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Full Length Article

Thrombomodulin alfa prevents the decrease in platelet aggregation in rat models of disseminated intravascular coagulation

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ABSTRACT

Introduction: Disseminated intravascular coagulation (DIC), a deadly complication characterized by uncontrolled hypercoagulation, causes a decrease in the platelet count and impairs platelet aggregation. Thrombomodulin (TM) alfa, a recombinant human soluble TM, reduces hypercoagulation in DIC patients. However, the effects of TM alfa on impaired platelet aggregation remain to be determined. In this study, we aim to investigate the effects of TM alfa on platelet aggregation using lipopolysaccharide (LPS)-induced and tissue factor (TF)-induced DIC rat models.

Materials and methods: Sprague–Dawley rats were administered TF or LPS intravenously, with or without TM alfa before the injection. Six hours after LPS injection or 1 h after TF infusion, blood samples were obtained, and platelet-rich plasma was prepared. Collagen or adenosine diphosphate-induced platelet aggregation was measured using an aggregometer. In the other experiments, platelets were transfused 1 h after the TF infusion. Five minutes after transfusion, collagen-induced platelet aggregation was also measured.

Results: The amplitude of platelet aggregation in platelet-rich plasma was decreased in LPS- and TF-treated rats. TM alfa inhibited the decrease in platelet aggregation in a dose-dependent manner. The washed platelet aggregation amplitude was not decreased in TF-treated rats. Suspension of normal platelets in plasma obtained from TF-treated rats reduced platelet aggregation. Platelet transfusion for TF-treated rats increased the platelet count but was unable to improve platelet aggregation.

Conclusions: TM alfa attenuated impairment of platelet aggregation in LPS- and TF-induced DIC rat models. The changes in plasma composition played a role in the decrease of platelet aggregation in TF-treated rats.

1. Introduction

Platelets are anucleate blood cells that play a vital role in maintaining hemostasis by forming clots at injury sites. At the injury sites, exposed extracellular matrix proteins, such as collagen and von Willebrand factor, initiate platelet activation, which results in secretion of secondary mediators including adenosine diphosphate (ADP) and thromboxane A2, to activate nearby platelets [1]. Accumulated platelets then contract to form a firm clot.

Disseminated intravascular coagulation (DIC) typically occurs as an acute complication in patients with underlying life-threatening illnesses such as sepsis, hematologic malignancies, severe trauma, or placental abruption. DIC is characterized by wide-spread, uncontrolled activation of coagulation, which leads to a pro-thrombotic state with disseminated formation of microthrombi and development of multiple organ dysfunction syndrome [2,3]. Additionally, consumption of platelets and coagulation factors leads to bleeding throughout the course of DIC [2,3]. Clinical studies revealed that the platelet aggregation response in DIC patients was lower than that in non-DIC patients, suggesting that not only the number of platelets is reduced but also the remaining platelets have impaired function in DIC patients [4–6]. Considering platelet function in hemostasis, attenuated platelet function may be detrimental for DIC patients. A previous study showed that the amplitude of the reduction in platelet aggregation capacity was related to mortality in DIC patients [7].

Thrombomodulin (TM) is a glycoprotein that is expressed on the

Abbreviations: ADP, adenosine diphosphate; APC, activated protein C; DIC, disseminated intravascular coagulation; FDPs, fibrinogen/fibrin degradation products; L-PRP, leukocyte- and platelet-rich plasma; LPS, lipopolysaccharide; PGI_2, prostaglandin I_2; PPP, platelet-poor plasma; PRP, platelet-rich plasma; RT, room temperature; SEM, standard error of the mean; TAT, thrombin-antithrombin complex; TF, tissue factor; TM, thrombomodulin

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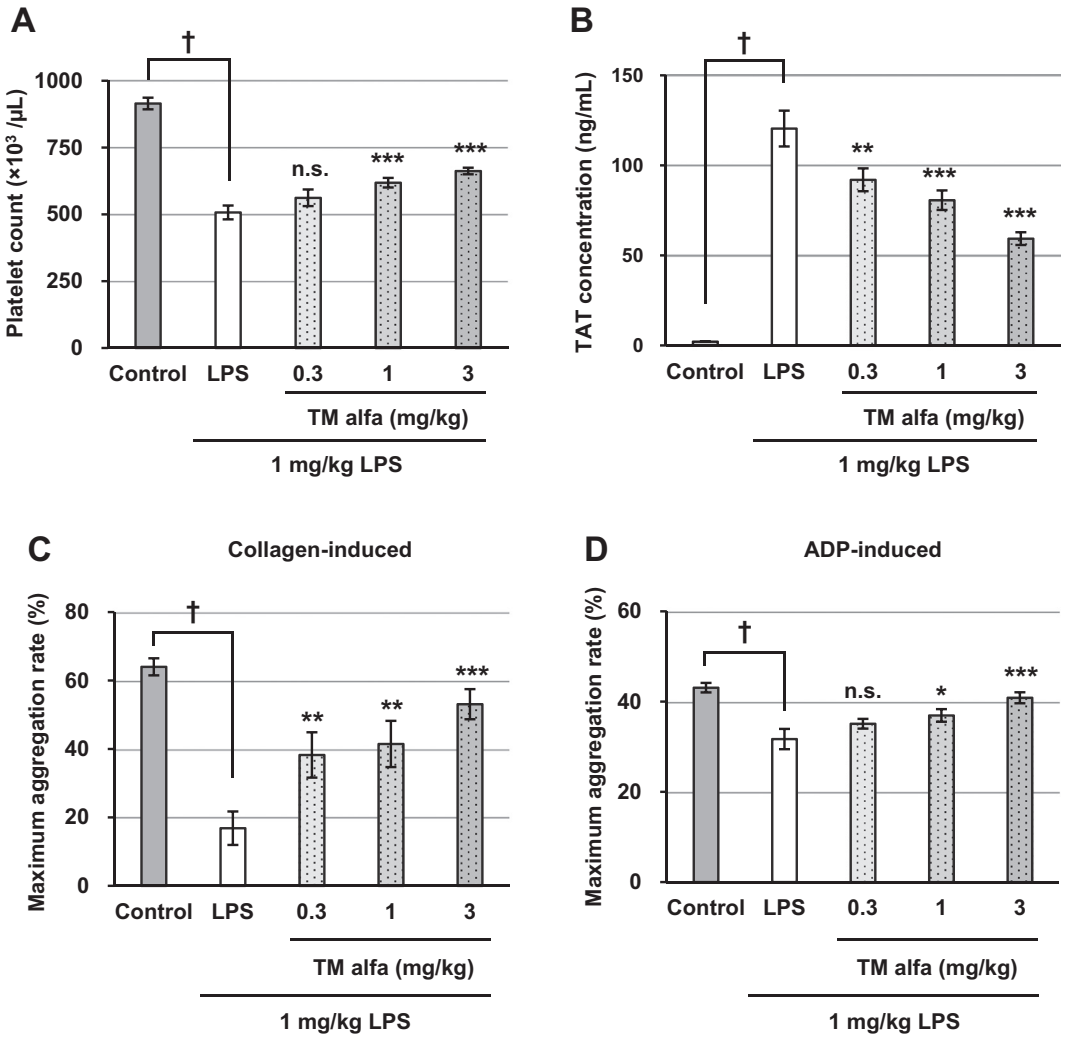

Fig. 1. Effects of TM alfa in LPS-induced DIC model rats. A, B: Platelet count in blood (A) or the plasma TAT concentration (B) was measured 6 h after intravenous administration of saline (control group), LPS (LPS group), or LPS and TM alfa (TM alfa groups). C, D: PRP, isolated from the control group, LPS group, or TM alfa groups, was stimulated with either 15 μg/mL collagen (C) or 2 μM ADP (D), and platelet aggregation was measured. The data are presented as the mean ± SEM (n = 12–13). $^{\dagger}P < 0.001$ vs. control group (unpaired t-test), $^{*}P < 0.025$, $^{**}P < 0.005$, $^{***}P < 0.0005$ vs. LPS group (Williams' test).

surface of vascular endothelial cells, and it accelerates protein C conversion to activated protein C (APC) in a thrombin-dependent manner [8]. TM is known to regulate coagulation, fibrinolysis, and inflammation in blood [9–11]. TM alfa, also known as ART-123, is a recombinant protein of the extracellular part of human TM that was approved for treating DIC in Japan [12]. In our previous studies, we have demonstrated that TM alfa exerts anti-coagulant effects via APC and anti-inflammatory effects via thrombin activatable fibrinolysis inhibitor [13,14]. Additionally, we have also shown that TM alfa inhibits thrombin-induced endothelial hyperpermeability and histone-induced endothelial cell death through APC [15,16]. Our findings suggest that TM alfa improves clinical outcomes in DIC patients through a pleiotropic mechanism of action.

Although a meta-analysis showed that administration of TM alfa was associated with a trend in the reduction of mortality at 28–30 days in septic DIC patients [17], the mechanism of action for TM alfa in the improvement of the patient's prognosis is not fully understood. As mentioned above, platelet function is reported to be decreased in non-survivors among DIC patients [7]. We have shown that TM alfa prevents the decrease in platelet count in several animal DIC models [18,19], but the effects of TM alfa on platelet function have not been examined. To investigate the effect of TM alfa on platelet aggregation impairment in DIC patients with life-threatening condition, strong rationale is ethically required. Thus, we evaluated the effects of TM alfa on platelet aggregation impairment with rat DIC models, which were lipopolysaccharide (LPS)-induced and tissue factor (TF)-induced DIC rat models. Then, we evaluated the effects of plasma exchange between control and TF-treated rats ex vivo and platelet transfusion in vivo to examine the mechanism of platelet aggregation impairment in a TF-induced DIC rat model.

2. Methods

2.1. Reagents

TM alfa was manufactured by Asahi Kasei Pharma (Tokyo, Japan). LPS from *Escherichia coli* (type 0111:B4), ADP, prostaglandin I_2 (PGI_2) sodium salt, apyrase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pentobarbital sodium salt was purchased from Tokyo Kasei (Tokyo, Japan). Collagen Reagens HORM was purchased from TAKEDA AUSTRIA GmbH (Linz, Austria). TF (thromboplastin) from rabbit brain was purchased from sysmex (Thrombocheck PT, Kobe, Japan). The TF suspension for injection in rats was prepared using the method described below. Thrombocheck PT was dissolved in 2 mL of distilled water, which is half the volume indicated in the manufacturer's instructions, and centrifuged at 16,000*g* for 20 min at 4 °C. The pellet

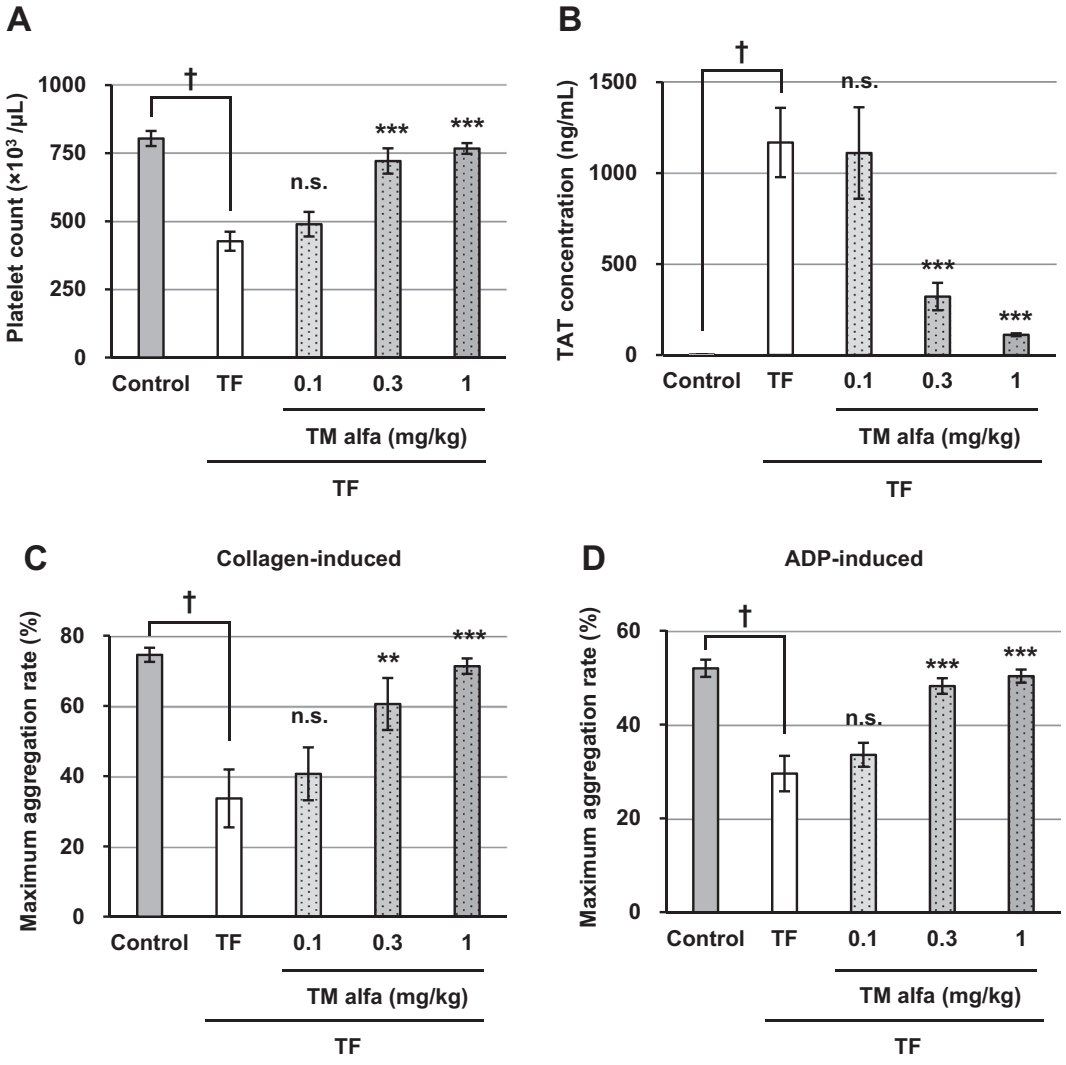

Fig. 2. Effects of TM alfa in TF-induced DIC model rats. A, B: Platelet count in blood (A) or the plasma TAT concentration (B) was measured after intravenous administration of saline (control group), TF (TF group), or TF and TM alfa (TM alfa groups). C, D: PRP, isolated from the control group, TF group, or TM alfa groups, was stimulated with either 15 μg/mL collagen (C) or 2 μM ADP (D), and platelet aggregation was measured. The data are presented as the mean ± SEM (n = 9 each). $^{\dagger}P < 0.001$ vs. control group (unpaired t-test), $^{**}P < 0.005$, $^{***}P < 0.0005$ vs. TF group (Williams' test).

containing TF was washed with saline once, and suspended in 2 mL of saline. The TF suspension was stored at −80 °C until use. In the experiment evaluating platelet transfusion, TF was diluted with the same volume of saline before use. In the other experiments, TF was diluted four times with saline before use. The concentration of TF was determined based on our preliminary study to evaluate the relationship between TF dose and platelet aggregation impairment.

2.2. Animals

Male Sprague–Dawley rats, 7–8 weeks old (Japan Slc, Inc., Hamamatsu, Japan) were used for experiments. Rats were housed one or two per cage under a 12-h light–dark cycle (photoperiod, 0700–1900 h). The rats were provided tap water and a standard laboratory diet CRF-1 (Oriental Yeast, Tokyo, Japan) ad libitum. All animal experiments were performed in accordance with the Basic Guidelines on Animal Care and Use in Asahi Kasei Pharma Corporation, which meets the ethical standards required by Japanese law and guidelines for the use of experimental animals. All animal procedures were approved by the Committee on Ethics in Animal Experiments of Asahi Kasei Pharma Corporation.

2.3. LPS-induced rat DIC model

Rats were divided into control, LPS, and TM alfa groups (n = 12–13 for each group). Rats were anesthetized using isoflurane (2.5%–4%). Saline (1 mL/kg, control group and LPS group) or different doses of TM alfa (0.3, 1, and 3 mg/kg, TM alfa groups) was injected via the tail vein. Then, saline (1 mL/kg, control group) or LPS (1 mg/kg, LPS group and TM alfa groups) was injected via the tail vein. Six hours after the end of these injections, rats were anesthetized using isoflurane (2.5%–4%), and 4.5-mL blood samples were collected from the abdominal aorta using syringes filled with 0.5 mL of 3.13% sodium citrate. Blood was used to measure the thrombin-antithrombin complex (TAT) concentration, the number of platelets, and platelet aggregation. The number of platelets in the blood was measured using an automated laser-based hematology analyzer (XT-2000i, Sysmex, Kobe, Japan) immediately after collection.

2.4. TF-induced rat DIC model

Rats were divided into control, TF, and TM alfa groups (n = 9 for each group). Rats were anesthetized using an intraperitoneal injection of 40 mg/kg pentobarbital sodium. Saline (1 mL/kg, control group and

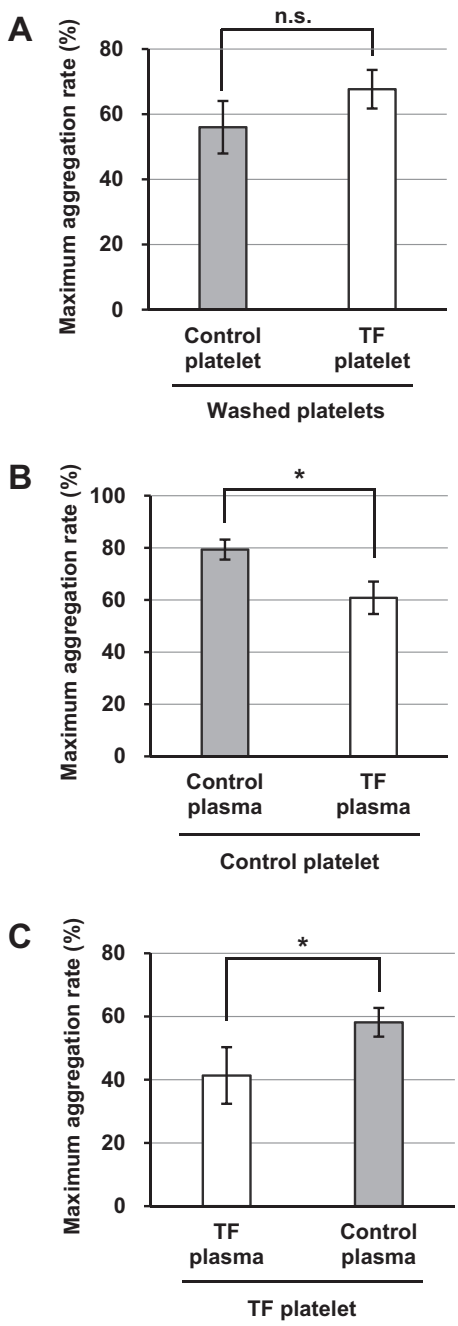

Fig. 3. Contribution of plasma components in impaired platelet aggregation in TF-induced DIC model rats. A: Washed platelets, prepared from rats treated with either saline (control group) or TF (TF group), were stimulated with 2 μg/mL collagen to measure platelet aggregation. The data were analyzed using an unpaired *t*-test. B: Platelets isolated from a normal rat were suspended in plasma obtained from either another normal rat (control group) or a TF-treated rat (TF group), then stimulated with 15 μg/mL collagen to measure platelet aggregation. *P < 0.05 (paired *t*-test) C: Platelets isolated from a TF-treated rat were suspended in autologous plasma (TF group) or plasma obtained from a normal rat (control group), then stimulated with 15 μg/mL collagen to measure platelet aggregation. *P < 0.05 (paired *t*-test). The data are presented as the mean ± SEM ($n = 6$ each).

TF group) or different doses of TM alfa (0.1, 0.3, and 1 mg/kg in the TM alfa groups) were injected via the tail vein. Then, saline (10 mL/kg/h, control group) or TF (10 mL/kg/h, TF group and TM alfa groups) was infused via the tail vein for 1 h. After the infusion, rats were anesthetized using isoflurane (2.5%–4%), and 4.5-mL blood samples were collected from the abdominal aorta using syringes filled with 0.5 mL of 3.13% sodium citrate. Blood was used to measure the TAT concentration, the number of platelets, and platelet aggregation.

2.5. Platelet transfusion

Leukocyte- and platelet-rich plasma (L-PRP) and platelet-poor plasma (PPP) for transfusion were prepared using a Mini GPSIII Kit (Zimmer-Biomet, Warsaw, Indiana, USA), in accordance with the manufacturer's instructions. Rats were anesthetized using isoflurane (2.5%–4%), and 6.5-mL blood samples were collected from the abdominal aorta using syringes filled with 1 mL of ACD-A solution. Blood was collected from four rats, and 30 mL of the pooled blood was loaded into the attached separation tube. The tube was centrifuged at 3200 rpm for 15 min at room temperature (RT) using a Biomet Biologics centrifuge (Zimmer-Biomet, Warsaw, Indiana, USA). The L-PRP and PPP fractions were collected and used for transfusion.

Rats were infused with either saline or TF as described above. After the infusion, an approximately 50-μL blood sample was collected from the subclavian vein using heparinized syringes to measure the number of platelets in the blood before the blood transfusion. Then, 10 mL/kg of L-PRP or PPP was slowly injected via the tail vein for 5 min. Five minutes after the end of the transfusion, rats were anesthetized using isoflurane (2%–2.5%), and 4.5-mL blood samples were collected from the abdominal aorta using syringes filled with 0.5 mL of 3.13% sodium citrate. The number of platelets in the blood and platelet aggregation were measured in these samples.

2.6. Measurement of TAT concentration

Citrated blood was placed on ice immediately after withdrawal, then centrifuged at 2000*g* for 20 min at 4 °C. The plasma supernatant was obtained and stored at −80 °C until use. The TAT concentration in plasma was measured using Enzygnost TAT micro (Siemens Healthcare Diagnostics, Marburg, Germany), in accordance with the manufacturer's instructions.

2.7. Platelets preparation

Citrated blood was allowed to stand for 5 min at RT, then centrifuged at 190*g* for 5 min at RT. The supernatant was transferred to a new tube and again centrifuged at 90*g* for 5 min at RT. The supernatant was collected as platelet-rich plasma (PRP). The bottom fraction of the first centrifugation was also centrifuged at 1600*g* for 10 min at RT. The supernatant was collected as PPP. In the experiment evaluating the effect of TM alfa on TF-treated rats, PRP was additionally centrifuged at 660*g* for 5 min at RT, and precipitated platelets were suspended using PPP to concentrate PRP. When washed platelets were prepared, PRP was centrifuged at 660*g* for 5 min at RT, and the pellet was washed using Tyrode's Buffer (5 mM HEPES, 136.5 mM NaCl, 2.68 mM KCl, 11.9 mM $NaHCO_3$, 0.43 mM Na_2HPO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% glucose, 0.35% bovine serum albumin, pH 7.4) containing 0.5 μM PGI_2. Then, platelets were centrifuged again at 660*g* for 5 min at RT and suspended with Tyrode's Buffer containing 0.02 U/mL apyrase to obtain the washed platelets. To prepare normal rat platelets suspended in TF-treated rat plasma, PRP was centrifuged at 660*g* for 5 min at RT, and the pellet was suspended in PPP prepared from the TF-treated rat or PPP prepared from another normal rat as a control. To prepare TF-treated rat platelets suspended in normal rat plasma, PRP was centrifuged at 660*g* for 5 min at RT, and the pellet was suspended with normal rat PPP or autologous PPP as a control. The platelet concentration was adjusted to 40×10^4/μL before measuring platelet aggregation.

2.8. In vitro TM alfa incubation

PRP were obtained from TF- or LPS-treated rats as described above.

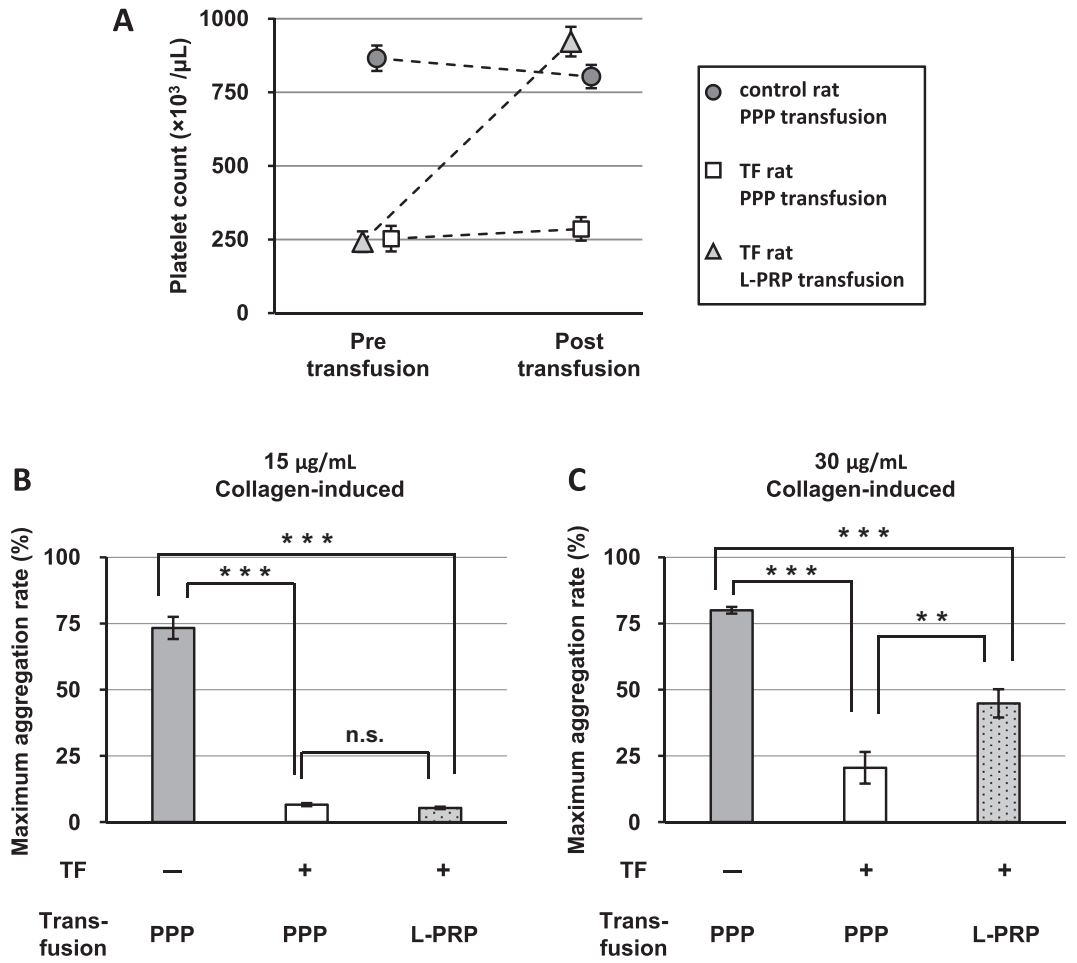

Fig. 4. The effect of platelet transfusion on platelet aggregation in TF-induced DIC model rats. A: Platelet count in the blood was measured before and after the transfusion of PPP or L-PRP in TF-treated or saline-treated rats. Saline-treated rats transfused with PPP (filled circle, $n = 6$), TF-treated rats transfused with PPP (open rectangle, $n = 11$) and TF-treated rats transfused with L-PRP (filled triangle, $n = 6$) were evaluated. B, C: PRP, isolated from TF-treated or saline-treated rats transfused with PPP or L-PRP, was stimulated with either 15 μg/mL collagen (B) or 30 μg/mL collagen (C), and aggregation was measured. The data are presented as the mean ± SEM. $n = 7$ (B) or $n = 10$ (C) for the group in which TF-treated rats were transfused with PPP, and $n = 6$ in the other groups. **P < 0.01, ***P < 0.001 (the Tukey's test).

Then, PRP, which platelet concentration was adjusted to 40×10^4/μL, was incubated with or without 100 μg/mL TM alfa for 30 min at 37 °C. Platelet aggregation was measured immediately after the incubation.

2.9. Platelet aggregation measurement

Platelet aggregation was measured using a PRP313M aggregometer (TAIYO Instruments Inc., Osaka, Japan) at 37 °C. Aliquots of PRP or washed platelets were preincubated in the aggregometer cuvettes for 1.5 min and platelet aggregation was then induced using the indicated concentration of collagen or ADP under continuous stirring followed by monitoring for 10 min. The maximum amplitude of aggregation was statistically analyzed.

2.10. Statistical analysis

All quantitative data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using EXSUS ver. 10.0 (CAC Croit, Osaka, Japan) based on SAS ver. 9.4 (SAS Institute Japan, Tokyo, Japan). Statistical significance was analyzed using a paired *t*-test, an unpaired *t*-test, Williams' test, or Tukey's test. P-values < 0.05 for the paired *t*-test, the unpaired *t*-test, and Tukey's test, and P-values < 0.025 for the Williams' test were considered statistically significant.

3. Results

3.1. The effect of TM alfa on impaired platelet aggregation in LPS-induced DIC model rats

To confirm the induction of hypercoagulation in rats by LPS administration, we measured the blood platelet count and plasma TAT concentration in the rats. The blood platelet count was significantly decreased and the plasma TAT concentration was significantly increased in the LPS group compared with the control group. TM alfa significantly inhibited the decrease in the platelet count and the increase in TAT in a dose-dependent manner (Fig. 1A, B).

To examine the effects of TM alfa on platelet function in LPS-treated rats, we evaluated collagen- and ADP-induced platelet aggregation in PRP, which included the same number of platelets among the experimental groups. The maximum amplitude of platelet aggregation induced by both collagen and ADP was significantly lower in the LPS group compared with the control group. TM alfa also significantly inhibited the decrease in the maximum amplitude of platelet aggregation in a dose-dependent manner (Fig. 1C, D). Incubation of PRP obtained from LPS-treated rats with TM alfa in vitro did not recover impaired platelet aggregation (without TM alfa; 36.2 ± 11.6%, with 100 μg/mL TM alfa; 44.4 ± 10.7%, $n = 5$, P > 0.05, paired *t*-test).

3.2. The effect of TM alfa on impaired platelet aggregation in TF-induced DIC model rats

To investigate whether hypercoagulation caused the decrease of platelet aggregation, we evaluated platelet aggregation in TF-induced DIC model rats, which present markedly different pathophysiology from LPS-induced DIC model rats [20]. First, we confirmed the induction of hypercoagulation in TF-treated rats. The platelet count in the blood was significantly decreased and the plasma TAT concentration was significantly increased in the TF group compared with the control group. TM alfa significantly inhibited the decrease of platelet count and the increase of TAT in a dose-dependent manner (Fig. 2A, B).

Then, to examine the effects of TM alfa on the platelet function in TF-treated rats, we evaluated collagen- and ADP-induced platelet aggregation in PRP which included the same number of platelets among the experimental groups. The maximum amplitude of platelet aggregation by both collagen and ADP was significantly lower in the TF group compared with the control group. TM alfa also significantly inhibited the decrease in the maximum amplitude of platelet aggregation in a dose-dependent manner (Fig. 2C, D). Incubation of PRP obtained from TF-treated rats with TM alfa in vitro did not recover impaired platelet aggregation (without TM alfa; 40.5 ± 17.5%, with 100 μg/mL TM alfa; 42.0 ± 17.5%, $n = 4$, $P > 0.05$, paired t-test).

3.3. Contribution of plasma to the TF-induced decrease in platelet aggregation

We then explored the mechanism of impaired platelet aggregation in DIC-model rats. Because impaired platelet aggregation for collagen and ADP stimulation and the effect of TM alfa for the impairment were commonly detected in both TF- and LPS-induced DIC model rats, we speculated that the main mechanism of impaired platelet aggregation is same between two models. Thus, we focused on TF-induced DIC model rats in subsequent mechanism analysis. Although we showed impaired platelet aggregation in DIC rat models, it is unclear whether the platelets itself or environmental factors, such as plasma components, played major roles in attenuating platelet aggregation. To evaluate platelet function apart from the effects of plasma components, we suspended platelets from control and TF-treated rats in Tyrode's Buffer to prepare washed platelets that were not contaminated with plasma components. The maximum amplitude of aggregation induced by collagen was almost comparable between the washed platelets derived from control and TF groups (Fig. 3A).

To evaluate the contribution of plasma components to impaired platelet aggregation in TF-treated rats, we suspended platelets, which were obtained from normal rats, in plasma from either TF-treated rats or control rats and measured collagen- and ADP-induced platelet aggregation. The maximum amplitude of platelet aggregation significantly decreased in platelets that were suspended in plasma from TF-treated rats compared with platelets suspended in plasma from control rats (Fig. 3B and Supplemental Fig. 1). Then, we suspended platelets from TF-treated rats in plasma obtained from either TF-treated rats or control rats. The maximum amplitude of collagen-induced platelet aggregation in the plasma from control rats was significantly higher than that in the plasma from TF-treated rats (Fig. 3C).

3.4. The effect of platelet transfusion on platelet aggregation in TF-induced DIC model rats

The above results suggest that changes in the plasma composition are the main causes of impaired platelet aggregation in the TF-induced DIC rat model. If this is the case, transfused platelets cannot maintain their ability to aggregate in recipient blood. To test this hypothesis, TF-treated rats were transfused with L-PRP or PPP and platelet aggregation was measured. L-PRP transfusion in TF-treated rats increased the platelet count in the blood to the same level as that of control rats, whereas the platelet count did not change when vehicle (PPP) was transfused into the control and TF-treated rats (Fig. 4A). However, the maximum amplitude of platelet aggregation caused by 15 μg/mL collagen in TF-treated rats transfused with L-PRP was significantly lower than that in control rats and was almost comparable to that in TF-treated rats transfused with PPP (Fig. 4B). The maximum amplitude of platelet aggregation induced with 30 μg/mL collagen, which more strongly stimulated platelet aggregation, in TF-treated rats transfused with L-PRP was significantly increased compared with that in TF-treated rats transfused with PPP (Fig. 4C). However, the maximum amplitude of platelet aggregation in TF-treated rats transfused with L-PRP did not reach that of control rats (Fig. 4C).

4. Discussion

In this study, we examined the effects of TM alfa on impairment of platelet aggregation using two different DIC rat models: the TF- and LPS-induced DIC models. We showed that TM alfa attenuated impaired platelet aggregation in both models. We also showed that suspension of normal platelets in plasma from TF-treated rats reduced platelet aggregation, which indicated changes in plasma composition impaired platelet aggregation. The contribution of plasma components to impaired platelet aggregation was further supported by the results that showed transfusion of normal platelets into TF-treated rats did not restore platelet aggregation.

TF and LPS are the most established inducers of hypercoagulation in animal models of DIC. In the present study, we confirmed that treatment with either TF or LPS caused a decrease of the blood platelet count and the increase in TAT concentration in rat plasma. Moreover, TM alfa treatment dose-dependently inhibited the decrease in the platelet count and the increase in the TAT concentration in both LPS- and TF-treated rats, which is consistent with previous studies [18,19]. These results suggest that DIC-like hypercoagulation was induced by TF and LPS treatment and TM alfa suppressed hypercoagulation in the rats.

Previous studies reported that platelet aggregation is suppressed in LPS-treated rats, but they did not investigate the involvement of hypercoagulation in the impairment of platelet aggregation [21]. Kander et al. showed that the amplitude of platelet aggregation is decreased in DIC patients [7]. Therefore, we hypothesized that hypercoagulation suppresses the aggregation function of platelets. In the present study, we confirmed that platelet aggregation was impaired in LPS-induced DIC model rats. We also showed, for the first time, that platelet aggregation was impaired in another DIC rat model induced with TF. Furthermore, we showed that TM alfa, which has anti-coagulation effects, attenuated the impairment of platelet aggregation in both TF-induced and LPS-induced DIC rat models. Our study suggests that hypercoagulation impairs the aggregation function of platelets, besides its well-known pathology of reducing the blood platelet count [3].

We demonstrated that platelet aggregation was impaired in PRP obtained from TF-treated rats. Impairment of platelet aggregation in PRP can be caused by impaired platelet function, changes in the plasma composition, or both. To define the major contributor of impaired platelet aggregation, we first evaluated the platelet aggregation ability by measuring aggregation of washed platelets, which are platelets suspended in Tyrode's buffer. In this study, the maximum amplitude of platelet aggregation was comparable between washed platelets from TF-treated rats and that from control rats. This result suggests that platelets are normal in TF-treated rats. Then, we evaluated the contribution of the plasma components to the impairment of platelet aggregation. We suspended normal platelets in PPP obtained from control or TF-treated rats and compared platelet aggregation. In this study, platelet aggregation was lower in the plasma from TF-treated rats compared with plasma from control rats. Moreover, aggregation of the TF-treated rat platelets suspended in control PPP was higher than that in PPP from TF-treated rats. These results suggest that the changes in plasma composition are the major contributor to platelet aggregation

impairment in TF-treated rats, and that TM alfa may suppress impaired platelet aggregation by inhibiting changes in plasma composition in TF-treated rats.

To verify the hypothesis, TF-treated rats were transfused with normal platelets and platelet aggregation was measured. We showed that platelet transfusion in TF-treated rats increased the platelet count, but did not increase the platelet aggregation induced with 15 μg/mL collagen. When platelets were stimulated with 30 μg/mL collagen, stronger stimuli, platelet aggregation was partially improved by platelet transfusion in TF-treated rats. These results suggest that platelet transfusion is able to restore the platelet counts, but only slightly improve the impaired platelet aggregation in TF-treated rats. These results strengthen our hypothesis that the changes in plasma composition mainly impair platelet aggregation in TF-treated rats.

We revealed that the plasma components may cause platelet dysfunction in TF-treated rats. Unfortunately, we could not identify the molecules causing impairment of platelet aggregation in this study. However, the molecules related to coagulation may be involved in this mechanism because two different DIC models decreased platelet aggregation. Some coagulation-related candidates can be proposed based on previous researches. One candidate molecule is fibrinogen, which is a coagulation factor that is converted into fibrin to form clots. Fibrinogen is also required for platelet aggregation. When fibrinogen was added to rabbit washed platelet solution, platelet aggregation was shown to be enhanced [22]. The other candidate is fibrinogen/fibrin degradation products (FDPs). When human FDPs are supplemented into human PRP, platelet aggregation is shown to be impaired [23]. Fibrinogen consumption, FDP accumulation, and their inhibition by TM alfa were already confirmed in DIC model rats [18,19]. The detailed analysis regarding involvement of these molecules in impaired platelet aggregation is the future issue. Also, evaluation with other anti-coagulants, such as unfractionated heparin, will provide further evidence that coagulation factors are involved in impaired platelet aggregation, and these studies are also our next issues of mechanism analysis.

In contrast to typical anti-coagulants, TM alfa is considered to be an agent with a low risk of bleeding even in DIC patients. Clinical trials of TM alfa in Japan in patients with DIC demonstrated that the incidence of bleeding-related adverse events was lower in the TM alfa group compared with the heparin group [24]. Additionally, in a global phase 2b study for sepsis and suspected DIC, no difference in the incidence of bleeding complications between TM alfa- and placebo-treated patients was observed [25]. The anti-fibrinolytic effect of TM alfa was proposed as one of the mechanism for a lower risk of bleeding complications with TM alfa [14,26]. In this study, we showed that TM alfa attenuated impairment of platelet function in DIC rat models. Impairment of platelet function leads to an increased risk of bleeding complications, as shown in patients with hereditary platelet dysfunction [27]. Therefore, the effect of TM alfa on platelet aggregation might partially contribute to the minimal bleeding risk of TM alfa in clinical settings.

In conclusion, we demonstrated that TM alfa attenuated impaired platelet aggregation in LPS- and TF-induced DIC rat models. The impairment of platelet aggregation in DIC models may mainly result from changes in the plasma composition, and this could not be reversed by transfusion of normal platelets. Our findings propose a novel pharmacological action of TM alfa on platelets. Further study will clarify whether TM alfa exerts its action on platelets in DIC patients.

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Conflicts of interest

All authors are employees of Asahi Kasei Pharma Corporation.

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