase 2b study of TM alfa showed no difference in the incidence of bleeding complications between TM alfa- and placebo-treated patients, rhAPC was associated with an increased bleeding risk. The mechanism of the difference in bleeding risk between these 2 agents has not been well elucidated. Our goal was to investigate the pharmacological differences between TM alfa and APC by conducting a comparative study *in vitro*. TM alfa has antithrombogenic effects, promoting generation of APC at clinically effective concentrations. Thus, the antithrombogenic mechanisms of TM alfa and APC are similar. APC shortens clot lysis time (CLT) in human plasma, indicating a profibrinolytic effect.<sup>15</sup> In contrast, TM alfa prolongs CLT in human plasma by activating TAFI, an effect independent from protein C activation, resulting in an antifibrinolytic effect.<sup>11</sup> However, these experiments were performed using only human plasma, and the 2 agents were evaluated separately under different experimental conditions. Therefore, to compare these 2 agents more reliably, it was necessary to investigate the effects of TM alfa and APC in the same experiment under more physiological conditions than those in previous reports. Therefore, in this study, we compared the effects of TM alfa and APC on thrombin generation and prothrombin fragment F1+2 (F1+2) generation to indicate their antithrombogenic effects and on the activated clotting time (ACT) and activated partial thromboplastin time (APTT) to indicate their anticoagulation effects. In addition, we investigated and compared the effects of TM alfa and APC on CLT

in human plasma with endothelial cells and thromboelastography (TEG) in human blood to evaluate the impact of these agents on the fibrinolytic process.

## Materials and Methods

### Materials

TM alfa was manufactured by Asahi Kasei Pharma (Tokyo, Japan). Tissue-type plasminogen activator (t-PA) and carboxypeptidase inhibitor (CPI) from potato tuber were from Sigma-Aldrich (St Louis, Missouri). Purified human APC was from Haematologic Technologies (Essex Junction, Vermont). Tranexamic acid (TA) was from Daiichi Sankyo (Tokyo, Japan). Citrated pooled human plasma was from George King Bio-Medical (Overland Park, Kansas). Dulbecco's modified Eagle's medium (DMEM) and heat-inactivated fetal bovine serum (FBS) were from Life Technologies Japan (Tokyo, Japan). PPP-Reagent and FluCa-Kit were from Thrombino-scope (Maastricht, the Netherlands).

The study using whole human blood was performed according to the protocols approved by the ethics committee on Human Research at Loyola University Medical Center.

### Thrombin Generation and Prothrombin Fragment F1+2 Generation Assays

Thrombin generation in human plasma was analyzed using the calibrated automated thrombogram platform (Thrombino-scope), according to the manufacturer's instruction. Briefly, 20  $\mu$ L PPP-Reagent and 74  $\mu$ L human plasma were mixed in a 96-well plate. Then, 6  $\mu$ L TM alfa or APC solutions at various concentrations were added to the reaction mixtures, while controls received 6  $\mu$ L Tris-buffered saline. After the samples were preincubated at 37°C for 10 minutes, 20  $\mu$ L FluCa reagent was added to each well and the measurements initiated. Continual thrombin generation was monitored with a Fluoroskan Ascent fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts) for 30 minutes, and data were analyzed with the Thrombino-scope software. Two representative parameters were derived from the thrombin generation curves: peak height (maximum thrombin concentration) and endogenous thrombin potential (ETP; the area under the thrombin generation curve).

F1+2 generation in human whole blood was measured with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Siemens Healthcare Diagnostics, Marburg, Germany). Whole blood was drawn into 3.2% sodium citrate containing either APC or TM alfa at various concentrations. Each of these samples (2.5 mL) was added to a plastic tube containing thromboplastin C diluted 1:4 in 20 mmol/L calcium chloride (CaCl<sub>2</sub>) or saline as a control. The tubes were gently mixed and incubated in a 37°C water bath for 10 minutes. Each reaction was then stopped by addition of 0.25 mL ethylenediaminetetraacetic acid/indomethacin (50  $\mu$ g/mL). The tubes were centrifuged to obtain plasma samples that were analyzed for prothrombin fragment F1+2 using the ELISA kit.

## Clotting Time Measurements

TM alfa and APC at various concentrations were supplemented into whole blood and citrated plasma. ACT was measured in whole blood using celite tubes on an ACT Hemochron machine (Accriva Diagnostics, San Diego, California). The APTT assay (TriniCLOT, Stago, Parsippany, New Jersey) was performed with the citrated plasma samples using the ACL Elite Analyzer (Instrumentation Laboratory, Lexington, Massachusetts).

## Clot Lysis Assays With and Without Endothelial Cells

EA.hy926 cells (ATCC, Manassas, Virginia) were cultured in DMEM + 10% FBS at 37°C in 95% air and 5% CO<sub>2</sub>. EA.hy926 cells ( $2 \times 10^5$  cells/mL in DMEM with 10% FBS) were seeded, at 100  $\mu$ L per well, in a 96-well plate and cultured for 2 days. After washing the cells with phosphate buffered saline, citrated pooled human plasma (25%), sodium chloride (NaCl; 5 mmol/L), t-PA (50 ng/mL), TM alfa (0.03-1  $\mu$ g/mL), APC (0.03-1  $\mu$ g/mL), and/or TA (10  $\mu$ g/mL) were added to the cells and to blank wells. The same concentrations of plasma, NaCl, and t-PA were added to some wells as a control; CaCl<sub>2</sub> (10 mmol/L) was added to initiate clot formation, and samples were incubated at 37°C. Clot formation/dissolution was monitored by measuring turbidity at 405 nm every 1 minute for 120 minutes using a VersaMax Microplate Reader (Molecular Devices Japan, Tokyo, Japan). The CLT was defined as the time from full clot formation to the midpoint of the maximum turbid-to-clear transition, as described previously.<sup>16</sup>

## Tissue-Type Plasminogen Activator Supplemented TEG

Tissue-type plasminogen activator supplemented TEG was performed as described previously.<sup>17</sup> Citrated whole blood was supplemented with t-PA (100 ng/mL) and/or TM alfa or APC at various concentrations and placed into a TEG cup. Clotting was initiated using TriniCLOT (Stago, Parsippany, New Jersey) and recalcification using 0.2 mol/L CaCl<sub>2</sub> and was measured using the TEG 5000 system (Haemonetics, Rosemont, Illinois). The following parameters were obtained from the tracings of the TEG: R time (time to clot initiation), K-time (total clotting time), maximal amplitude (MA, strength of the clot), angle (clot kinetics), LY30 (lysis 30 minutes after MA), and LY60 (lysis 60 minutes after MA).

## Statistical Analysis

All quantitative data are presented as mean (SD). Statistical analyses were performed using EXSUS version 8.1 (CAC Croit, Osaka, Japan) based on SAS version 9.2 (SAS Institute Japan, Tokyo, Japan). For the CLT and TEG parameters, comparisons between control and TM alfa groups and control and APC groups were performed using Dunnett test with the control group as a reference. In the CLT assay, with and without EA.hy926 and CPI, comparisons were performed using unpaired *t* tests. Differences were considered statistically significant at  $P < .05$ .

## Results

### Effects of TM Alfa and APC on Thrombin Generation and Coagulation

The effects of TM alfa and APC on thrombin generation and coagulation were measured in terms of TF-induced thrombin generation, F1+2 generation, ACT, and APTT. TM alfa and APC inhibited thrombin generation in human plasma and F1+2 generation in human whole blood, in a concentration-dependent manner (Figure 1). The 50% inhibitory concentration (IC<sub>50</sub>) values for TM alfa and APC on thrombin and F1+2 generation were 0.77 and 0.22  $\mu$ g/mL (peak height), 0.66 and 0.23  $\mu$ g/mL (ETP), and 1.25 and 0.72  $\mu$ g/mL (F1+2), respectively.

TM alfa slightly prolonged ACT and APTT, but did not cause a doubling of clotting time, at concentrations up to 10  $\mu$ g/mL. In contrast, APC clearly prolonged ACT and APTT in a concentration-dependent manner (Figure 2), doubling the clotting times at 4.70 and 2.13  $\mu$ g/mL, respectively.

### Effects of TM Alfa, APC, and TA on Clot Lysis in Human Plasma

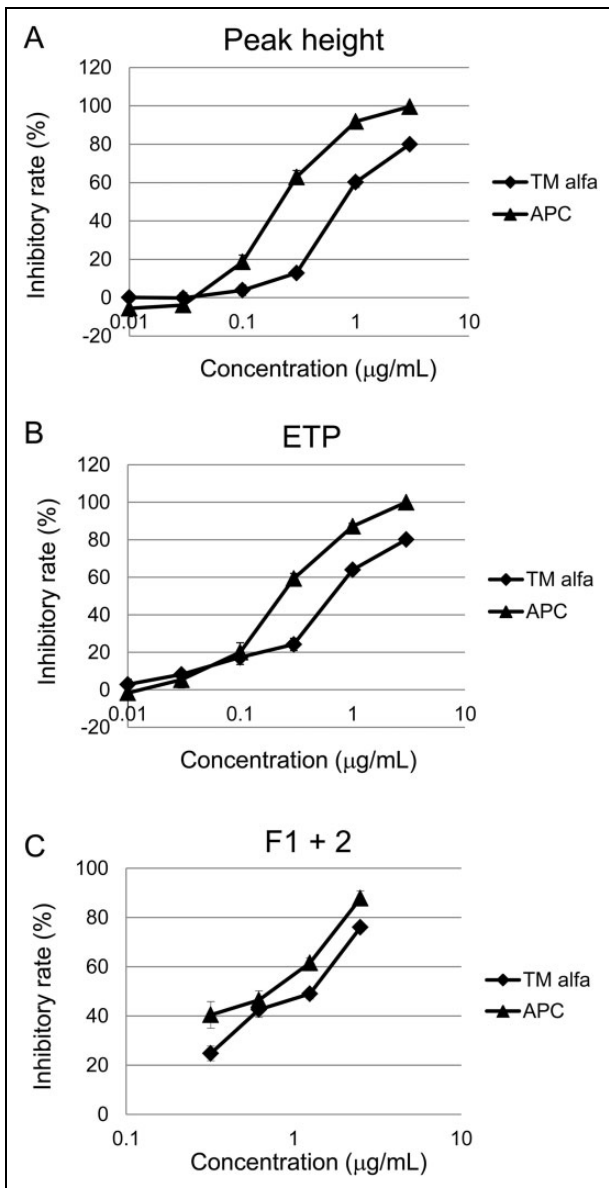
We examined the effects of TM alfa and APC, along with that of TA, a plasmin generation inhibitor serving as a positive control, on CLT in human plasma. TM alfa (0.03-1  $\mu$ g/mL) significantly prolonged CLT in human plasma, while APC (1  $\mu$ g/mL) significantly shortened it. Tranexamic acid (10  $\mu$ g/mL) completely inhibited clot lysis (Figure 3).

### Effects of TM Alfa and APC on Clot Lysis in Human Plasma With Endothelial Cells

To examine the effects of TM alfa and APC on fibrinolysis under more physiological conditions than those reported previously, we investigated their effects on t-PA-induced clot lysis in human plasma in the presence of EA.hy926 cells. The EA.hy926 cells significantly prolonged CLT, compared to the absence of cells. CPI, a TAFIa inhibitor, strongly shortened CLT, with or without EA.hy926 cells (Figure 4A). In the presence of EA.hy926 cells, TM alfa at concentrations of 0.03 to 1  $\mu$ g/mL also significantly prolonged CLT in human plasma, while APC at concentrations of 0.3 and 1  $\mu$ g/mL significantly shortened this parameter (Figure 4B and C).

### Effects of TM Alfa and APC on Coagulation and Fibrinolysis in Human Blood

To investigate the effects of TM alfa and APC on coagulation and fibrinolysis, simultaneously, in human whole blood, we performed the t-PA supplemented TEG assay using human blood containing TM alfa or APC. TM alfa had no effect on coagulation parameters measured by TEG at 0.1 or 1  $\mu$ g/mL, while at 10  $\mu$ g/mL, there was a trend toward prolonged R time and decreased angle and MA. APC did not affect the coagulation parameters measured by TEG at 0.1  $\mu$ g/mL, but its

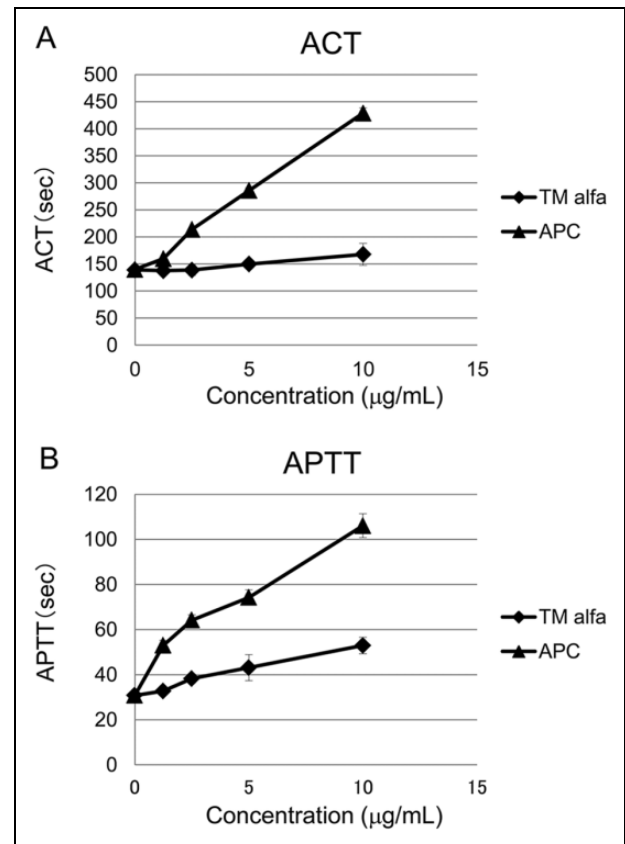


**Figure 1.** Effects of thrombomodulin (TM) alfa and activated protein C (APC) on tissue factor-induced thrombin generation in human plasma and prothrombin fragment F1+2 generation in human whole blood. Inhibition rates of TM alfa and APC on peak height (maximum thrombin concentrations; A), endogenous thrombin potential (ETP; B) and F1+2 (C) are shown. Data are means (standard deviation [SD]),  $n = 3$  to 5 each.

concentration dependently prolonged the R time and decreased the angle and MA at 1 and 10 µg/mL. In addition, 0.1, 1, and 10 µg/mL TM alfa significantly decreased LY30 and LY60, whereas 0.1 and 1 µg/mL APC showed a trend toward increased LY30 and did not affect LY60 (Table 1).

## Discussion

In this study, we examined the pharmacological differences among the effects of TM alfa and APC on thrombogenic,

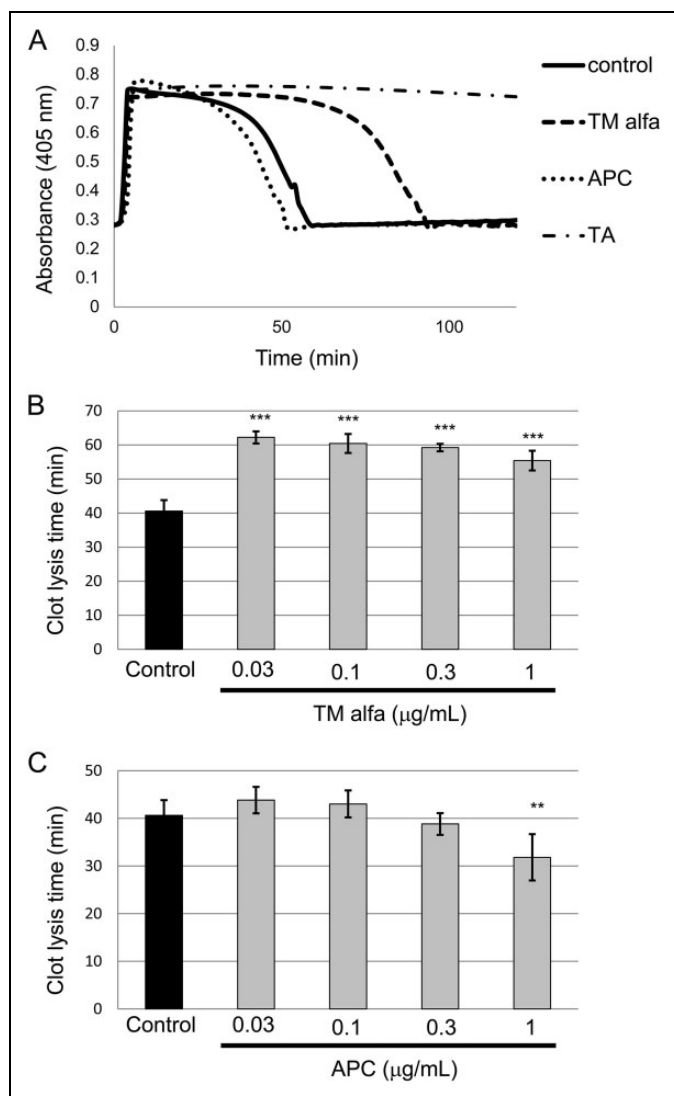


**Figure 2.** Effects of thrombomodulin (TM) alfa and activated protein C (APC) on activated clotting time (ACT) in human whole blood (A) and activated partial thromboplastin time (APTT) in human plasma (B). Data are means (standard deviation [SD]),  $n = 3$  to 4 each.

coagulation, and fibrinolytic processes. We demonstrated that TM alfa and APC inhibited thrombin generation over the same concentration range. However, the effective concentrations of TM alfa on ACT and APTT prolongation and TEG coagulation parameters were clearly higher than those of APC. We also showed that TM alfa prolonged, whereas APC shortened, CLT in human plasma and whole blood.

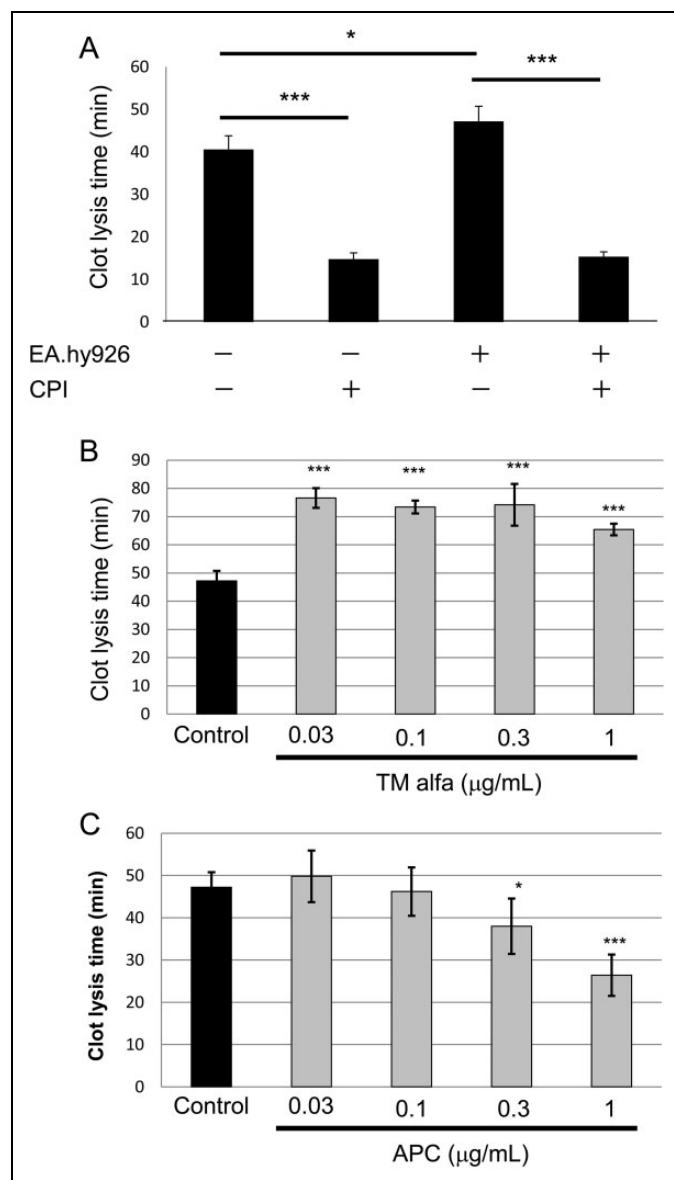
The thrombin generation assay is considered to be a broad functional test of hemostatic–thrombotic mechanisms in the blood and is useful to monitor therapeutic effects of antithrombotic drugs.<sup>18</sup> F1+2 is an activation peptide released from prothrombin during thrombin generation and is useful for evaluating enhanced coagulability.<sup>19</sup> Therefore, we chose thrombin generation and F1+2 generation as appropriate indicators of the antithrombotic effects of TM alfa and APC. In our study, both TM alfa and APC inhibited thrombin and F1+2 generation in a concentration-dependent manner, with similar  $IC_{50}$  values. These results suggested that the antithrombotic effects of TM alfa and APC in human blood were comparable.

In contrast to their antithrombotic effects, the actions of TM alfa on ACT, APTT, and coagulation parameters of TEG appeared to be weaker than those of APC. ACT and APTT are commonly used tests for monitoring anticoagulation with heparin. With antithrombin agents such as dabigatran, a direct



**Figure 3.** Effects of thrombomodulin (TM) alfa, activated protein C (APC), and tranexamic acid (TA) on tissue-type plasminogen activator (t-PA)-induced clot lysis in human plasma. A, Representative clot lysis curves, monitored by measuring turbidity at 405 nm, are shown. B, Clot lysis times in human plasma with TM alfa (gray bar) and without TM alfa as a control (black bar). C, Clot lysis times in human plasma with APC (gray bar) and without APC as a control (black bar). Data are means (standard deviation [SD]), n = 5 each. *\*\*P* < .01 and *\*\*\*P* < .001 versus control group (Dunnett test).

oral thrombin inhibitor, an elevated APTT provided qualitative evidence for an increased risk of bleeding, making APTT a valuable parameter for identifying patients at high risk of major bleeding during dabigatran treatment.<sup>20,21</sup> Moreover, low MA values were strongly associated with a high-bleeding risk in patients with thrombocytopenia.<sup>22</sup> Therefore, the differing effects of TM alfa and APC on coagulation may determine, at least in part, their different bleeding risks. TM alfa directly inhibited thrombin, while its effective concentration was higher than that which promotes protein C activation.<sup>9</sup> In addition, enhancement of APC generation by TM alfa



**Figure 4.** Effects of thrombomodulin (TM) alfa and activated protein C (APC) on tissue-type plasminogen activator (t-PA)-induced clot lysis in human plasma with EA.hy926 cells. A, Clot lysis times in human plasma, with or without EA.hy926 cells and/or carboxypeptidase inhibitor (CPI; activated thrombin activatable fibrinolysis inhibitor [TAFI] inhibitor). *\*P* < .05 and *\*\*\*P* < .001 (unpaired *t* test). B, Clot lysis times in human plasma with TM alfa (gray bar) and without TM alfa as a control (black bar) in the presence of EA.hy926 cells. C, Clot lysis times in human plasma with APC (gray bar) and without APC as a control (black bar) in the presence of EA.hy926 cells. Data are means (standard deviation [SD]), n = 5 each. *\*P* < .05 and *\*\*\*P* < .001 versus control group (Dunnett test).

depends on the presence of thrombin, but we could not measure the APC concentrations in the plasma and blood containing TM alfa because of technical limitations. Therefore, we speculated that the different effects of TM alfa and APC on coagulation processes were caused by direct actions on thrombin and/or APC levels in the plasma. However, further studies

**Table 1.** Thromboelastography Parameters in Normal Human Blood Supplemented With t-PA and/or TM Alfa or APC.<sup>a</sup>

Treatment	n	R Time (minutes)	K Time (minutes)	Angle	MA (mm)	LY30 (%)	LY60 (%)
Control	4	11.3 (7.9)	4.0 (2.5)	46.2 (18.6)	50.2 (11.3)	8.1 (4.1)	32.0 (12.3)
0.1 µg/mL TM alfa	4	9.1 (5.1)	3.4 (2.3)	51.5 (15.1)	53.0 (11.7)	3.3 (2.0)	13.9 (4.4) <sup>b</sup>
1 µg/mL TM alfa	4	10.3 (6.4)	2.9 (1.3)	52.9 (13.4)	49.5 (9.1)	2.7 (1.8) <sup>c</sup>	12.1 (3.9) <sup>b</sup>
10 µg/mL TM alfa	4	14.0 (7.2)	5.7 (3.9)	38.2 (18.6)	43.3 (11.9)	2.3 (1.0) <sup>c</sup>	14.6 (4.5) <sup>c</sup>
Control	3	10.0 (3.9)	4.0 (0.6)	43.8 (4.8)	42.9 (14.2)	10.7 (3.6)	40.6 (9.2)
0.1 µg/mL APC	3	11.0 (5.0)	3.9 (0.3)	44.7 (3.5)	43.2 (12.2)	13.3 (4.0)	42.8 (12.7)
1 µg/mL APC	3	16.6 (6.1)	6.1 (1.6)	36.7 (9.0)	38.3 (16.3)	17.2 (12.8)	42.6 (25.1)
10 µg/mL APC	3	77.4 (20.6) <sup>d</sup>	NA	4.9 (5.9) <sup>d</sup>	19.0 (26.9)	NA	NA

Abbreviations: APC, activated protein C; NA, not applicable; SD, standard deviation; t-PA, tissue-type plasminogen activator; TM, thrombomodulin.

<sup>a</sup>Data are means (SD).

<sup>b</sup>*P* < .01.

<sup>c</sup>*P* < .05.

<sup>d</sup>*P* < .001 versus control group (Dunnett test).

will be needed to elucidate the differences between TM alfa and APC.

In this study, TM alfa prolonged, while APC shortened, CLT in human plasma. These results were consistent with previous independent reports.<sup>11,15</sup> In the TEG assays, TM alfa decreased LY30 and LY60, whereas APC showed a trend toward increased LY30 and did not effect LY60. These results suggested that the effects of TM alfa and APC on fibrinolysis were quite different from one another. This difference likely resulted from their effects on TAFI activation. TAFI is a plasma carboxypeptidase synthesized in the liver and is converted to the active enzyme (TAFIa) in the presence of thrombin, thrombin-TM complex, and plasmin.<sup>23</sup> TAFIa removes the C-terminal lysine from partially degraded fibrin, thereby decreasing t-PA and plasminogen binding to the clot and, consequently, plasmin formation.<sup>23</sup> We previously showed that TM alfa prolonged CLT in human plasma through TAFI activation.<sup>11</sup> Bajzar et al reported that APC decreased TAFI activation by inhibiting thrombin generation and decreasing levels of thrombin-TM complex.<sup>15</sup> Therefore, differences in their impacts on TAFI activation may explain why TM alfa and APC have distinct effects on fibrinolysis.

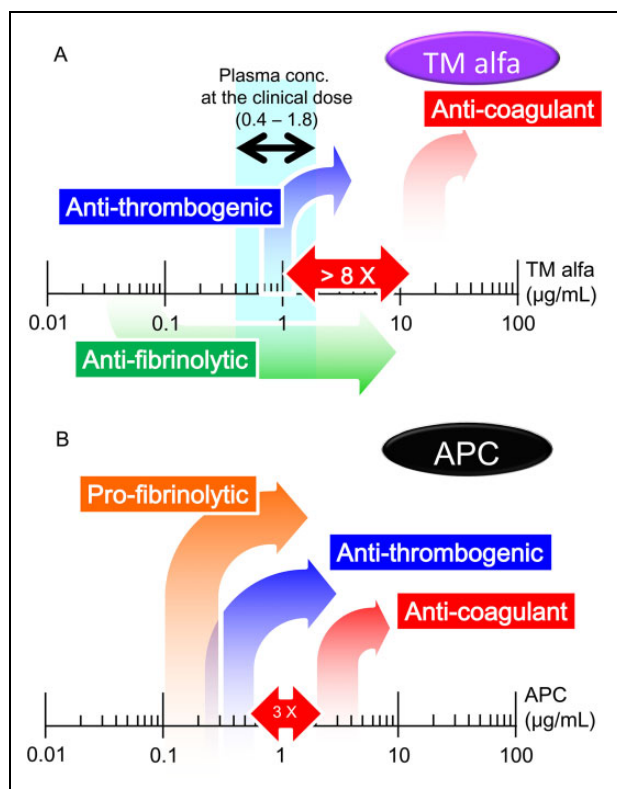
To explore the potential antifibrinolytic effects of TM alfa, we simultaneously evaluated its effect, along with that of the clinically used plasmin activation inhibitor TA, in the clot lysis assay. We found that the effect of TM alfa on clot lysis was clearly weaker than that of 10 µg/mL TA, a plasma concentration achievable in the clinic. These results suggested that the antifibrinolytic effect of TM alfa was low compared with that of antifibrinolytic agents directly inhibiting plasmin activation. Asakura et al reported that TA caused tissue damage in TF-induced DIC rats by inhibiting fibrinolysis.<sup>24</sup> We confirmed that TM alfa prevented hematological changes induced by TF, including decreased levels of fibrinogen and platelets and increased levels of fibrin and fibrinogen degradation products and did not induce detectable tissue damage in rats with TF-induced DIC rats (unpublished data). Moreover, Nagato et al reported that TM alfa attenuated liver damage in rats with lipopolysaccharide-induced DIC.<sup>25</sup> Therefore, we concluded

that it was unlikely that TM alfa would cause or exacerbate thrombosis in patients with sepsis and DIC via the antifibrinolytic effects detected in our experiments.

The EA.hy926 cells prolonged CLT in human plasma. CPI shortened CLT, with or without at the same level of EA hy926 cells. We previously showed that EA.hy926 cells expressed TM and enhanced protein C activation in the presence of thrombin, mimicking physiological conditions.<sup>26</sup> These results suggested that TAFI was partially activated under basal conditions and that EA.hy926 cells enhanced TAFI activation through thrombin-TM complexes in the clot lysis assay.

APC shortened CLT at lower concentrations in the presence of EA.hy926 cells, compared to their absence. Our results suggested that EA.hy926 cells enhanced TAFI activation through thrombin-TM complexes and that APC decreased TAFI activation by inhibiting thrombin generation and decreasing levels of thrombin-TM complexes. Therefore, we speculated that APC would decrease TAFI activation more efficiently in the presence of EA.hy926 cells. Additionally, TM alfa prolonged CLT, with or without EA.hy926 cells. Thus, TM alfa has potential antifibrinolytic effects, even in the presence of endothelial cells. This difference in the effects of TM alfa and APC on fibrinolysis may also explain the differences in their bleeding risks in patients with sepsis.

The effective concentrations of TM alfa on thrombogenic and fibrinolytic processes are thought to be similar. However, the current studies demonstrate that the effects of TM alfa on the coagulation process clearly required concentrations that were higher than plasma concentrations observed clinically (0.4-1.8 µg/mL) after administration of TM alfa at 0.06 mg/kg/d, the dose used to treat patients with DIC in Japan and patients with sepsis in the global P3 study.<sup>13,14,27</sup> Thus, we would expect TM alfa to have antithrombogenic and antifibrinolytic effects, but possibly not to show anticoagulant effects, in patients with sepsis and DIC. A previous clinical study demonstrated that TM alfa improved thrombin generation markers, such as F1+2 and thrombin-antithrombin (TAT) complex, in patients with sepsis<sup>14</sup> but did not affect PT and APTT in healthy volunteers.<sup>28</sup>



**Figure 5.** Summary of the concentrations of thrombomodulin (TM) alfa (A) and activated protein C (APC; B) effective on thrombogenic, coagulation, and fibrinolytic processes. Antithrombogenic (blue arrow):  $IC_{50}$  values for TM alfa and APC on thrombin and F1+2 generation. Anticoagulant (red arrow): doubling concentration for TM alfa and APC on activated partial thromboplastin time (APTT) and activated clotting time (ACT). Antifibrinolytic (green arrow): prolonged concentration for TM alfa on clot lysis time (CLT). Profibrinolytic (orange arrow): shortened concentration for APC on CLT. Two-headed arrow: minimum ratio of effective concentration on thrombogenic and coagulation processes. Thrombomodulin alfa did not cause a doubling of clotting time, even at maximum concentration (10  $\mu\text{g/mL}$ ), thus the ratio indicates greater than 8 times ( $>8\times$ ).

In this study, we used purified human APC instead of rhAPC, as the latter was withdrawn from the market. The effective concentrations of purified human APC on thrombogenic, coagulation, and fibrinolytic processes were comparable, approximately 1  $\mu\text{g/mL}$ . rhAPC also inhibited the increases in F1+2 and TAT and prolonged APTT by 1.5-fold in patients with sepsis at 24  $\mu\text{g/kg/h}$ , a dosing regimen used to treat patients with sepsis.<sup>4</sup> Thus, the effects of purified human APC on thrombogenic and coagulation processes may be similar to those of rhAPC in patients with sepsis. This suggested that purified human APC would be a useful research tool for investigating the effects of rhAPC in such experiments. The results of our *in vitro* study may, thus, reflect potential effects of TM alfa and APC in the blood of patients with sepsis.

In conclusion, TM alfa exerted antithrombogenic effects at plasma concentrations achievable in the clinic and, simultaneously, demonstrated antifibrinolytic effects at much lower concentrations. TM alfa did not double ACT and APTT, even at

levels more than 8 times higher than its antithrombotic concentrations (Figure 5A). In contrast, APC exhibited antithrombotic and profibrinolytic effects over the same concentration range and doubled ACT and APTT at levels only 3 times higher than its antithrombotic concentrations (Figure 5B). Such differences may potentially contribute to the differences in bleeding risks associated with TM alfa and APC administration to patients with sepsis.

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### Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: KT is an employee of Asahi Kasei Pharma America Corporation. ST and KT are employees of Asahi Kasei Pharma Corporation.

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