Elevated plasma factor VIII enhances venous thrombus formation in rabbits: Contribution of factor XI, von Willebrand factor and tissue factor

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Summary

Elevated plasma levels of factor VIII (FVIII) are associated with increased risk of deep venous thrombosis. The aim of this study is to elucidate how elevated FVIII levels affect venous thrombus formation and propagation *in vivo*. We examined rabbit plasma FVIII activity, plasma thrombin generation, whole blood coagulation, platelet aggregation and venous wall thrombogenicity before and one hour after an intravenous infusion of recombinant human FVIII (rFVIII). Venous thrombus induced by the endothelial denudation of rabbit jugular veins was histologically assessed. Thrombus propagation was evaluated as indocyanine green fluorescence intensity. Argatroban, a thrombin inhibitor, and neutralized antibodies for tissue factor (TF), factor XI (FXI), and von Willebrand factor (VWF) were infused before or after thrombus induction to investigate their effects on venous thrombus formation or propagation. Recombinant FVIII (100 IU/kg) increased rabbit plasma FVIII activity two-fold and significantly enhanced whole blood coagulation and total plasma thrombin generation, but did not affect initial thrombin generation time, platelet aggregation and venous wall thrombogenicity. The rFVIII infusion also increased the size of venous thrombus one hour after thrombus induction. Argatroban and the antibodies for TF, FXI or VWF inhibited such enhanced thrombus formation and all except TF suppressed thrombus propagation. In conclusion, elevated plasma FVIII levels enhance venous thrombus formation and propagation. Excess thrombin generation by FXI and VWF-mediated FVIII recruitment appear to contribute to the growth of FVIII-driven venous thrombus.

Key words

deep venous thrombosis, factor VIII, factor XI, von Willebrand factor

Introduction

Venous thromboembolism (VTE) that comprises deep venous thrombosis (DVT) and pulmonary embolism (PE) has become a major medical problem, with an overall age- and sex-adjusted annual incidence of > 1:1,000. The incidence of VTE noticeably increases with advancing age, and PE represents an increasing proportion of total VTE with high morbidity and mortality rates (1). Many acquired and inherited risk factors have been identified including surgery, long-term immobilization, age, malignancy, and deficiencies of antithrombin, protein C and protein S (2). In addition to these, recent epidemiological studies suggest that high plasma levels of factor VIII (FVIII) are associated with increased risk of DVT (3-5).

Factor VIII is a glycoprotein that is an essential cofactor for blood coagulation, and it circulates in plasma as a complex with von Willebrand factor (VWF). This complex is protected from proteolysis by activated protein C (6), and thrombin causes FVIII to dissociate from VWF (7). Activated FVIII (FVIIIa) binds to activated factor IX on negatively charged phospholipids such as the surface of activated platelets (8), where it subsequently activates factor X. The regulation of plasma FVIII levels and activity is complex, and FVIII coagulation activity ranges from < 50% in controls to > 300%, and 500% in patients with VTE (9).

Venous thrombus is initiated by endothelial injury and/or slow or static blood flow. It is generally recognized that the initiation phase is mainly modulated by procoagulant activity of the venous wall, and the propagation phase is regulated by blood flow and procoagulant factors in circulating blood. We previously demonstrated a close association among FVIII, platelets, VWF and fibrin in venous thrombi derived from human DVT, and that FVIII contributes to platelet aggregation and fibrin

formation on a collagen surface under low shear conditions *in vitro* (10). Animal studies have also shown that FVIII plays a significant role in venous thrombus formation (11,12). However, whether elevated FVIII levels promote venous thrombus formation and/or propagation, and its association with other coagulation factors *in vivo* remain unclear.

The present study investigates this issue in a rabbit model of venous thrombosis.

Materials and methods

Affinity of human rFVIII for rabbit VWF

The affinity between recombinant human FVIII (rFVIII, Kogenate-FS, Bayer Healthcare, Leverkusen, Germany) and rabbit VWF was examined using an enzyme-linked immunosorbent assay (ELISA) (13). Rabbit plasma (100 μl) with or without rFVIII and diluted 10,000-fold was added to microtiter plates coated with anti-rabbit VWF antibody and incubated at 37°C for two hours. The contents of each well were removed, the plates were incubated with 100 μl of horseradish peroxidase (HRP)-conjugated anti-human FVIII antibody for one hour at room temperature, washed and then perborate/3, 3', 5, 5'-tetramethylbenzidine substrate (100 μl) was added. After 20 minutes at room temperature, the enzymatic reaction was stopped by adding 0.5 N sulfuric acid. The amount of FVIII bound to rabbit VWF was measured at 450 nm.

Coagulation parameters

Blood samples were collected from the central ear arteries of rabbits into 3.8%

sodium citrate (9:1, v/v). Plasma samples were prepared by centrifugation at 2,580 g for 10 minutes at room temperature, and incubated with rFVIII (final concentrations: 0, 0.025, 0.25, 2.5 IU/ml) for 10 minutes. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured using a coagulation timer (Thrombotrack, AXIS-SHIELD; PoC AS, Oslo, Norway).

Coagulant activity of FVIII in rabbit plasma

The FVIII coagulant activity in rabbit plasma was measured using a one-stage clotting assay with a coagulation timer (Thrombotrack). Plasma samples were collected before, 30, 60 and 120 minutes after an intravenous infusion of rFVIII. The FVIII activity was assessed by measuring aPTT in diluted human FVIII-deficient plasma (Haematologic Technologies Inc., Essex Junction, VT, USA) (13). Pooled rabbit plasma served as the standard.

Thrombin generation assay

Thrombin generation in rabbit plasma was measured by calibrated automated thrombography as follows. Rabbit plasma collected before and 30 and 60 minutes after the rFVIII infusion was incubated with (final concentrations) 0.3 μM ellagic acid (Sysmex, Kobe, Japan), 0.5 pM recombinant human TF (Innovin®; Dade, Marburg, Germany) and 4 μM synthetic PL vesicles comprising phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine at a ratio of 1:6:3 as trigger reagents. Thrombin generation was started by adding $CaCl_2$ and the fluorogenic substrate Z-Gly-Gly-Arg-AMC. Lag time (LT), peak thrombin (Peak Th), time to peak (ttPeak) and endogenous thrombin potential (ETP), were calculated using Thrombinoscope

software (Thrombinoscope BV, Maastricht, Netherlands) (14).

Thromboelastogram assay

Whole blood hemostatic parameters before, 30 and 60 minutes after rFVIII infusion were measured using a ROTEM analyzer (Pentapharm GmbH, Munich, Germany). Blood samples (300 μl) in 3.8% sodium citrate (9:1, v/v) were transferred into the ROTEM reaction chamber. The blood re-calcified with 20 μl of 0.2 M $CaCl_2$ before clot formation was measured in duplicate using the standard NATEG evaluation parameters provided by the manufacturer (15). We assessed clotting time (CT) as the elapsed time from re-calcification to the start of clot formation, maximum clot firmness (MCF) and alpha angle, and the slope of clot formation. These parameters describe the following phases of the clotting process: initiation (CT), termination/final clot strength (MCF), and reaction velocity of clot formation (alpha angle).

Measurement of platelet aggregation

Blood samples in 3.8% sodium citrate (9:1, v/v) were separated by centrifugation at 130 g for 10 minutes and at 2,580 g for 10 minutes for platelet-rich plasma (PRP) and -poor plasma (PPP), respectively. We measured thrombin-induced platelet aggregation by mixing blood samples with acid citrate dextrose solution (9:1, v/v), and adding the mixture to acid citrate dextrose and modified suspension buffer (137 mM NaCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 2.7 mM KCl, 1.1 mM $MgCl_2$, 5.6 mM dextrose, pH 7.3). The mixtures were separated by centrifugation at 100 g for 10 minutes (16). Platelets sedimented from PRP by centrifugation at 1,010 g for 10 minutes with 0.7 μM (final concentration) of prostaglandin I_2 (Sigma-Aldrich Corp., St. Louis,

MO, USA) were re-suspended in modified suspension buffer.

The number of platelets was adjusted to 2×10^5 μl^{-1} with PPP (for PRP aggregation) or suspension buffer (for washed platelet aggregation). Adjusted plasma was incubated with rFVIII (1.25 IU/ml) or with distilled water (control) for 10 minutes. Thereafter, collagen (Nycomed Austria GmbH, A-4020 Linz, Austria), adenosine 5'-diphosphate (ADP, Sigma-Aldrich Corp.), botrocetin (American Diagnostica Inc., West Avenue, CT, USA) or thrombin (Sigma-Aldrich Corp.) was added as agonists. Platelet aggregation was measured using a PA-20 aggregation analyzer (Kowa, Aichi, Japan). Changes in light transmittance caused by each agonist were recorded for 10 minutes (5 minutes for thrombin-induced platelet aggregation) and maximal aggregation was estimated. The extent of aggregation was expressed as a ratio (%) of the maximum light transmittance obtained with PPP or suspension buffer.

Tissue factor activities in normal jugular veins

To evaluate TF activities in the vascular walls, rabbit plasma clotting time initiated by the vessel homogenate was measured using a coagulation timer (Thrombotrack) (17). Jugular veins removed from rabbits that had been infused with rFVIII (1.25 IU/ml) or saline were homogenized in Tris-buffered saline (pH 7.4) containing 10 mM $CaCl_2$ and 0.1% Triton X-100 (Nacalai Tesque Inc., Kyoto, Japan) using a Polytron PT3100 (Kinematica, Littau, Switzerland). After centrifugation at 2,580 g for 10 minutes, the supernatant (vessel sample; 100 μl containing 100 μg protein) was incubated with rabbit plasma (100 μl) for one minute. Clotting assays were then started by adding 20 mM $CaCl_2$ (100 μl). Protein concentrations were determined using bicinchoninic acid (BCA) protein assay kits (Pierce, Rockford, IL, USA). We also

measured TF protein levels in the walls of rabbit veins and in plasma using a TF ELISA kit (USCN Life Science Inc., Houston, TX, USA).

Preparation of anti-rabbit TF antibody, R37

A monoclonal anti-rabbit TF neutralizing antibody, R37, was generated using a standard procedure. Briefly, lymph node cells were isolated from five female SD rats (Japan Charles River, Yokohama, Japan) which had been immunized with recombinant rabbit TF (American Diagnostica Inc.), and fused with murine myeloma SP2/0 cells to establish hybridoma clones. Monoclonal hybridomas that secreted an antibody binding to rabbit TF were selected by an ELISA assay. Monoclonal antibodies were respectively purified with recombinant Protein A from the culture supernatants of the selected hybridoma clones. R37 exhibited a potent neutralizing activity against TF/factor VII-catalyzed factor X activation which was measured by an enzymatic assay using purified rabbit coagulation factors (American Diagnostica Inc. and Enzyme Research Laboratories, Swansea, UK) and S-2222 (Chromogenix Co., Milan, Italy). The neutralizing ability of R37 was assessed using a diluted PT assay with modification (18). The PT reagent Thromboplastin C plus (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) diluted 100-fold with 25 mM $CaCl_2$ or rabbit plasma was incubated with R37 for 10 minutes at 37°C. R37 dose-dependently prolonged PT under both conditions (Supplementary Figure 1).

Thrombus formation in rabbit jugular veins

The Animal Care Committee of the University of Miyazaki (No.2010-511-3) approved the study protocols. Fifty male Japanese white rabbits weighing 2.5 - 3.0 kg

were fed with a conventional diet. All surgical operations proceeded under aseptic conditions and general anesthesia was induced via an intravenous infusion of pentobarbital (25 mg/kg body weight). Thrombi were induced in the jugular veins by endothelial denudation using a 3F balloon catheter (Edwards Lifesciences, Irvine, CA, USA) (19). Saline (control) or rFVIII (100 IU/kg) was infused into the ear vein before balloon injury. Sixty minutes thereafter, the rabbits were infused with heparin (500 U/kg, i.v.) and then sacrificed with an overdose of pentobarbital (60 mg/kg, i.v.). The animals were perfused with 50 ml of phosphate buffered saline (0.01 mol/l) and perfusion-fixed with 50 ml of 4% paraformaldehyde to evaluate thrombus size and content.

We investigated the roles of thrombin, TF, factor XI (FXI) and VWF in thrombus formation by infusing 50 μg/kg/min of the thrombin inhibitor, argatroban (Argaron, Nichiiko Co. Ltd., Toyama, Japan), 3.0 mg/kg of anti-TF antibody R37 prepared by us, 3.0 mg/kg of XI-5108 anti-FXIa antibody (19,20), or 1.0 mg/kg of AJW200 anti-VWF antibody (a gift from Ajinomoto Pharmaceutical Co. Ltd., Tokyo, Japan) (21) immediately before endothelial denudation. Argatroban was continuously infused i.v. because it has a short half-life (22).

Fluorescent imaging of venous thrombus

We observed thrombus formation and propagation in the jugular vein in real time by exposing the rabbit jugular vein and then infusing 3 μg of indocyanine green (ICG; Sigma-Aldrich Corp.) into the ear vein before, and 5, 15, 30, 45 and 60 minutes after endothelial denudation (23). A PDE-neo C10935-11 near-infrared camera (Hamamatsu Photonics Co. Ltd., Shizuoka, Japan) was placed 5 cm over the vein. Four regions of interest (ROIs, 20 × 20 pixels) were established at regular intervals on the

vessel with one adjacent ROI as background (Fig. 6A). The signal intensity was measured in all images from all animals. The vessels were washed with saline one minute after ICG infusion and the average fluorescence intensity in each ROI was analyzed using dedicated U11437 software (Hamamatsu Photonics Co. Ltd.). Data are expressed as ratios of the signal intensity of the vein to the adjacent background. The removed venous thrombi emitted fluorescence. To assess the effect of antithrombotic agents on thrombus propagation under elevated FVIII levels, argatroban, anti-TF, anti-FXI or anti-VWF antibodies were infused to the point where the average fluorescence intensity of ICG in each ROI exceeded > 3-fold the background (when mural thrombus developed).

Immunohistochemistry of rabbit venous thrombus

One hour after thrombus induction, jugular veins were fixed in 4% paraformaldehyde for 24 hours at 4°C and embedded in paraffin. Sections (3-μm thick) were stained with hematoxylin and eosin and immunohistochemically examined using antibodies against glycoprotein (GP) IIb/IIIa (Affinity Biologicals Inc., Ancaster, CA, USA), fibrin (a gift from Takeda Chemical Industries Ltd., Osaka, Japan) and human FVIII (VIII-3776, Chugai Pharmaceutical Co. Ltd., Shizuoka, Japan) (10). The sections were stained with Envision (Dako, Glostrup, Denmark) or donkey anti-sheep IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Horseradish peroxidase activity was visualized using 3, 3'-diaminobenzidine tetrahydrochloride and the sections were faintly counterstained with Mayer's hematoxylin. Areas of venous thrombus and positive immunostaining for GPIIb/IIIa and fibrin were analyzed using a color imaging morphometric system (WinROOF, Mitani, Fukui, Japan) (17).

Statistical analysis

All data are expressed as means ± standard error. Differences between or among individual groups were compared using paired or unpaired t-tests, respectively, or ANOVA with the Bonferroni post-hoc test (GraphPad Prism 5, GraphPad Software Inc., San Diego, CA). A *P*-value of < 0.05 was considered to indicate statistical significance.

Results

Recombinant human FVIII binds rabbit VWF and shortens aPTT, but not PT in rabbit plasma

We found that rFVIII dose-dependently bound rabbit VWF (Fig. 1). Adding rFVIII (final concentration, 0.025-2.5 IU/ml) to rabbit plasma significantly and dose-dependently shortened aPTT, but did not affect PT (Fig. 2A). We infused rabbits with 20, 40 or 100 IU/kg of rFVIII and measured PT and aPTT *ex vivo*. We found that 100, but not 20 or 40 IU/kg of rFVIII significantly shortened aPTT one hour after the infusion (Fig. 2B).

Recombinant human FVIII enhanced rabbit plasma FVIII activity one hour after infusion

Plasma FVIII activity in rabbit plasma measured using a one-stage clotting assay before, and at 30, 60 and 120 minutes after rFVIII (100 IU/kg) increased about

2-fold for up to one hour after, compared with that before rFVIII infusion (198 ± 58 %, $p < 0.05$; Fig. 2C).

Recombinant human FVIII enhances thrombin generation and whole blood coagulation

Tables 1 and 2 show thrombin generation parameters triggered by ellagic acid, TF and PL and whole blood coagulation parameters initiated by $CaCl_2$. The infusion of rFVIII enhanced peak Th and ETP and shortened ttPeak, but did not affect LT. The results of whole blood coagulation assays showed that the infusion of rFVIII significantly shortened CT, and increased MCF and alpha angle. These findings indicate that the rFVIII infusion enhanced whole blood coagulability via increased thrombin generation.

Recombinant human FVIII did not affect platelet aggregation

To determine whether rFVIII directly affects platelet function, we assessed the effect of rFVIII on rabbit platelet aggregation initiated by collagen, ADP, botrocetin or thrombin. Recombinant FVIII did not affect platelet aggregation induced by these agents (Fig. 3).

Recombinant human FVIII did not affect vascular wall thrombogenicity

To determine whether rFVIII directly affects venous wall thrombogenicity, we measured TF activities in rabbit jugular veins one hour after rFVIII infusion. Rabbit plasma clotting time initiated by the jugular vein homogenate did not differ regardless of rFVIII infusion (104 ± 32 or 108 ± 25 seconds, respectively, $p = 0.82$, $n = 6$ each).

Levels of TF antigen were 156 ± 20 pg/ml in the venous wall (n = 5), but undetectable in rabbit plasma.

Recombinant human FVIII increased thrombus size in rabbit jugular veins one hour after endothelial denudation

To determine whether high plasma levels of FVIII promote venous thrombus formation, we histologically assessed rabbit jugular veins after rFVIII (100 IU/kg) infusion. Endothelial denudation caused by balloon insertion induced the formation of small mural thrombus, whereas balloon insertion together with rFVIII (100 IU/kg) infusion enhanced venous thrombus formation (Fig. 4A) and 11 of 24 jugular veins (46%) became occluded within one hour. The mean thrombus areas was about 200-fold larger in the rFVIII, than in the control group (Fig. 4B). The rFVIII infusion alone did not induce either endothelial denudation or thrombus formation.

All venous thrombi were immunopositive for both GPIIb/IIIa and fibrin, and the rFVIII infusion increased both GPIIb/IIIa and fibrin immunopositive areas in thrombi (Fig. 4C). The ratio of GPIIb/IIIa and fibrin immunopositive to thrombus areas did not differ between the rFVIII-infused and control groups. Thrombi were immunopositive for human FVIII in the rFVIII-infused, but not in the control group (Fig. 4D).

Thrombin, TF, FXI, and VWF are required for venous thrombus formation enhanced by rFVIII

We infused rabbit jugular veins with the thrombin inhibitor argatroban, or anti-TF, anti-FXI, or anti-VWF antibodies immediately before endothelial denudation to

evaluate the contribution of thrombin, TF, FXI, and VWF to venous thrombus formation enhanced by rFVIII. All of these agents suppressed venous thrombus formation within one hour of endothelial denudation (Fig. 5A-C).

Thrombin, FXI and VWF are required for FVIII-driven venous thrombus growth but not TF

We assessed the contribution of these thrombotic factors during thrombus propagation in rabbit jugular veins using ICG fluorescence imaging. Figure 6B shows representative images and corresponding fluorescence intensity 15 minutes after endothelial denudation in the control and rFVIII-infusion groups. Fluorescence emission elicited by ICG in each ROI immediately disappeared in the control group after the ICG infusion (Fig. 6B and C; Movie 1), but persisted in jugular veins due to ICG incorporation by thrombi in the rFVIII group. Thus the fluorescence intensity was significantly higher in the rFVIII, than in the control group (Fig. 6B and C, Movie 2). Figure 7 shows the average fluorescence intensity in ROIs before and after endothelial denudation in each group. Fluorescence emission gradually became more intense in the rFVIII-infused group, but did not change in the control group. We investigated the relationship between fluorescence intensity and thrombus formation by comparing fluorescence intensity in ROIs with thrombus size in corresponding histological sections. The ratio of ICG fluorescence intensity to the background positively correlated with thrombus area ($r = 0.84$, $p < 0.0001$, $n = 4$).

We administered argatroban, anti-TF, anti-FXI or anti-VWF antibodies when fluorescence intensity exceeded 3-fold the background level to evaluate the role of thrombin, TF, FXI and VWF during venous thrombus propagation in the presence of

high FVIII levels. At this point thrombi occupied about one sixth of the area of the vessel lumen. The amount of time taken to infuse argatroban, anti-TF, anti-FXI and anti-VWF antibodies did not significantly differ (11 ± 6, 15 ± 6, 13 ± 3, and 14 ± 5 minutes, respectively). An infusion of argatroban or anti-FXI antibody initially prevented the increase in the fluorescence intensity, and then gradually reduced the intensity (Movie 3 and 4). Anti-VWF antibody also suppressed the increase in intensity, but to a lesser extent than argatroban or anti-FXI antibody (Fig. 8A), whereas anti-TF antibody did not affect fluorescence intensity (Fig. 8A, Movie 5 and 6). These findings indicate that argatroban and antibodies for FXI or VWF significantly suppressed thrombus propagation, whereas anti-TF antibody did not. Histological and immunohistochemical studies also showed that argatroban and antibodies for FXI or VWF significantly reduced areas of thrombus, platelet (GPIIb/IIIa) and fibrin in thrombus one hour after endothelial denudation. Anti-TF antibody slightly suppressed thrombus and fibrin areas, but the difference did not reach significance (Fig. 8B and C).

Discussion

The present findings showed that elevated levels of FVIII enhance thrombin generation in plasma and promote thrombus formation and propagation in the injured jugular veins of rabbits. Thrombin, FXI, and VWF also significantly contributed to thrombus propagation.

Studies have historically focused on a deficiency of FVIII in patients with haemophilia associated with a significant bleeding diathesis. However, increasing evidence suggests that high plasma FVIII levels might constitute a clinically important risk factor for thrombosis. Several cohort and case-control studies have confirmed a high prevalence of elevated FVIII levels and coagulant activities in patients with DVT or PE and that the increased risk of VTE is dose-dependent upon plasma FVIII levels (3-5,24,25). Although levels of plasma FVIII activity widely vary, they are significantly higher in patients VTE than controls (200.1 ± 75.9% vs. 151.9 ± 57.7%) (9). The present study found that an infusion of rFVIII elevated rabbit plasma FVIII activity to 200%. This increase is within the range of controls and patients with DVT and corresponds to a 5-fold increase in the risk of DVT (3). The aPTT of the rabbit decreased 60 but not 30, 120 minutes after rFVIII infusion. Although the reason is obscure, it could be due to the sensitivity of the aPTT assay and interspecies differences with respect to the half-life of rFVIII. Only normal rabbits were included in this study, which might have made shortening the aPTT difficult. The half-life of rFVIII varies according to species, being 4.1, 5.5 and 15.8 hours in mice, rats, and humans, respectively (26).

High plasma levels of FVIII promote venous thrombosis and FVIII inhibition reduces venous thrombosis in mice model with $FeCl_3$-induced thrombosis (11,12).

However, the $FeCl_3$-injury model is to a large extent dependent on blood platelet activation and venous wall injury. Machlus et al. reported that elevated FVIII did not affect carotid arterial thrombus formation after extensive vascular damage caused by $FeCl_3$ (12). Slow or static blood flow together with endothelial injury comprises a fundamental risk factor for DVT. We therefore used a more accurate model of DVT pathophysiology and found that high FVIII levels enhanced venous thrombus formation and propagation. The results also indicated that endothelial denudation alone is insufficient to generate large thrombi even under slow flow, and that combination with a hypercoagulability state is essential for thrombus formation resulting in overt VTE/PE.

A cohort study demonstrated that persistently high FVIII activity actually increased thrombin generation in patients with DVT (27). The present study found that infused rFVIII increased whole blood coagulation and total plasma thrombin generation, but did not affect the initiation time of thrombin generation. Elevated FVIII activity might contribute mainly to the propagation of thrombin generation. The rFVIII did not affect platelet aggregation by thrombin *in vitro* (Fig. 3). The direct effect of rFVIII on platelet aggregation is less likely and excess thrombin generation enhanced by rFVIII plays a critical role in venous thrombus formation and propagation in our model.

Factor VIII is recruited by binding to VWF on surfaces comprising collagen and thrombus (28). We previously reported that FVIII colocalizes with VWF in venous thrombi of patients with DVT and that FVIII inhibition reduces thrombus formation under low shear conditions *in vitro* (10). Interrupting VWF-platelet interaction prevents venous thrombus formation in rabbit and mouse models of DVT (29,30). A shortage of VWF also reduces $FeCl_3$-induced thrombus formation in mesenteric venules, and rFVIII

infusion in mice does not restore thrombus stability (31). These lines of evidence suggest that VWF plays an important role in FVIII-driven venous thrombus formation. The present study showed that anti-VWF antibody, which interrupts interactions between VWF and GPIbα, suppressed venous thrombus formation under high FVIII levels (Fig. 5A-C). As reported (32), a blockade of VWF and GPIbα interaction abolished platelet adhesion on a collagen surface at a high, but not at a low shear rate, indicating that VWF might recruit FVIII on the surface of platelets but not of collagen under conditions of venous flow. This antibody also significantly suppressed venous thrombus propagation, but to a lesser extent than argatroban and anti-FXI antibody (Fig. 8B and C). These results suggest that VWF-platelet interaction contributes principally to the initiation and somewhat to propagation of venous thrombus under high FVIII levels.

Factor XI is generally considered to be less important in normal haemostasis, because a bleeding tendency is mild or absent in patients with an inherited or acquired FXI deficiency (33,34). However, recent studies indicate that FXI is activated during blood coagulation and that even small amounts of FXI induce thrombus growth by generating thrombin and by protecting thrombi from fibrinolysis via thrombin activatable fibrinolysis inhibitor (35,36). Therefore, FXI apparently plays a significant role in thrombus growth and stability. Animal studies using $FeCl_3$- or vessel clamp-induced venous thrombosis models have shown that FXI plays a crucial role in thrombus propagation and stability (37-39). We and others (20,40) have also demonstrated that FXI contributes to arterial thrombus propagation rather than to initiation. The present study found that anti-FXIa antibody reduced venous thrombus formation and propagation. FXI is mainly activated by thrombin and FXIa but not by factor XII on negatively charged surfaces (41). As thrombin generation is significantly

promoted under high FVIII levels, FXIa could largely contribute to the initiation of thrombus formation as well as thrombus propagation in venous thrombosis.

Although studies have shown that TF contributes to venous thrombus formation and propagation (42,43), the source of TF in venous thrombosis remains obscure. Mice with a severe TF deficiency have impaired thrombus formation after inferior vena cava ligation (44). Transplanting wild-type mice with low-TF bone marrow does not suppress venous thrombus formation and transplanting wild-type marrow into such mice does not accelerate thrombosis (44). This indicates that vascular wall TF rather than circulating TF is critical for venous thrombus formation. On the other hand, Von Bruhl et al. reported that TF derived from myeloid leukocytes contributes to venous thrombosis initiated by restricting blood flow in the inferior vena cava (45). In addition, TF derived from hematopoietic cells or neutrophils was responsible for thrombus formation and propagation in a laser-induced arteriolar injury model (46,47). We found here that anti-TF antibody reduced the formation, but not the propagation of thrombus even in the presence of high FVIII levels. Plasma TF protein was undetectable in the rabbits and TF inhibition in blood did not affect whole blood coagulation (data not shown). Our results suggest that venous thrombus formation in this model mainly depends on venous wall TF rather than blood-derived TF. These controversial results could be due to differences among triggers of venous thrombus formation (endothelial denudation, vessel ligation, flow restriction or laser-injury), flow condition (absence, presence or restriction), and vascular bed (jugular vein, inferior vena cava, or arteriole).

Venous thrombi have been created in various animal models. Ferric chloride (48) and electrolytic model (49) are reproducible, but such chemical and physical

reactions are far removed from the actual pathophysiology of DVT. Vein ligation with or without endothelial denudation (19,50) allows the assessment of interaction between the venous wall and progression from acute to chronic thrombus, but has a disadvantage for evaluating the efficacy of therapeutic agents. The vein stenosis model (30) can form laminar thrombus in the presence of blood flow and mimics the clinical situation, but it has the disadvantage of variations in thrombus size and stability. Endothelial denudation without flow restriction induces small venous, but not occlusive thrombi. We applied this model to evaluate thrombus propagation under conditions of elevated FVIII levels.

In conclusion, our results suggest that elevated plasma levels of FVIII enhance venous thrombus formation, and that excess thrombin generation by FXI and VWF-mediated FVIII recruitment might contribute to FVIII-driven venous thrombus growth.

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Conflict of interest

A. Harada, T. Kitazawa, K. Hattori are employed by Chugai Pharmaceutical Co., Ltd. C. Sugita, A. Yamashita, Y. Matsuura, T. Iwakiri, N. Okuyama, S. Matsuda, T. Matsumoto, O. Inoue, M. Shima, and Y. Asada have no conflicts of interest.

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Figure legends

Fig. 1. Affinity of rFVIII for rabbit VWF.

Affinity between rFVIII and rabbit VWF was measured by ELISA. Diluted rabbit plasma with or without rFVIII was added to microtiter plates coated with anti-rabbit VWF antibody. The rFVIII dose-dependently bound to rabbit VWF and was detected using anti-human FVIII antibodies. (*$p < 0.0001$; $n = 5$ each).

Fig. 2. Effects of rFVIII on PT and aPTT *in vitro* and *ex vivo*, and plasma FVIII activity.

(A) Recombinant human FVIII shortened aPTT *in vitro* (*$p < 0.0001$), but did not affect PT ($p = 0.37$; $n = 4$ each).

(B) Three doses of rFVIII (20, 40 or 100 IU/kg) were infused into rabbits. Blood samples were collected before, 30, 60 and 120 min after rFVIII infusion. Infused rFVIII at 100 (filled circles) but not at 20 (open circles) or 40 (open triangles) IU/kg shortened aPTT within one hour (*$p < 0.05$ vs. before rFVIII infusion). However, no rFVIII doses affected PT ($p = 0.18$; $n = 4$ each).

(C) Plasma FVIII activity was measured using one-stage clotting assays in rabbit plasma diluted with human FVIII-deficient plasma. Blood samples were collected before and after rFVIII (100 IU/kg) infusion. Infused rFVIII increased plasma FVIII activity for up to 60 minutes ($n = 4$ each; *$p < 0.05$ vs. before rFVIII infusion).

Fig. 3. Platelet aggregation with or without rFVIII *in vitro*.

Rabbit PRP and washed platelets with (filled columns) or without (opened

columns) rFVIII were prepared as described in Materials and methods. Platelet aggregation was initiated by adding collagen, ADP, botrocetin or thrombin. Infused rFVIII did not affect platelet function (n = 4 each).

Fig. 4. Infused rFVIII enhances thrombus formation in rabbit jugular vein.

(A) Representative light microphotographs of venous thrombi one hour after endothelial denudation with or without rFVIII infusion.

(B) Area with thrombi formed in rabbit jugular veins one hour after endothelial denudation. Recombinant human FVIII was infused just before endothelial denudation (*$p < 0.0001$, n = 16 sections each).

(C) GPIIb/IIIa and fibrin immunopositive areas in thrombi of jugular veins. Thrombi were immunochemically stained using anti-GPIIb/IIIa and anti-fibrin antibodies (*$p < 0.0001$, n = 16 sections each).

(D) Recombinant human FVIII in venous thrombi localized by staining with anti-human FVIII antibody.

Fig. 5. Thrombin, TF, FXI, and VWF are required for thrombus formation enhanced by rFVIII in rabbit jugular veins.

(A) Representative light microphotographs of venous thrombi one hour after endothelial denudation that proceeded immediately after infusions of rFVIII and inhibitor of thrombin, TF, FXI and VWF.

(B) Area with thrombi formed in rabbit jugular veins one hour after endothelial denudation (*$p < 0.0001$ vs. rFVIII group, n = 16 sections each).

(C) GPIIb/IIIa and fibrin immunopositive areas in thrombi of jugular veins (*p <

0.0001 vs. rFVIII group, n = 16 sections each).

Fig. 6. Fluorescence intensity of ICG at 15 minutes after endothelial denudation.

(A) Four ROIs (yellow, green, blue, and pink frames) were set at regular intervals on rabbit jugular vein before rFVIII infusion. One adjacent ROI (white frame) was set beside ROIs as background.

(B) Fluorescent images at 15 minutes after endothelial denudation and at 15 seconds after ICG infusion with saline (left) or 100 IU/kg of rFVIII (right).

(C) Ratio of fluorescence intensity in ROIs to background after infusing ICG into saline (open circles) and rFVIII (closed circles) groups (*$p < 0.05$ vs. control, n = 4 each).

Fig. 7. Ratio of ICG to background fluorescence intensity before and after endothelial denudation.

Saline (open circles) or 100 IU/kg of rFVIII (closed circles) was infused immediately before endothelial denudation. This graph shows average fluorescence intensity before, and 5, 15, 30, 45 and 60 minutes after endothelial denudation (*$p < 0.01$ vs. control, n = 4 each).

Fig. 8. Thrombin, FXI and VWF, but not TF are required for thrombus growth after mural thrombus formation in rabbit jugular vein.

(A) Venous growth was monitored over time as fluorescence intensity of ICG after endothelial denudation with rFVIII infusion. Argatroban, anti-TF, anti-FXI and anti-VWF antibodies were infused when average fluorescence intensity of ICG exceeded three-fold background. Differences in average fluorescence intensity of

ICG before inhibitor administration and one hour after endothelial denudation are evident (*$p < 0.05$, †$p < 0.01$, ‡$p < 0.0001$, n = 4 each).

(B) Representative light and immunohistochemical microphotographs of venous thrombi. Administration of argatroban, anti-FXI, and anti-VWF antibodies after mural thrombus formation reduced further thrombus formation enhanced by rFVIII infusion.

(C) Areas of thrombi, and GPIIb/IIIa and fibrin immunopositive areas in thrombi of jugular veins (*$p < 0.05$, †$p < 0.01$, ‡$p < 0.0001$ vs. rFVIII group, n = 10 sections each).

Movie 1. Fluorescence imaging of rabbit jugular vein one hour after saline infusion and endothelial denudation.

Marginal ear veins were infused with ICG one hour after saline infusion and thrombus induction, and then ICG fluorescence in rabbit jugular veins was visualized using a near-infrared camera. The fluorescence disappeared after infusion.

Movie 2. Fluorescence imaging of rabbit jugular vein one hour after rFVIII infusion and endothelial denudation.

Marginal ear veins were infused with ICG one hour after rFVIII infusion and thrombus induction. Persistent, intense fluorescence emitted by ICG indicates disturbed flow and stasis in jugular vein.

Movie 3. Fluorescence imaging of rabbit jugular vein immediately before anti-FXI antibody infusion.

Marginal ear veins of rabbits were infused with ICG 15 minutes after rFVIII infusion and thrombus induction and then fluorescence emitted by ICG in rabbit jugular veins was visualized with a near-infrared camera. Image shows slow or abnormal venous flow and filling defects along venous wall.

Movie 4. Fluorescence imaging shows effects of anti-FXI antibody after mural thrombus formation.

Anti-FXI antibody was infused immediately after Movie 3. Marginal ear veins of rabbits were infused with ICG one hour after rFVIII infusion and thrombus induction. Fluorescence emitted by ICG in jugular veins indicates slow or abnormal venous flow and filling defects along venous wall but no apparent flow disturbance or stasis.

Movie 5. Fluorescence imaging of rabbit jugular vein immediately before anti-TF antibody infusion.

Marginal ear veins of rabbits were infused with ICG 15 minutes after rFVIII infusion and thrombus induction. Fluorescence emitted by ICG in jugular vein indicates slow or abnormal venous flow and filling defects along venous wall.

Movie 6. Fluorescence images of rabbit jugular vein show effects of anti-TF antibody after mural thrombus formation.

Anti-TF antibody was infused immediately after Movie 5. ICG was infused into marginal ear vein one hour after rFVIII infusion and thrombus induction. Image shows obvious flow disturbance and stasis. Fluorescence intensity of ICG increased compared with that before antibody infusion and persisted in jugular vein.

Table 1. Thrombin generation in rabbit plasma *ex vivo* before and after rFVIII infusion.

Parameters	Before (n = 6)	30 min after (n = 6)	60 min after (n = 6)
LT (min)	3.4±0.2	3.2±0.2	3.2±0.1
Peak Th (nM)	250±23	295±26	313±40*
ttPeak (min)	6.4±0.3	5.6±0.2†	5.6±0.3†
ETP (nM × min)	1243±116	1291±100	1485±139‡

ETP, endogenous thrombin potential; LT, lag time; Peak Th, peak Thrombin; ttPeak, time to peak. *$p < 0.05$, †$p<0.001$ ‡$p < 0.01$ vs. before rFVIII infusion. Data are shown as means ± standard error.

Table 2. Whole blood coagulation in rabbit plasma *ex vivo* before and after rFVIII infusion.

Parameters	Before (n = 6)	30 min after (n = 6)	60 min after (n = 6)
CT (sec)	1034±74	519±46†	299±58†
MCF (mm)	59±1	66±1*	67±1†
Alpha angle (°)	47±3	65±3*	75±2†

CT, clotting time; MCF, maximum clot firmness. *$p < 0.01$, †$p < 0.001$ vs. before rFVIII infusion. Data are shown as means ± standard error.

Supplementary Figure 1. Neutralizing activity of monoclonal anti-rabbit TF antibody, R37.

Thromboplastin C plus (PT reagent) diluted 100-fold with 25 mM $CaCl_2$ (A) or rabbit plasma (B) was incubated with anti-TF antibody (R37) for 10 minutes at 37°C. R37 dose-dependently prolonged PT under both conditions (*$p < 0.0001$, $n = 4$ each).

Fig. 1

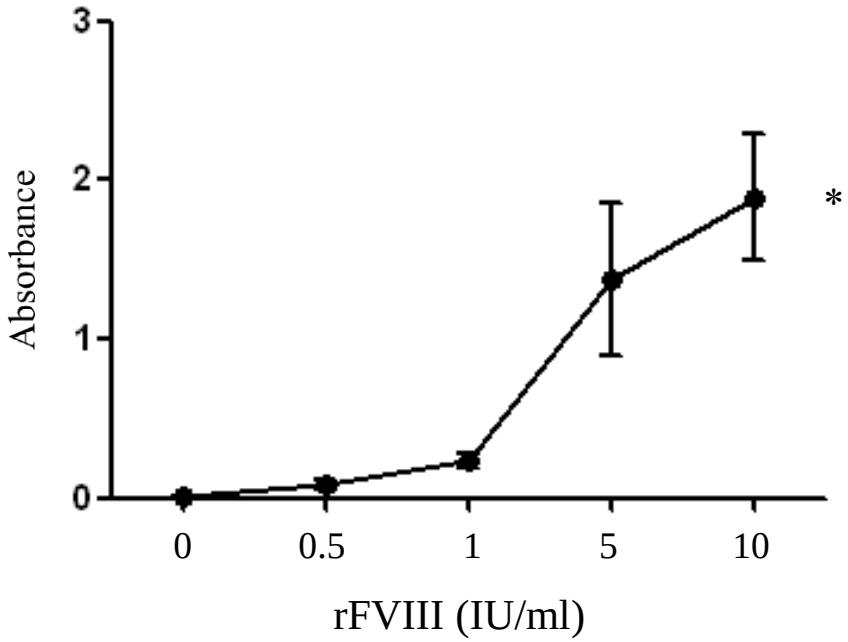
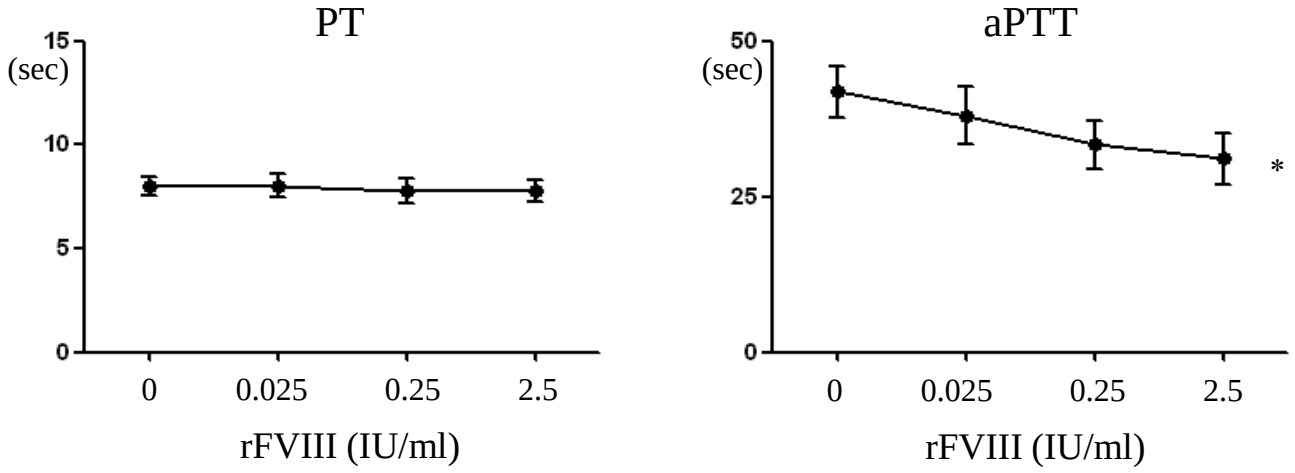


Fig. 2A



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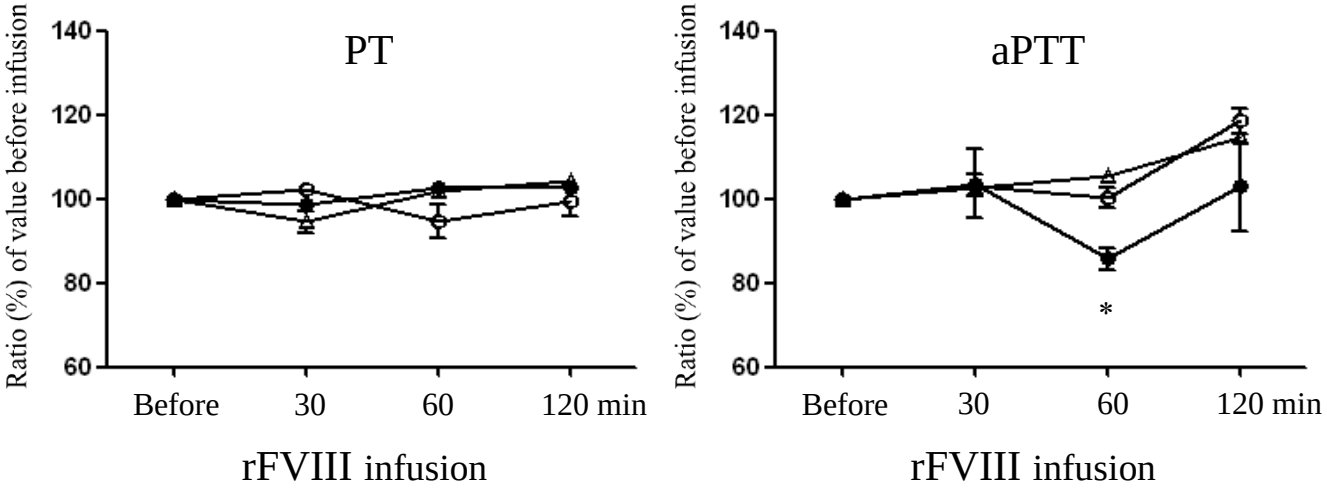


Fig. 2C

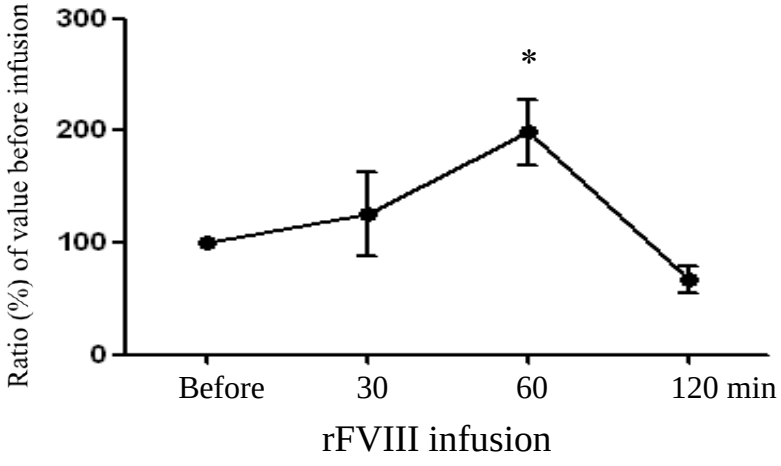


Fig. 3

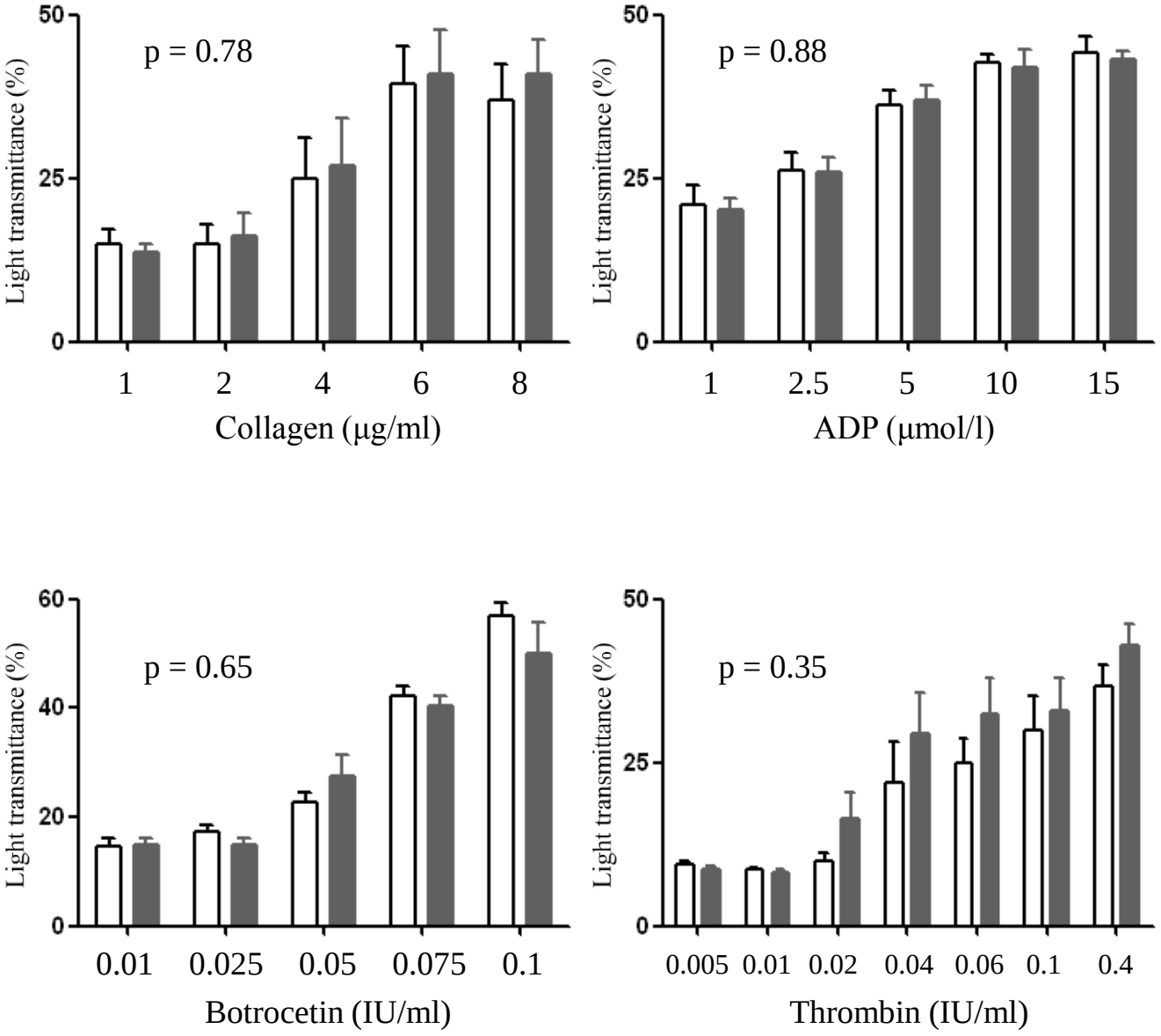
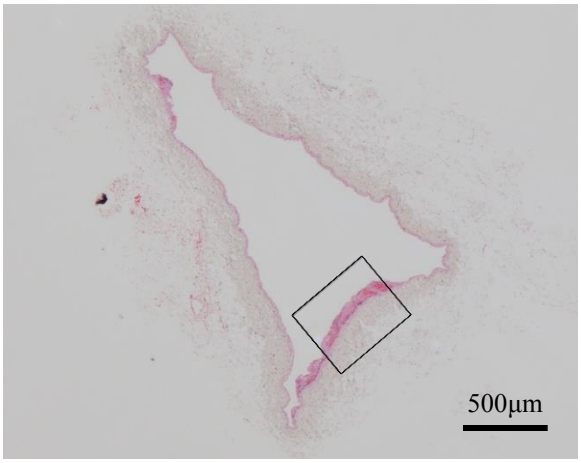
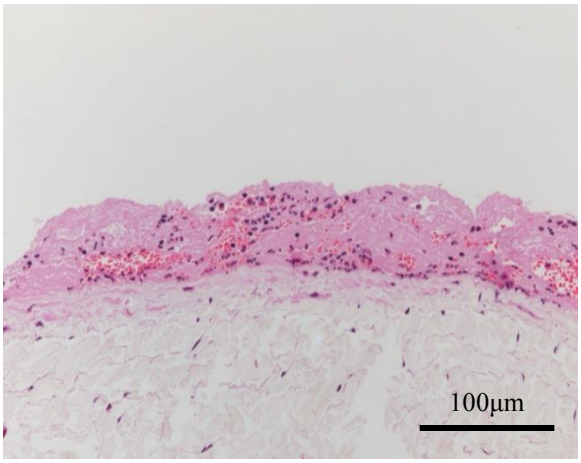

p = 0.78
Light transmittance (%)
50
25
0
1
2
4
6
8
Collagen (µg/ml)
p = 0.88
Light transmittance (%)
50
25
0
1
2.5
5
10
15
ADP (µmol/l)
p = 0.65
Light transmittance (%)
60
40
20
0
0.01
0.025
0.05
0.075
0.1
Botrocetin (IU/ml)
p = 0.35
Light transmittance (%)
50
25
0
0.005
0.01
0.02
0.04
0.06
0.1
0.4
Thrombin (IU/ml)

Fig. 4A

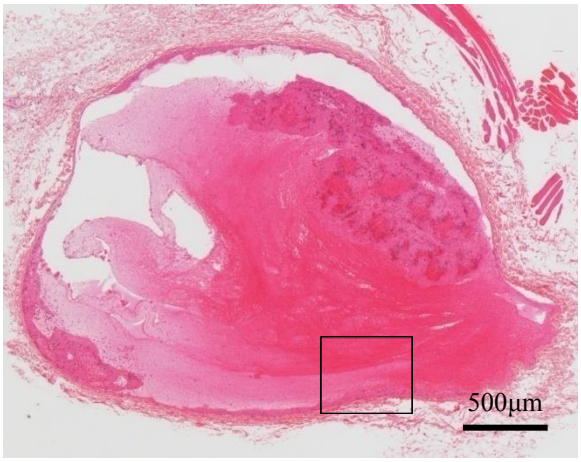
Enlarged image

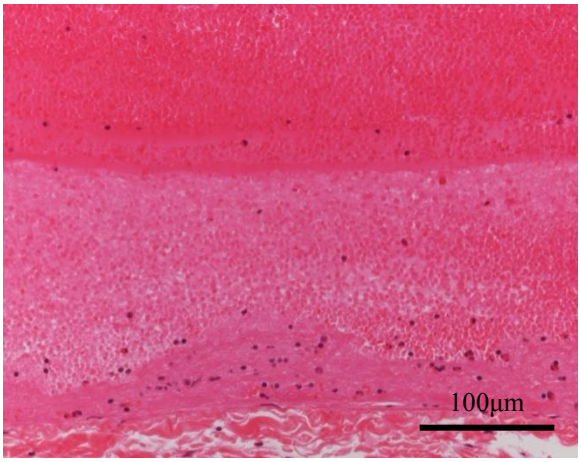
Saline





rFVIII





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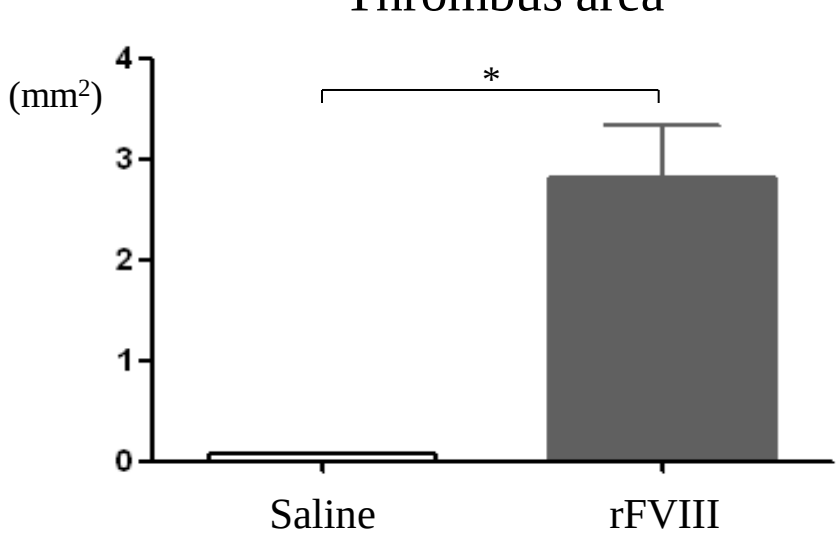


Fig. 4C

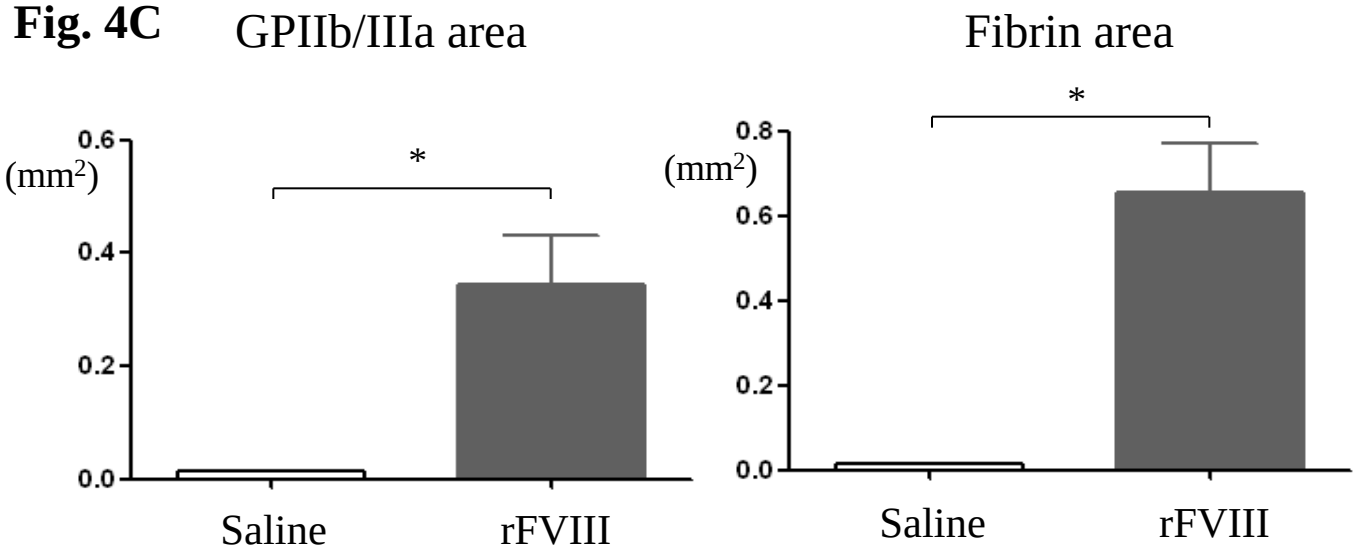


Fig. 4D

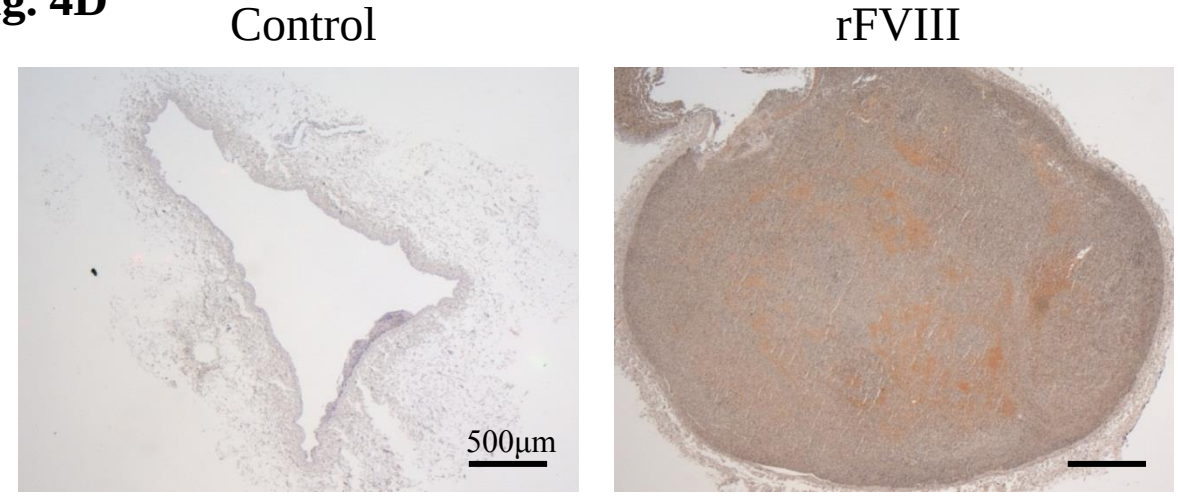


Fig. 5A

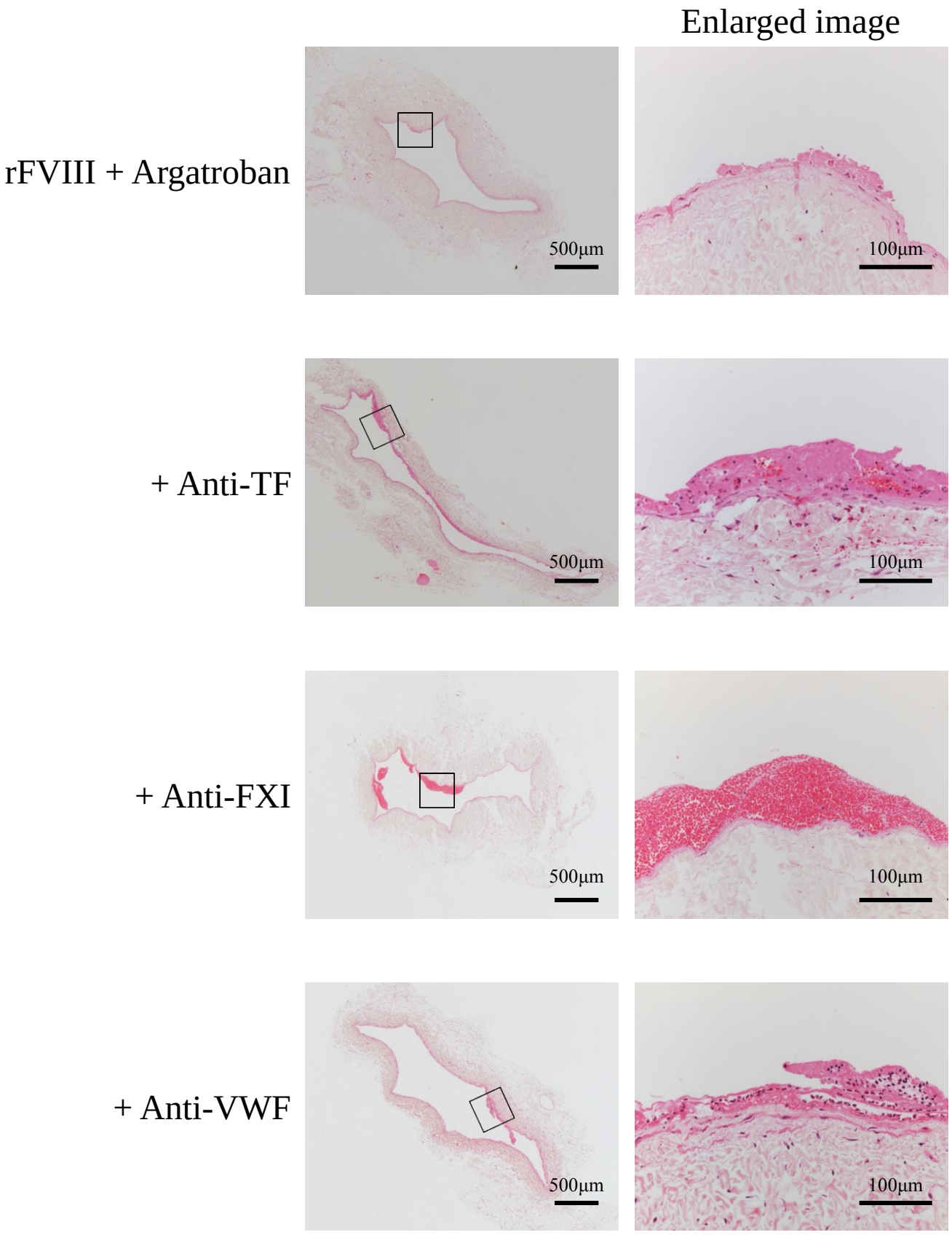

Enlarged image
rFVIII + Argatroban
500μm
100μm
+ Anti-TF
500μm
100μm
+ Anti-FXI
500μm
100μm
+ Anti-VWF
500μm
100μm

Fig. 5B

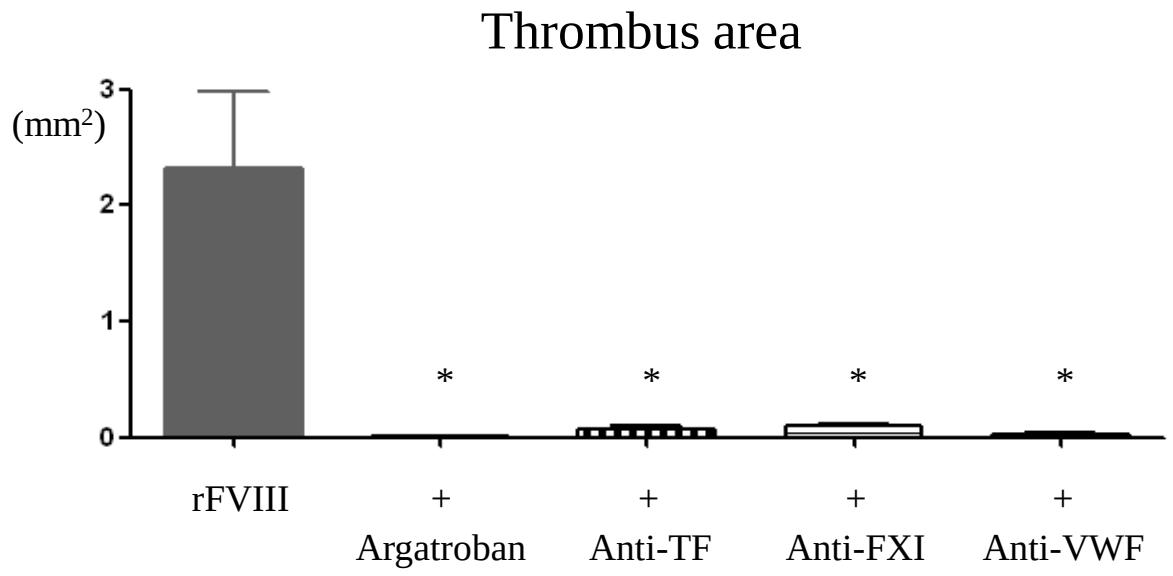
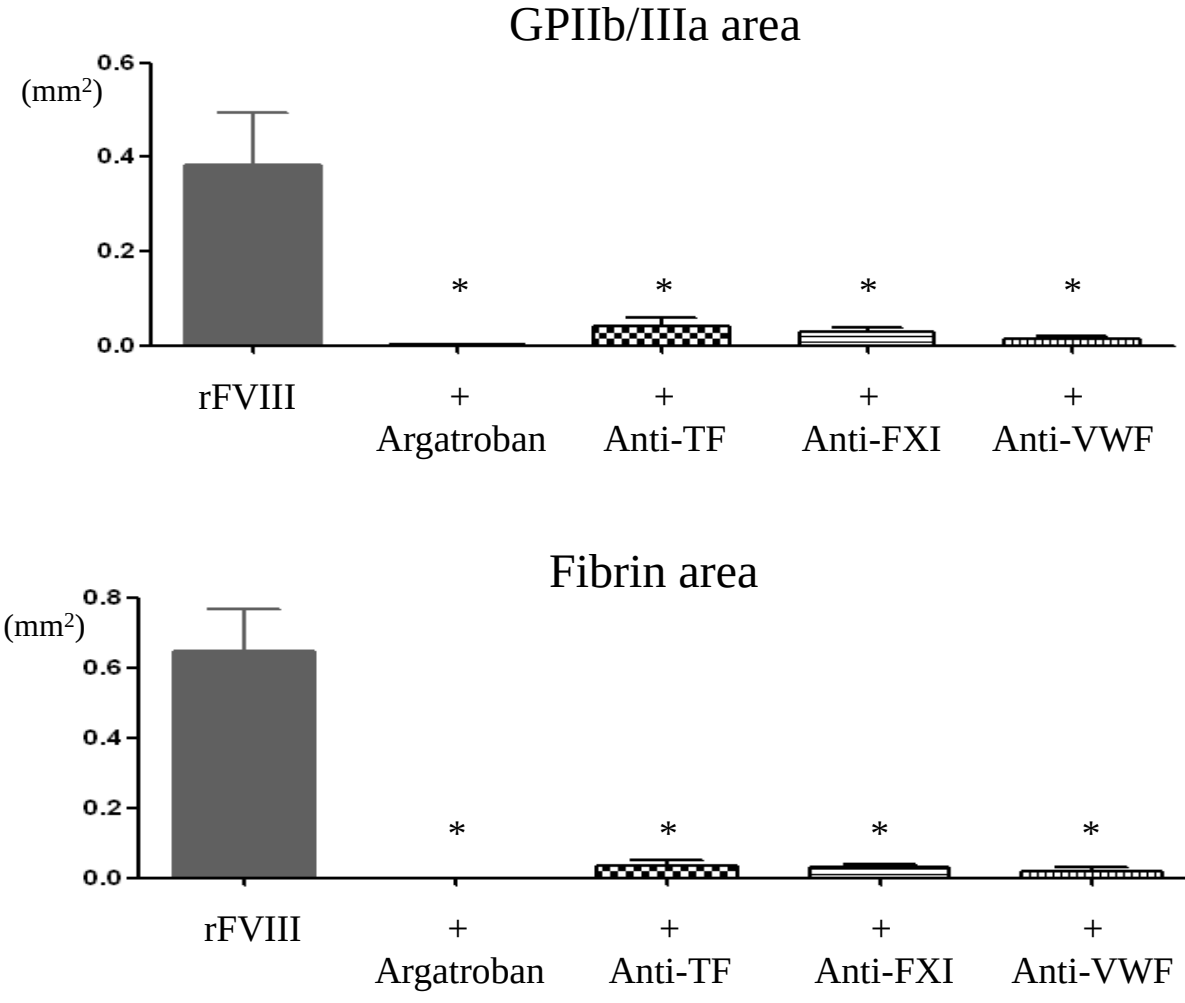

Thrombus area
(mm2)
3
2
1
0
*
*
*
*
rFVIII
+
Argatroban
+
Anti-TF
+
Anti-FXI
+
Anti-VWF

Fig. 5C


GPIIb/IIIa area
(mm2)
0.6
0.4
0.2
0.0
*
*
*
*
rFVIII
+
Argatroban
+
Anti-TF
+
Anti-FXI
+
Anti-VWF
Fibrin area
(mm2)
0.8
0.6
0.4
0.2
0.0
*
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*
rFVIII
+
Argatroban
+
Anti-TF
+
Anti-FXI
+
Anti-VWF

Fig. 6A

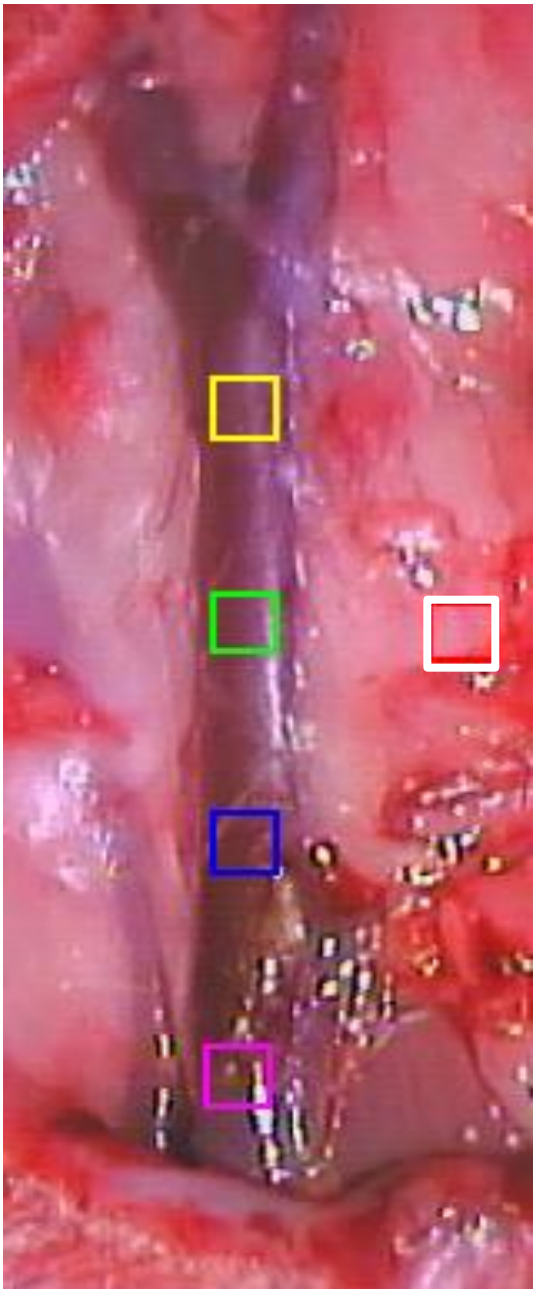


Fig. 6B

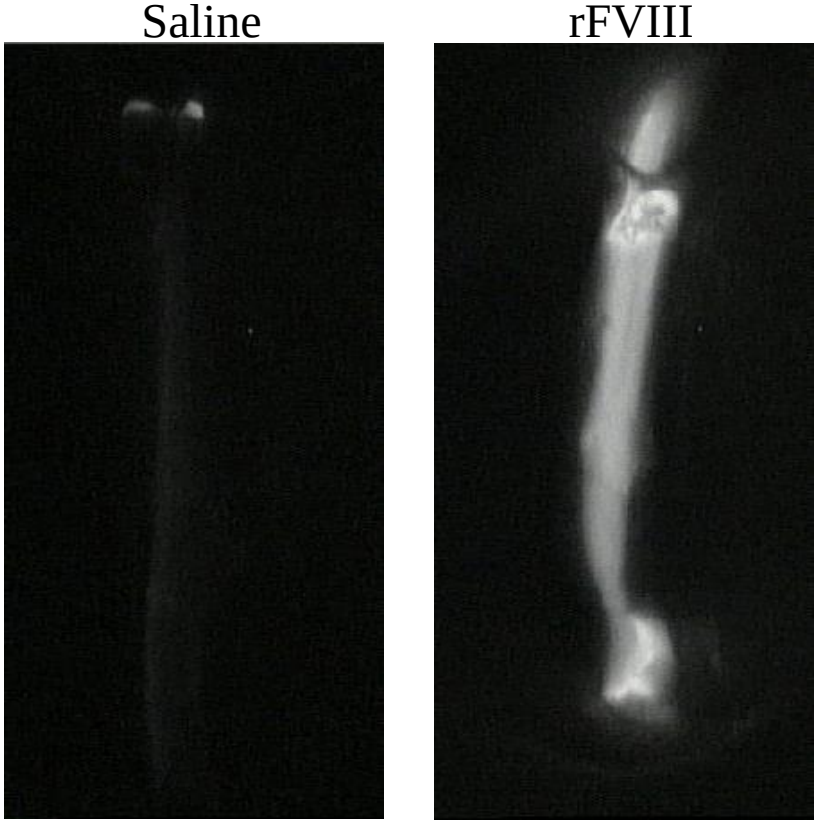

Saline
rFVIII

Fig. 6C

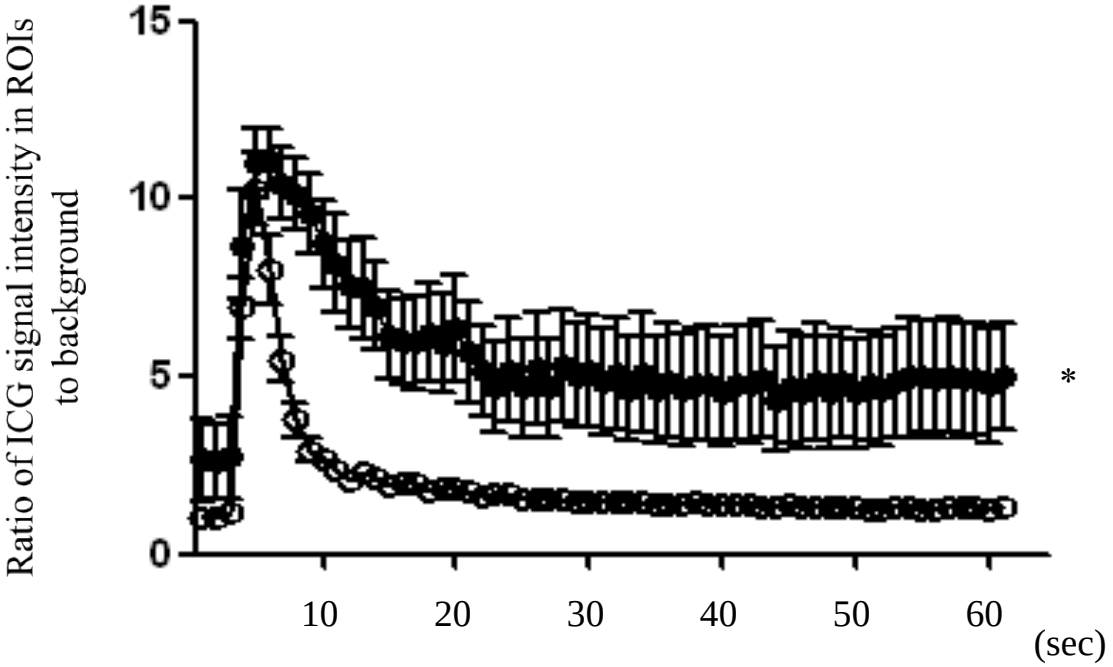
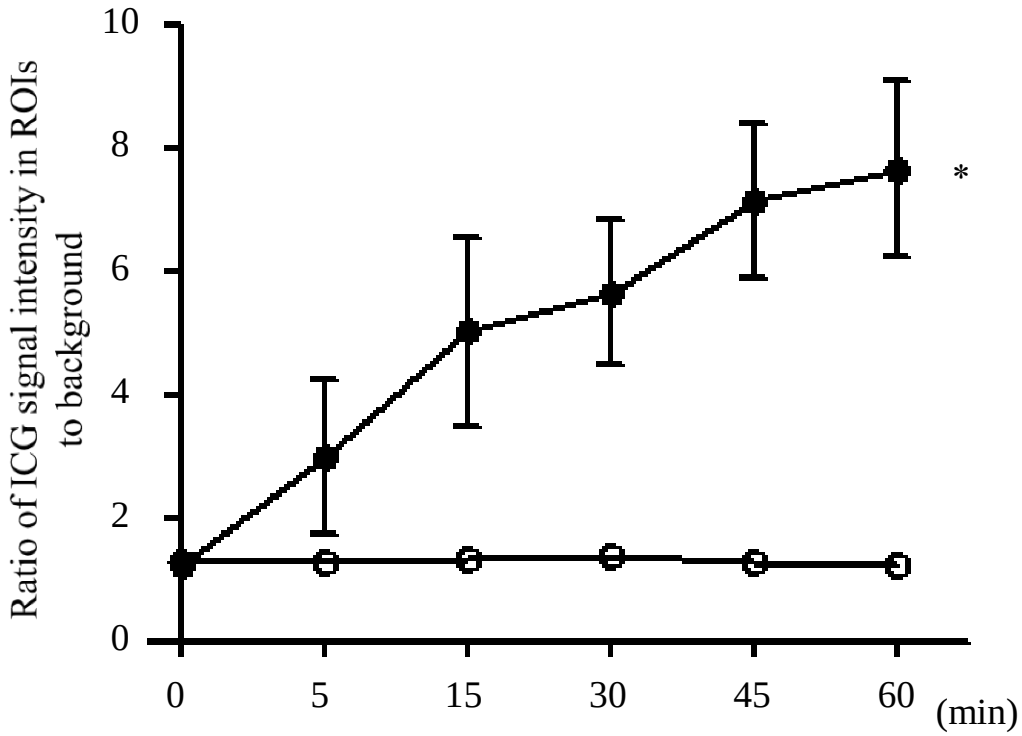

Ratio of ICG signal intensity in ROIs to background
15
10
5
0
10
20
30
40
50
60
(sec)
*

Fig. 7


Ratio of ICG signal intensity in ROIs to background
10
8
6
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2
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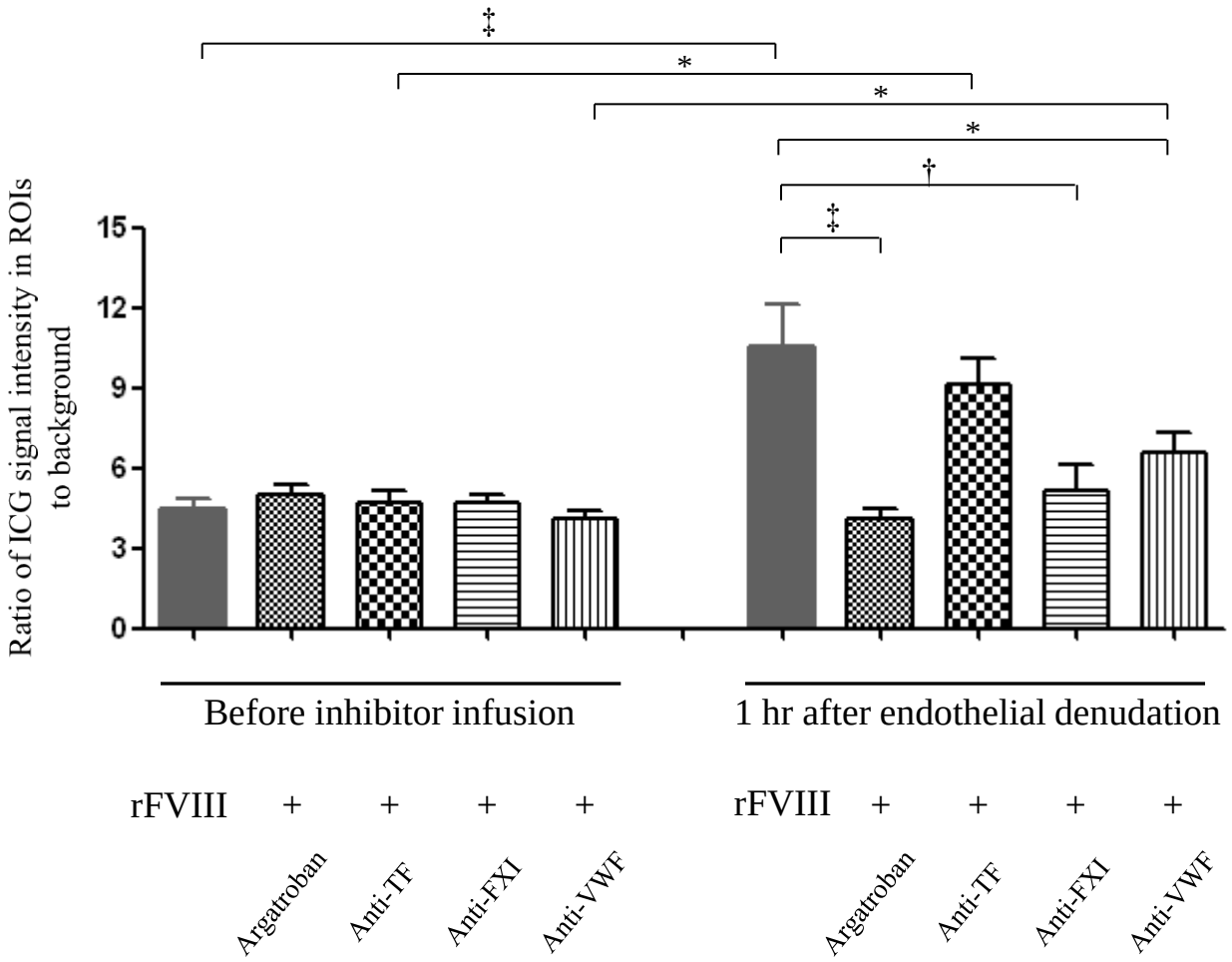
45
60
(min)
*

Fig. 8A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Ratio of ICG signal intensity in ROIs to background
15
12
9
6
3
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Before inhibitor infusion
1 hr after endothelial denudation
rFVIII + + + + rFVIII + + + +
Argatroban
Anti-TF
Anti-FXI
Anti-VWF
Argatroban
Anti-TF
Anti-FXI
Anti-VWF

Fig. 8B

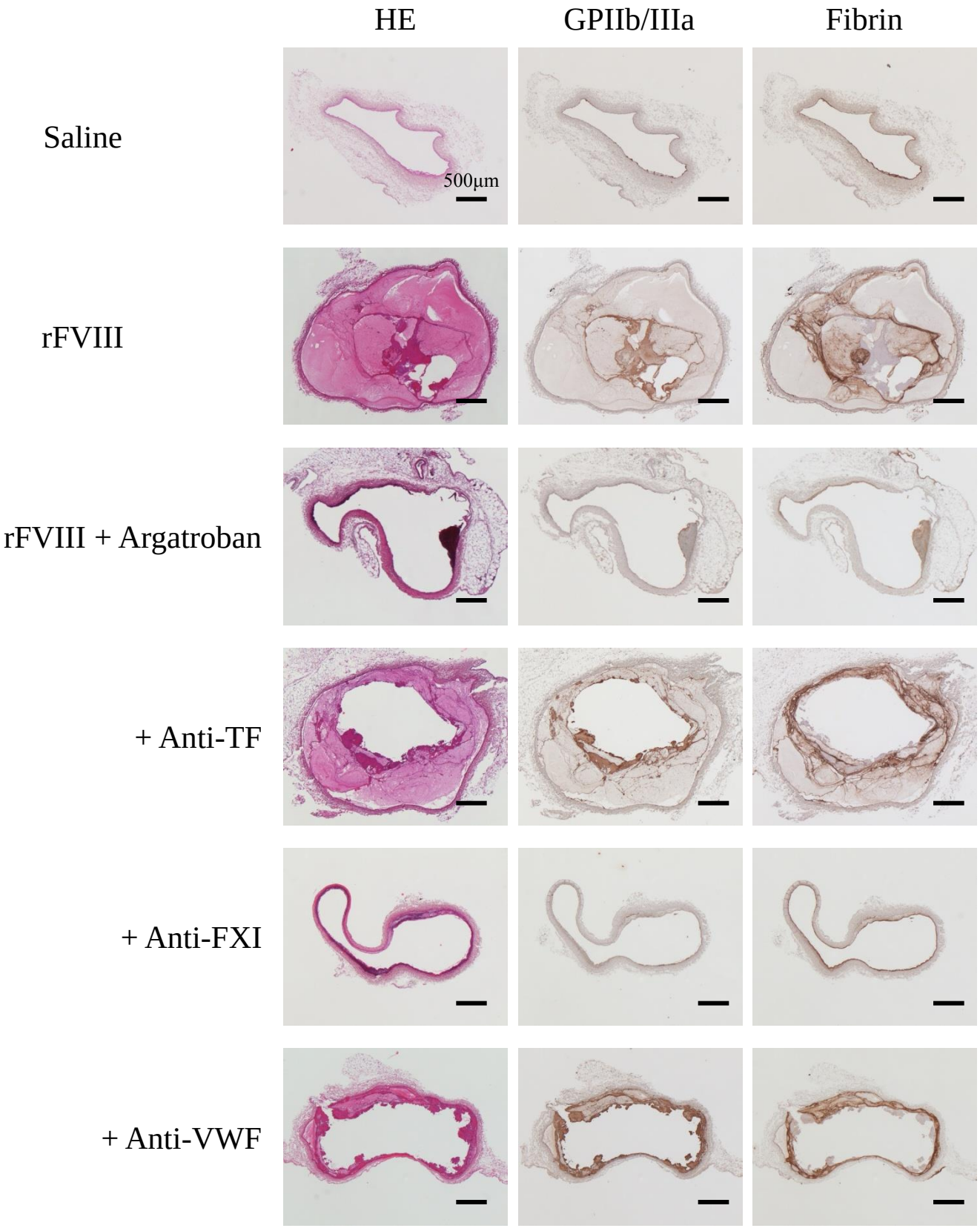
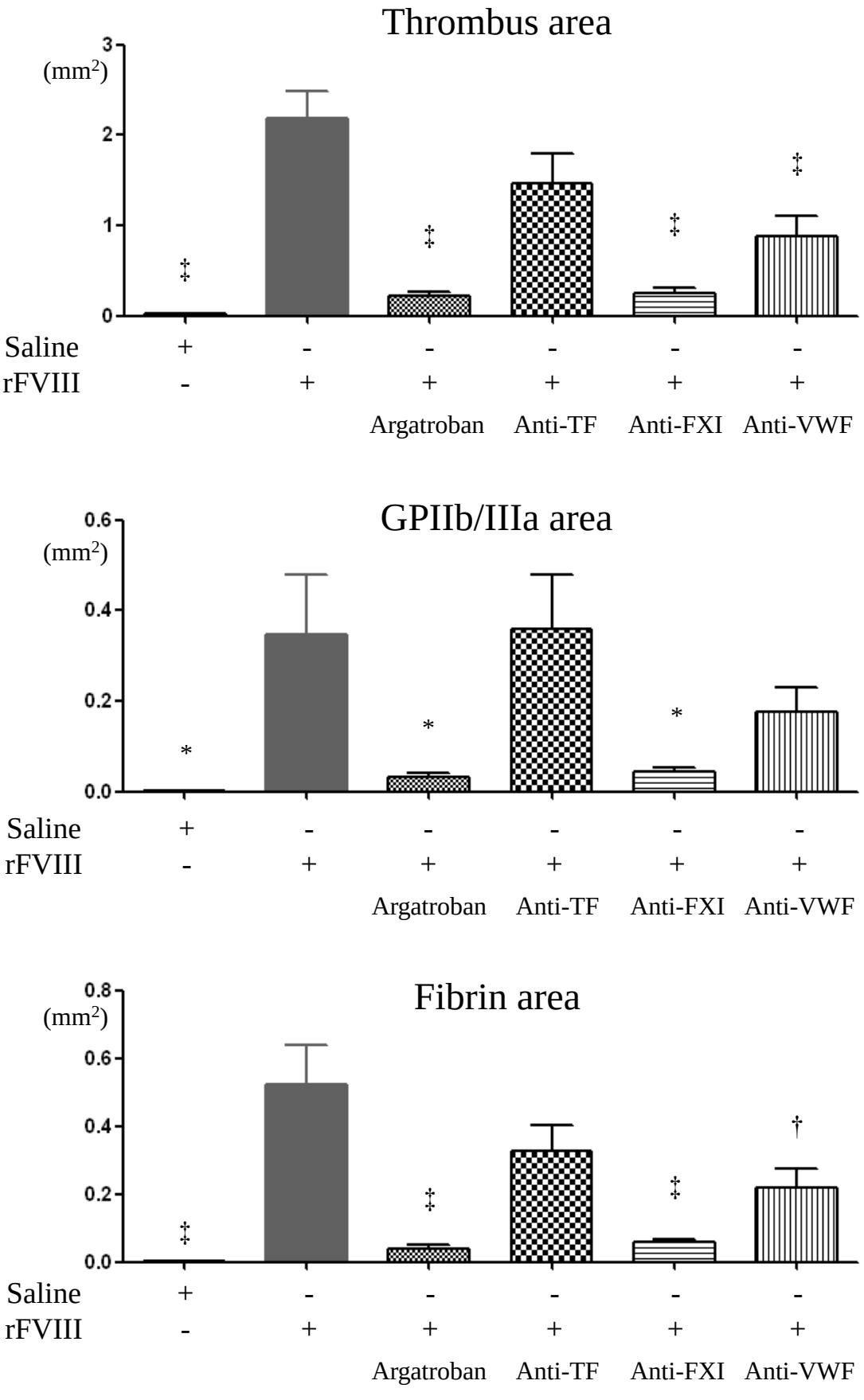
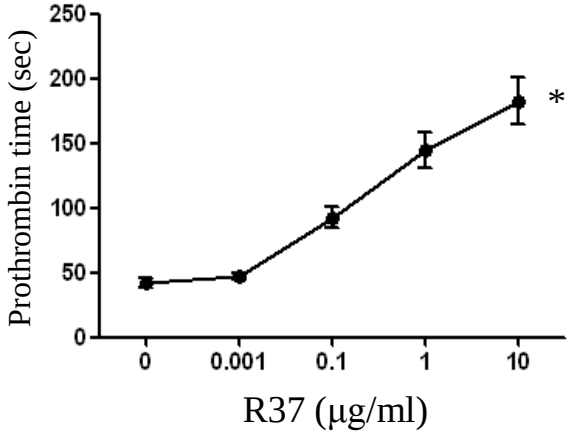

HE
GPIIb/IIIa
Fibrin
Saline
500μm
rFVIII
rFVIII + Argatroban
+ Anti-TF
+ Anti-FXI
+ Anti-VWF

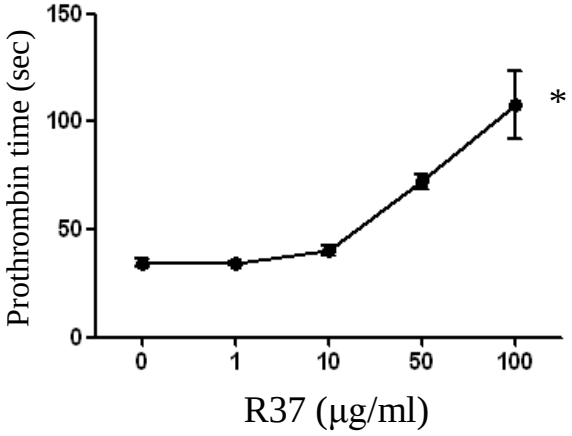
Fig. 8C


Thrombus area
(mm2)
3
2
1
0
‡
‡
‡
‡
Saline + - - - - -
rFVIII - + + + + +
Argatroban Anti-TF Anti-FXI Anti-VWF
GPIIb/IIIa area
(mm2)
0.6
0.4
0.2
0.0
*
*
*
Saline + - - - - -
rFVIII - + + + + +
Argatroban Anti-TF Anti-FXI Anti-VWF
Fibrin area
(mm2)
0.8
0.6
0.4
0.2
0.0
‡
‡
‡
†
Saline + - - - - -
rFVIII - + + + + +
Argatroban Anti-TF Anti-FXI Anti-VWF

Supplementary Figure 1



A



B