



A direct oral anticoagulant edoxaban accelerated fibrinolysis via enhancement of plasmin generation in human plasma: dependent on thrombin-activatable fibrinolysis inhibitor

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Abstract

A direct oral anticoagulant, edoxaban, is as effective as vitamin K antagonists for the treatment of venous thromboembolism (VTE). However, the mechanism underlying the treatment effect on VTE remains to be determined. The aims of this study were to evaluate the effect of edoxaban on tissue plasminogen activator (t-PA)-induced clot lysis in human plasma and to determine the roles of plasmin and thrombin-activatable fibrinolysis inhibitor (TAFI) in the profibrinolytic effect by edoxaban. Pooled human normal plasma or TAFI-deficient plasma (containing 180 ng/mL t-PA and 0.1 nM thrombomodulin) was mixed with edoxaban or an activated TAFI inhibitor, potato tuber carboxypeptidase inhibitor (PCI). Clot was induced by adding tissue factor and phospholipids. Clot lysis time and plasma plasmin- α 2 antiplasmin complex (PAP) concentration were determined. Clot structure was imaged with a scanning electron microscope. In normal plasma, edoxaban at clinically relevant concentrations (75, 150, and 300 ng/mL) and PCI significantly shortened clot lysis time. PCI increased PAP concentration and a correlation between PAP concentration and percent of clot lysis was observed. Edoxaban also dose-dependently elevated PAP concentration. In TAFI-deficient plasma, the effects of edoxaban and PCI on clot lysis and PAP concentration were markedly diminished as compared with normal plasma. Fibrin fibers were thinner in clots formed in the presence of edoxaban. In conclusion, edoxaban at clinically relevant concentrations accelerates t-PA-induced fibrinolysis via increasing plasmin generation in human plasma. The effects of edoxaban is mainly dependent on TAFI. The profibrinolytic effect of edoxaban might contribute to the efficacy for the treatment of VTE.

Keywords Fibrinolysis · Direct factor Xa inhibitor · Plasmin · Thrombin-activatable fibrinolysis inhibitor · Edoxaban

Highlights

- Edoxaban significantly shortened clot lysis time and elevated plasma plasmin- α 2 antiplasmin complex (PAP) concentration in plasma at clinically relevant concentrations.
- In thrombin-activatable fibrinolysis inhibitor (TAFI)-deficient plasma, the effects of edoxaban and a TAFIa inhibitor on clot lysis and plasma PAP concentration

were markedly diminished, suggesting that the profibrinolytic effect of edoxaban largely depends on TAFI.

- Fibrin fibers were thinner and the pores were larger in clots formed in the presence of edoxaban. The profibrinolytic effect of edoxaban might contribute to the efficacy for the treatment of venous thromboembolism.

Introduction

The appropriate balance between coagulation and fibrinolysis is essential to maintain blood flow. Once clots are formed in vessels, fibrinolysis is initiated through a number of orchestrated interactions among fibrin, plasminogen, and tissue-plasminogen activator (t-PA) [1]. Partial cleavage of fibrin at the C-terminal by plasmin and subsequent exposure of lysine residues accelerates fibrinolysis, because t-PA and

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plasminogen bind to the C-terminal lysine residues and further facilitate plasmin generation.

Activation of the coagulation system triggers the formation of clots and thrombi through the generation of Xa and thrombin. Thrombin is a multifunctional serine protease that not only converts fibrinogen to fibrin but also activates thrombin-activatable fibrinolysis inhibitor (TAFI) through the binding to thrombomodulin (TM) [2, 3]. Once TAFI is converted to its active form TAFIa, it removes the C-terminal lysine from partially degraded fibrin and reduces the binding of t-PA and plasminogen to the fibrin and suppresses the fibrinolytic system. Therefore, the coagulation system also contributes to the stability of clots.

Anticoagulants are used not only for prevention but also for treatment of venous thromboembolism (VTE). The 2016 American College of Chest Physicians guidelines for antithrombotic therapy in patients with VTE recommend the use of direct oral anticoagulants (DOACs) over vitamin K antagonists [4].

The amount of thrombus is defined by the balance between thrombus formation and fibrinolysis. Thus, when anticoagulants are administered even after the onset of VTE, the suppression of thrombus formation by anticoagulants may lead to dominance of fibrinolysis and consequently results in a decrease in thrombus volume. Moreover, anticoagulants not only inhibit thrombus formation, but also may indirectly enhance the fibrinolytic system. The profibrinolysis may also contribute to the VTE treatment effect by anticoagulants. The resolution of thrombi after treatment with DOACs in clinical settings [5–8] and the enhancement of clot lysis by DOACs *in vitro* [9–11] has been reported.

Edoxaban is one of the DOACs and a direct inhibitor of activated factor X with a rapid onset of action [12]. It is administered orally once daily and has proven antithrombotic efficacy [13, 14]. The phase III study for the treatment of VTE has demonstrated that edoxaban administered once daily after initial treatment with enoxaparin or unfractionated heparin was noninferior to standard therapy with warfarin after initial heparin, with significantly less bleeding [15]. However, the effect of edoxaban on clot lysis *in vitro* remains to be evaluated.

In this study, we determined the effect of edoxaban on t-PA-induced clot lysis in human plasma and the roles of plasmin and TAFI to clarify the mechanism underlying the profibrinolytic effect of edoxaban.

Methods

Materials

Human normal plasma and human TAFI-deficient plasma were purchased from George King Bio-Medical, Inc.

(Overland Park, KS, USA) and Affinity Biologicals, Inc. (Ontario, Canada), respectively. Edoxaban tosylate (edoxaban) was synthesized by Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Potato tuber carboxypeptidase inhibitor (PCI) was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). Recombinant t-PA and recombinant human thrombomodulin were purchased from Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan) and Asahi Kasei Pharma Corporation (Tokyo, Japan), respectively. PPP reagent (a mixture of tissue factor and phospholipids) and Fluo buffer (102.5 mM CaCl₂ in Hepes buffer, pH 7.35) were purchased from Thrombinoscope BV (Maastricht, Netherlands). DRG PAP micro ELISA was purchased from DRG International, Inc. (Springfield, NJ, USA). Phospholipid reagent (60% phosphatidylcholine, 20% phosphatidylethanolamine, 20% phosphatidylserine) was prepared at Daiichi Sankyo. Benzamidine hydrochloride monohydrate (benzamidine) and aprotinin were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and Sekisui Diagnostics, LLC (Lexington, MA, USA), respectively.

Clot lysis assay

Clot lysis assay was performed as follows: 70 μL of plasma (containing 180 ng/mL t-PA and 0.1 nM TM), 5 μL of edoxaban solution (or 1.92% dimethyl sulfoxide-saline solution as a vehicle), 5 μL of PCI solution (or HEPES buffer as a vehicle), and 20 μL of PPP reagent (2.5 pM tissue factor/4 μM phospholipids as final concentrations) were added to a 96-well microplate. The solution was preincubated at 37 °C for 10 min and clotting was initiated by adding 20 μL of Fluo buffer (17.1 mM CaCl₂ as a final concentration). Absorbance at 405 nm (OD_{405 nm}) was measured every 30 s with a spectrophotometer SpectraMax 190 (Molecular Devices, LLC., Sunnyvale, CA). Clot lysis time was defined as the interval between the time of the midpoint of the clear to maximum turbidity transition and the midpoint of the maximum turbidity to clear transition. In the time course experiment, the percent of clot lysis was calculated from the absorbance of each sample using the following formula.

$$\text{Clot lysis(\%)} = \left(\frac{\text{Maximum OD} - \text{OD at each time point}}{\text{Maximum OD}} \right) \times 100$$

The final concentrations in plasma of edoxaban and PCI were 75–300 ng/mL and 3 μg/mL, respectively. The edoxaban concentrations were decided based on plasma concentrations at the standard therapeutic dose 60 mg, peak plasma concentration was 302 ng/mL [16].

Measurement of plasmin— α 2-antiplasmin complex concentration

The generated plasmin binds to α 2-antiplasmin and forms plasmin— α 2-antiplasmin complex (PAP) to terminate the activity of plasmin. Therefore, PAP concentration can be used as an indicator of plasmin generation [17].

Clot formation and lysis were induced as mentioned above. The reaction was stopped at the sample collection time by adding 15 μ L of the stop solution (2000 kIU/mL aprotinine/20 mM benzamidine/0.048 M citrate). Clot was removed from the reaction mixture by centrifugation, and the supernatant was stored at -30 °C until measurement. The concentration of PAP in the sample was measured using an ELISA kit DRG PAP micro ELISA.

Scanning electron microscopy

Clots were formed in a 96-well microplate as follows: 75 μ L of plasma (containing 10 nM TM), 5 μ L of edoxaban solution (final concentration 164 ng/mL) or vehicle, and 20 μ L of PPP reagent were mixed and preincubated at 37 °C for 10 min. Clotting was initiated by adding 20 μ L of Fluo buffer and OD_{405 nm} of plasma was measured every 30 s. Clots were harvested 1 or 2 min after OD_{405 nm} reached a plateau. The resulting clots were washed in HEPES buffer and fixed in 1% glutaraldehyde solution.

The clots were stained with 1% osmic acid and 1% tannic acid, then dehydrated with increasing concentrations of ethanol, and substituted with *t*-butyl alcohol. After freeze-drying (ID-3 type, Eiko Engineering Co., Ltd., Hitachinaka, Japan), the clots were coated with platinum/palladium (IB-5 type, Eiko Engineering Co., Ltd.). The platinum/palladium-coated clots were imaged with a scanning electron microscope (S-4500, Hitachi High-Technologies Corporation, Tokyo, Japan).

Statistical analysis

SAS System Release 9.2 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis. Data are presented as the mean \pm standard error of the mean (SEM).

In the study using normal plasma, the statistical significance between control and edoxaban groups or control and PCI groups was evaluated by paired *t* test (with Bonferroni correction in case of 3 or more groups). In the study using TAFI-deficient plasma, Student *t* test was carried out for comparison between two groups. A *P* value of <0.05 (two-tailed) was considered as a statistically significant difference.

Results

Effects of edoxaban and PCI on clot lysis

We examined the effect of the direct FXa inhibitor, edoxaban, at clinically relevant concentrations (75, 150, and 300 ng/mL) on clot lysis by t-PA in human plasma. The typical time course of changes in OD_{405 nm} in the clot lysis assay is shown in Fig. 1a. Edoxaban enhanced the clot lysis in a concentration-dependent manner. The shortening effect of edoxaban on clot lysis time was statistically significant at concentrations of 75, 150 and 300 ng/mL (Fig. 1b).

Next, we determined the effect of the TAFIa inhibitor, PCI, on t-PA-induced clot lysis. PCI at 3 μ g/mL significantly shortened clot lysis time (Fig. 1c and d) as reported previously [18].

Effects of PCI and edoxaban on plasmin generation during clot lysis

We measured plasma PAP concentration as a marker of plasmin generation during clot lysis both in the absence and presence of PCI. Samples for the measurement of plasma PAP concentration was collected at 0, 8, 20, 30, 40, 50, 54 min in the absence of PCI, and 8, 15, 22, 29, 33, 40 min in the presence of 3 μ g/mL PCI during the course of clot lysis.

Plasma PAP concentration increased as clot lysis progressed in both groups; however, the rate of increase in PAP concentration was more rapid in the PCI group than control group (Fig. 2a). A scatter plot of % of clot lysis and plasma PAP concentration for each sample is shown in Fig. 2b. Plasma PAP concentration in both control and PCI groups highly correlated with % of clot lysis regardless of the presence or absence of PCI, suggesting that plasma PAP concentration is a good marker of clot lysis.

Next we determined the effect of edoxaban on plasma PAP concentration. Plasma PAP concentration was measured 33 min after the initiation of the reaction, at the time when clots were almost completely lysed in the presence of 300 ng/mL edoxaban. Edoxaban significantly increased the PAP concentration in a concentration-dependent manner (Fig. 2c), correlating with shortening the clot lysis time shown in Fig. 1.

Effects of edoxaban and PCI on clot lysis and plasmin generation in TAFI-deficient plasma

Next, we evaluated the role of TAFI using the TAFI-deficient plasma. In normal plasma, 3 μ g/mL PCI and 300 ng/mL edoxaban significantly shortened the clot lysis time by both 44% (Fig. 3a and c) and increased plasma PAP

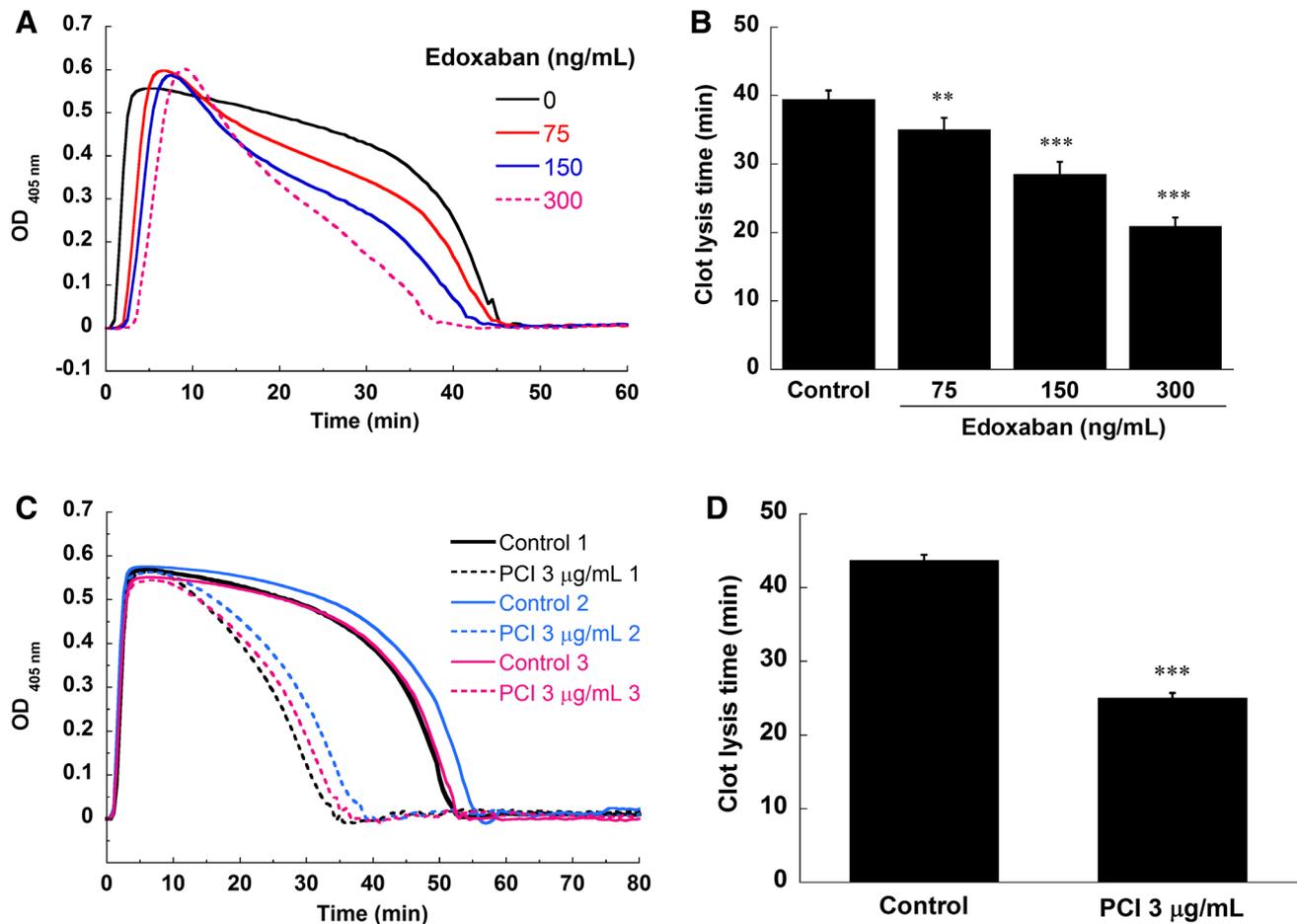


Fig. 1 Effects of edoxaban and PCI on clot lysis induced by t-PA in human normal plasma. **a** and **c** Typical optical density traces at 405 nm. **b** Effect of edoxaban on clot lysis time (n=8). **d** Effect of

PCI on clot lysis time (n=6). Data represent mean \pm SE. ** $P < 0.01$, *** $P < 0.001$ versus control

concentration 1.7- and 1.3-fold, respectively (Fig. 3d). In TAFI-deficient plasma in the absence of any drugs, the clot lysis time was significantly shortened compared with normal plasma (Fig. 3a and c). PCI had no effect on clot lysis time in TAFI deficient-plasma (Fig. 3b and c). Similarly, a large part of the shortening effect of edoxaban on clot lysis time disappeared; however, the drug slightly but significantly shortened clot lysis time (19%) in TAFI-deficient plasma (Fig. 3b and c). In TAFI-deficient plasma in the absence of any drugs, PAP concentration significantly elevated compared to normal plasma (Fig. 3d). Neither edoxaban nor PCI had effects on PAP concentration in TAFI-deficient plasma.

Effect of edoxaban on clot structure

Using a scanning electron microscope, the structure of clots formed in the absence and presence of 164 ng/mL edoxaban were characterized. Compared with control clots, the fibrin

fibers were thinner and the pores were larger in clots formed in the presence of edoxaban (Fig. 4).

Discussion

This study demonstrated that the oral direct FXa inhibitor, edoxaban, enhanced t-PA-induced clot lysis at clinically relevant concentrations in human plasma for the first time. To determine the mechanism of action of edoxaban, we measured plasma PAP concentration as the marker of plasmin generation [18]. We confirmed that plasma PAP concentration increased associated with the clot lysis and by a TAFIa inhibitor PCI. Similarly, we demonstrated that edoxaban significantly elevated plasma PAP concentration, indicating that plasmin generation is involved in the profibrinolytic effect of edoxaban.

Furthermore, to clarify the role of TAFI, we examined the effects of edoxaban and PCI on clot lysis time and

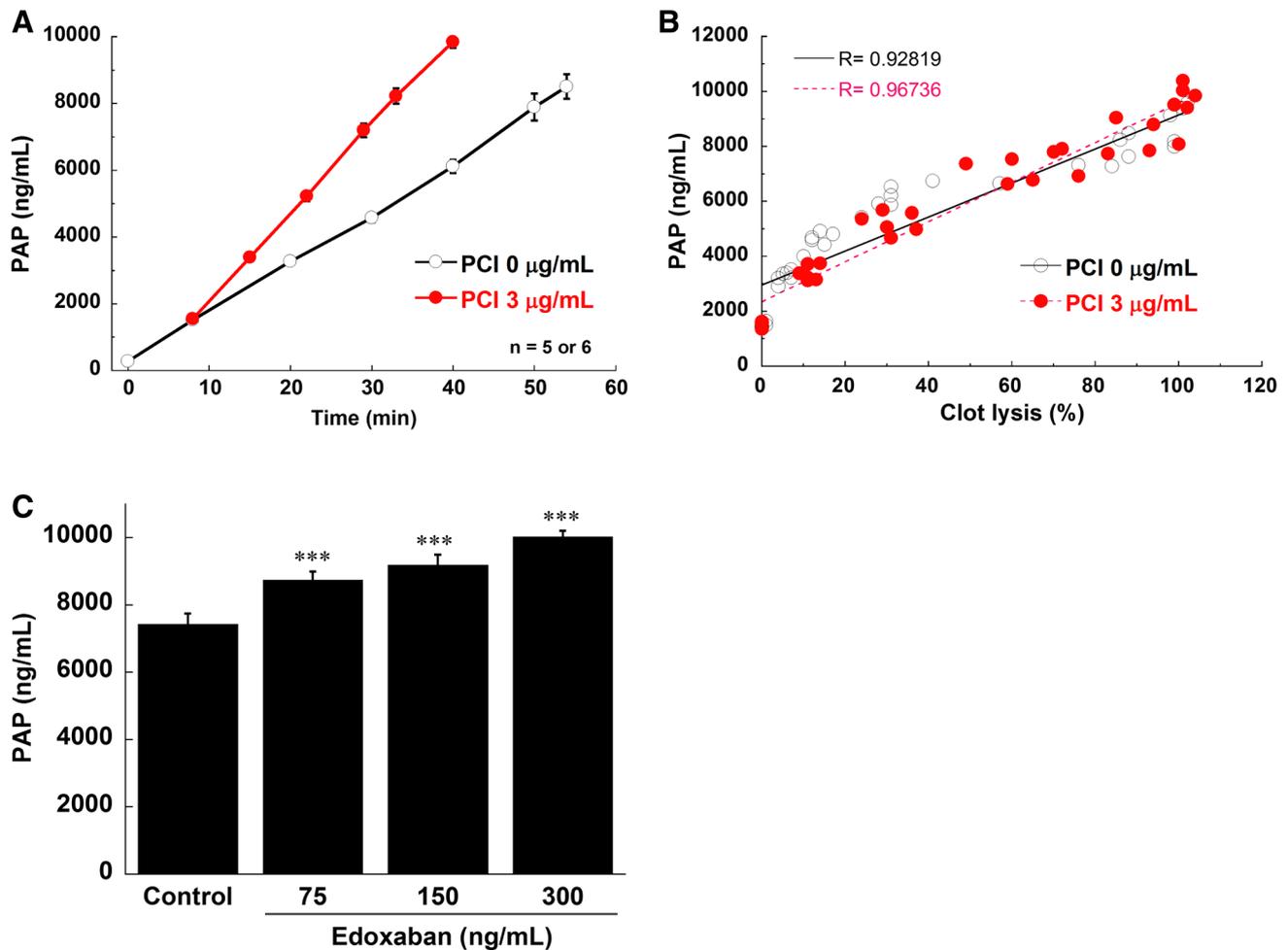


Fig. 2 Effects of edoxaban and PCI on plasma PAP concentration during t-PA-induced clot lysis in human normal plasma. **a** Effect of PCI on plasma PAP concentration ($n=5-6$). **b** Relationship between percent of clot lysis and plasma PAP concentration in the absence and

presence of PCI. **c** Effect of edoxaban on plasma PAP concentration ($n=8$). Plasma PAP concentration was measured 33 min after the initiation of the reaction. Data represent mean \pm SE. *** $P < 0.001$ versus control

plasma PAP concentration in TAFI-deficient plasma. The data clearly indicated that the acceleration of clot lysis and increase in plasma PAP concentration by edoxaban and PCI was largely dependent on TAFI. Thus it is suggested that inhibition of thrombin generation by edoxaban [19] would attenuate the activation of TAFI and suppress the cleavage of the C-terminal Lys residue on fibrin, thereby promoting plasmin generation and finally making clots more susceptible to fibrinolysis.

In terms of TAFI-dependency; however, Fig. 3 showed that the significant enhancement of clot lysis by edoxaban was still observed (19% shortened) even in TAFI-deficient plasma, whereas PAP concentration did not change at all. These data suggested that there would be a TAFI-independent mechanism. It has been reported that the microfibril density and the thickness of fibrin polymers influence the stability of clots and resistance to fibrinolytic dissolution

[20]. Thus, changes in clot structure shown in Fig. 4 might be involved in the TAFI-independent mechanism.

The phase III study of edoxaban for the treatment of VTE has demonstrated that it has at least similar efficacy to warfarin after initial treatment with enoxaparin or unfractionated heparin [15]. Moreover, we have reported that edoxaban significantly decreases the thrombus weight when it is administered 1 h after thrombus formation in a rat model of venous thrombosis [21]. These data suggest that edoxaban has a thrombus resolution effect. Actually, in clinical settings, edoxaban reduces thrombus volume in patients with symptomatic deep vein thrombosis [6].

It has already been reported that other DOACs, dabigatran, rivaroxaban, and apixaban, accelerate clot lysis [9–11]. A direct thrombin inhibitor dabigatran enhances clot susceptibility to fibrinolysis by two mechanisms: by reducing TAFIa generation and by altering the properties

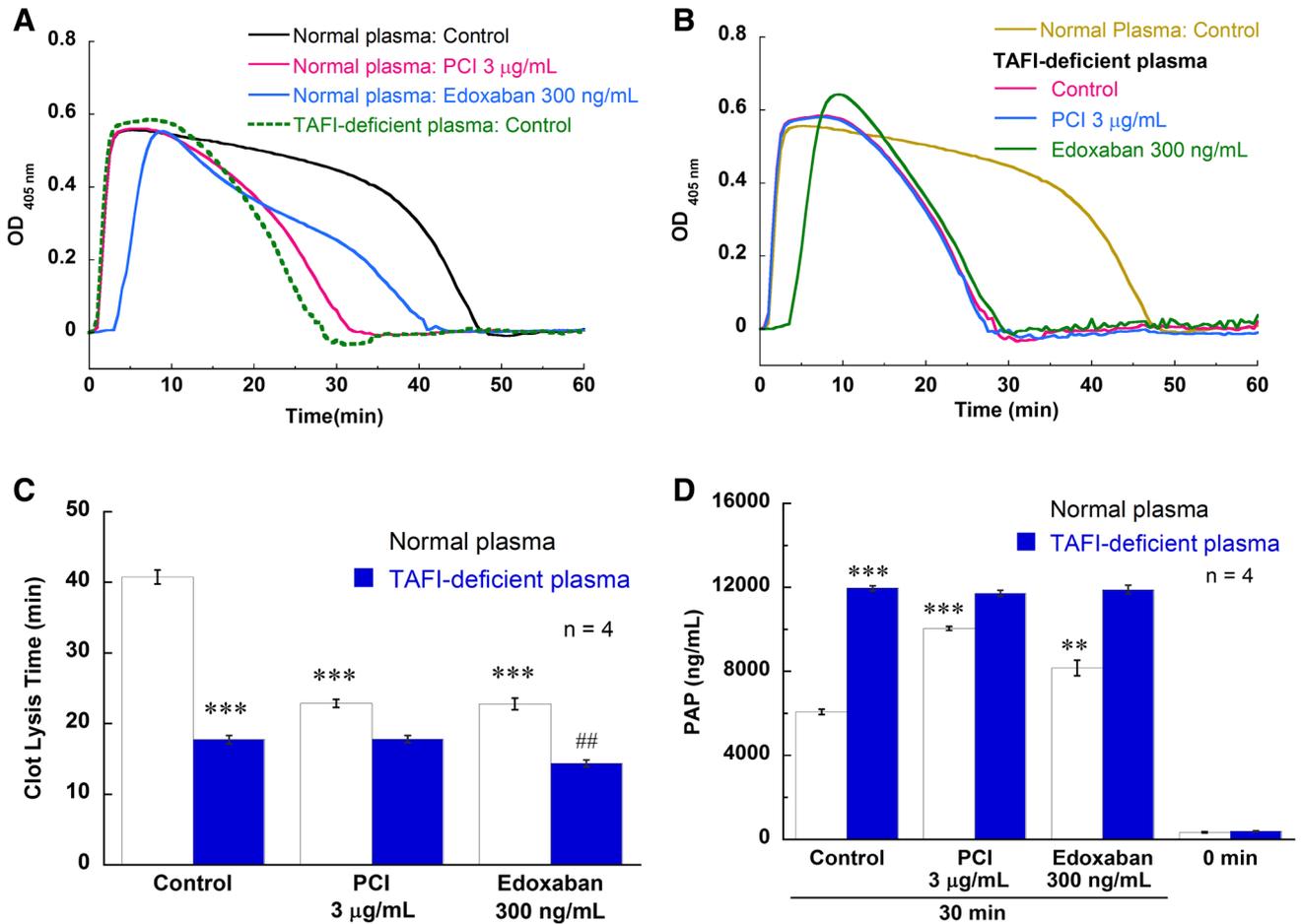
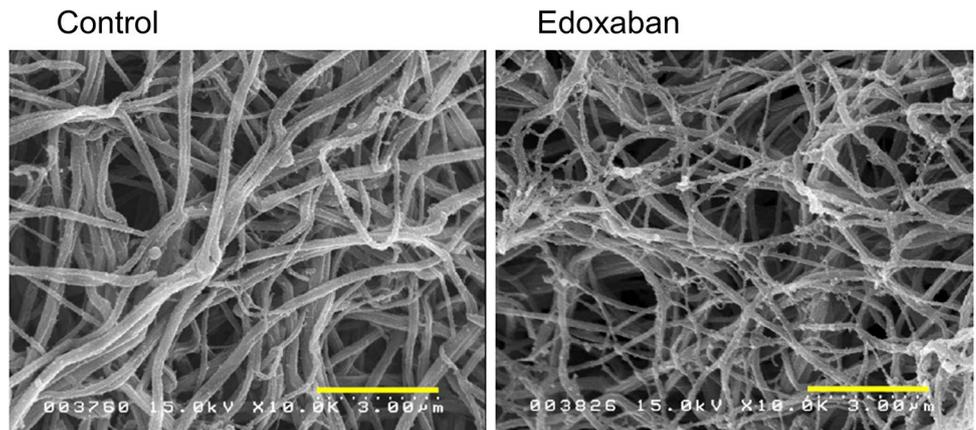


Fig. 3 Effects of edoxaban and PCI on clot lysis time and plasma PAP concentration in normal human plasma and TAFI-deficient plasma. Plasma PAP concentration was measured 30 min after the initiation of the reaction. **a** and **b** Typical optical density traces at

405 nm. **c** Clot lysis time. **d** Plasma PAP concentration. Data represent mean \pm SE (n=4). NHP: normal human plasma. TAFI-DP: TAFI-deficient plasma ** $P < 0.01$, *** $P < 0.001$ vs control in normal plasma. ## $P < 0.01$ versus control in TAFI-deficient plasma

Fig. 4 Representative scanning electron micrographs of clots prepared in human plasma spiked with vehicle or 164 ng/mL edoxaban. The magnification: $\times 10,000$ (yellow bar = 3 μ m)



of the clot [9]. The findings of the present study are similar to that of dabigatran. As additional mechanism of action, it has been reported that direct FXa inhibitors rivaroxaban

and apixaban enhance fibrinolysis by preventing the cleavage of FXa β , which has a t-PA cofactor function, to further fragments Xa33/13 [10]. This effect appears to be common

to all FXa inhibitors, thus a similar study using edoxaban would be necessary.

There are some limitations in this study. First is that we used soluble TM in our in vitro assay. In vivo, a majority of TM is located on the surfaces of endothelial cells, but not as a soluble form. We consider that the use of soluble TM in the microplate assay system is necessary to mimic the physiological conditions in vessels. However, the concentration of TM varies from 1 to 100 nM depending on the size of vessels [22]. Thus the impact of enhancement of fibrinolysis by edoxaban in vivo might vary among vessels. Second, we did not examine a role of factor XIII in this study. It is well known that activation of factor XIII by thrombin crosslinks fibrin strands and stabilizes clots [23]. Thus a further study would be needed to clarify the role of factor XIII.

In conclusion, the present results suggest that edoxaban at clinically relevant concentrations enhances t-PA-induced fibrinolysis via increasing plasmin generation in human plasma. The effects of edoxaban is mainly dependent on TAFI. The profibrinolytic effect of edoxaban might contribute to the efficacy for the treatment of VTE.

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Compliance with ethical standards

Conflict of interest The authors are the employees of Daiichi Sankyo Co., Ltd.

Ethical approval This was an in vitro investigation and did not involve any living subjects.

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