of 36 patients with severe HA, significant correlations between min2 and very low levels of FVIII:C were confirmed [4]. These results indicated that in some patients, the presence of trace amounts of FVIII mediated higher coagulation acceleration, characterized by the steeper slope, although it was possible that factors other than FVIII:C alone may have influenced clotting kinetics reflected in the waveform profile. Nevertheless, the data suggested that CWA could discriminate between different levels of FVIII:C in this critical category of severe HA, defined as having < 1.0 IU dL^{-1} FVIII:C by conventional assays (Figs S3 and S4). The evidence suggests that CWA can provide more specific data on global hemostasis in such patients, which could correlate better with the clinical phenotype.

Correlation between clinical severity and CWA parameters

Some HA patients, classified as severe on the basis of standard coagulation assays, exhibit milder clinical symptoms. It appeared possible, therefore, that CWA might provide valuable data for evaluating *in vivo* clotting function in various types of hemophilia A. To investigate this possibility, severe hemophilia A patients based on < 1 IU dL^{-1} of FVIII:C were divided into clinically severe and non- severe groups [4]. Clinically severe patients were characterized by the presence of spontaneous bleeding episodes at the age of < 1 year, the onset of joint or muscular bleeding before the age of 3 years old, or the presence of severe bleeding such as intracranial bleeding or refractory oral bleeding. The differences between the severe and the non-severe phenotype were significant for four CW parameters: clot time, maximal coagulation velocity (Min1), maximal coagulation acceleration (Min2) and maximal coagulation deceleration (Max2), These results strongly suggested, therefore, that CW parameters reflect clinical severity (Fig. S5).

Monitoring hemostatic therapy in the patients with inhibitors

The hemostatic benefits of various agents used for bypassing therapy, including activated prothrombin complex concentrates (APCC) and recombinant factor VIIa (rFVIIa), can be monitored by CWA [4,5]. In addition, CWA was also utilized effectively in a recent clinical phase 1 study for the assessment of a new bypassing agent based on mixtures of plasma-derived FVIIa and X [6]. In two hemophilia A patients with high responding inhibitors, CWA demonstrated improved hemostasis. Moreover, CWA was shown to reflect the prophylactic effect of regular infusions of FVIII during immune tolerance induction therapy (ITI) [7]. The findings confirmed that CWA is very sensitive to low levels of clotting factors, and suggested that the technique could also be useful for monitoring therapy using FVIII or FIX concentrates in patients with inhibitor.

Clotting function of acquired hemophilia

FVIII:C levels do not reflect clinical severity in many cases of acquired hemophilia A, and it may be difficult to determine clotting function precisely in these patients. CWA illustrates severely impaired patterns in these cases, however, characterized by a remarkably prolonged pre-coagulation phase and low values for maximum coagulation velocity and acceleration [8]. Assessment of clotting function by aPTT CWA, in addition to the measurement of FVIII activity, can be useful, therefore, to confirm decisions on hemostatic treatment and the monitoring of bypass therapy in these complicated clinical circumstances.

Advantages and limitations

There are several advantages to the use of CWA. The method has broad utility as a simple global test of hemostasis and is capable of providing sensitive, quantitative parameters as well as qualitative waveform patterns. Furthermore, CWA can be usefully applied in various difficult clinical settings. Not all current coagulation analyzers can be used for CWA, however, although the number of appropriate analyzers is increasing. Finally, the CWA is based on APTT-based coagulation mechanisms using an 'intrinsic' trigger. A modified CWA using trace amounts of tissue factor may extend the application of this technique.

Among the global hemostasis tests, CWA is perhaps the simplest to establish and standardize. It therefore needs to be tested more widely using standardized methods in different clinical situations to decide its place in the assessment of hemostasis and its disorders.

Addendum

M. Shima chaired the working party, performed the research, analyzed the data and wrote the manuscript. J. Thachil and S.C. Nair performed research and collected data. A. Srivastava supervised the study.

Disclosure of Conflict of Interests

M. Shima is supported for APTT reagents from Sysmex.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Data sheet of the transmittance and presentation by waveform.

Figure S2. APTT clot waveforms of various clotting factor deficiencies.
Figure S3. Dose-dependent waveform changes in plasma containing various concentrations of FVIII.
Figure S4. Waveform changes in hemophilia A with various levels of FVIII.
Figure S5. CWA parameters and clinical severity of severe hemophilia A.
Table S1. Coagulation analyzers and APTT reagents for clot waveform analysis.

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Simultaneous measurement of thrombin and plasmin generation to assess the interplay between coagulation and fibrinolysis

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Summary
Normal haemostasis is maintained by a controlled balance between coagulation and fibrinolysis, involving thrombin and plasmin the respective key enzymes. Simultaneous evaluation of both enzymes facilitates, therefore, an overall understanding of normal and pathological haemostasis. Combined thrombin and plasmin generation (T/P-G) assays have been recently described, and we have adapted the technique to investigate the interplay between coagulation and fibrinolysis in patients with various haemostatic disorders. Our modified T/P-G was initiated by the addition of a mixture of optimised lower concentrations of tissue factor and tissue-type plasminogen activator. Thrombin generation (TG) and plasmin generation (PG) were monitored simultaneously using individual fluorescent substrates in separate microtitre wells. The relationship between coagulation and fibrinolysis was demonstrated by analysing the effects of thrombin inhibitors, activated protein C and thrombomodulin. The most evident impairments in TG were observed with plasma samples deficient of coagulation factors participating in the prothrombinase complex. Defects in PG were observed with deficiencies of factor (F)V, FX, fibrinogen, and plasminogen. TG appeared to be a prerequisite for the initiation of PG, and overall PG was governed by fibrinogen concentration. TG in patients with haemophilia A correlated with levels of FVIII activity, but there was no significant relationship between PG and FVIII:C, confirming that the abnormal haemostasis in haemophilia A results in a severe imbalance between coagulation and fibrinolysis. The findings demonstrate that global haemostasis depends on a sensitive balance between coagulation and fibrinolysis, and that the modified T/P-G assay could provide an enhanced understanding of haemorrhage and thrombosis in clinical practice.

Keywords
Thrombin generation, plasmin generation, coagulation, fibrinolysis, global haemostatic assay

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Introduction

Normal haemostasis is maintained by a controlled balance of pro- or anti-coagulation and fibrinolysis. Abnormalities of these mechanisms lead to various clinical symptoms of haemorrhage, thrombosis, or both. Many laboratory techniques have been devised to evaluate haemostasis, using separate assays for the measurement of the activities of individual coagulation factors. Thrombin and plasmin are the key enzymes involved in these processes, however, and in order to understand overall haemostatic function, simultaneous assessment of the kinetics of thrombin and plasmin generation could offer significant advantages.

Other assays of global haemostasis, such as thromboelastography (TEG) (1) and the thrombin generation assay (TGA) (2) are known to be useful methods that reflect the individual process of coagulation and/or fibrinolysis. TEG evaluates both blood coagulation and subsequent clot lysis in whole blood by measuring viscoelastic changes in fibrin polymerisation. With this method, however, it is difficult to clearly identify the individual phases of coagulation and fibrinolysis. In addition, samples of whole blood are required within a very restricted time after venepuncture. TGAs can be performed using platelet-rich plasma (3), whole blood (4) or platelet-poor plasma, and the thrombogram parameters reflect global coagulation capacity. Enhanced thrombin generation suggests a tendency towards thrombosis (5, 6), whilst depressed thrombin generation is evident in patients with a bleeding tendency such as haemophilia A and B (7, 8). The TGA, however, is not significantly affected by fibrinolytic activity, and appears unlikely to reflect overall haemostasis *in vivo*.

In recent years, global assays of coagulation and/or fibrinolysis that evaluate coagulation capacity (thrombin generation; TG), fibrinolytic ability (plasmin generation; PG), or both, have been developed and utilised (4, 9, 10). More recently, simultaneous assays of thrombin and plasmin generation, using trigger reagents con-

taining mixtures of tissue factor (TF) and tissue-type plasminogen activator (tPA), have been reported by two groups (11, 12). The assay described by Simpson et al. (11), termed the STP-assay (simultaneous thrombin and plasmin generation), measures TG and PG in separate microtitre wells. Van Geffen et al. (12) utilised single fluorometer wells in their novel haemostasis assay (NHA). The concentrations of TF and tPA used as trigger in the NHA were less than those in the STP-assay, and both assays provided simultaneous assessment of TG and PG kinetics. The NHA, however, appeared to demonstrate the interplay between coagulation and fibrinolysis more clearly than the STP-assay (12). In the present study, we have established a simultaneous thrombin and plasmin generation (T/P-G) assay in separate wells similar to the STP-assay, using optimised, low concentrations of TF and tPA in order to retain sensitivity to the haemostatic interactions.

Materials and methods

Reagents

Recombinant human TF (TF; Innovin®, Dade, Marburg, Germany), recombinant tissue-type plasminogen activator (tPA; American Diagnostica Inc., Stamford, CT, USA), thrombin-specific fluorogenic substrate (Z-Gly-Gly-Arg-AMC, Bachem, Bubendorf, Switzerland), plasmin-specific fluorogenic substrate (BOC-Glu-Lys-Lys-MAC, Peptide Institute Inc., Osaka, Japan), carboxypeptidase inhibitor (CPI; Calbiochem, San Diego, CA, USA), recombinant thrombomodulin (TM, Recomodulin®, Asahi-Kasei Phama Corp., Tokyo, Japan), argatroban (Slonnon®, Daiichi Sankyo Co. Ltd., Tokyo, Japan) were obtained from the indicated vendors. Plasma samples deficient of factor (F)II, FV, FVII, FVIII, FIX, FX, FXII, and FXIII were obtained from George King Inc. (Overland Park, KS, USA). Plasma samples deficient of fibrinogen, plasminogen, and α2-plasmin inhibitor (α2-PI) were purchased from Affinity Biologicals Inc. (Ancaster, ON, Canada). Fibrinogen, FV, FX, plasminogen and plasmin (Hematologic Technologies, Burlington, VT, USA) and α-thrombin (Sigma-Aldrich, St. Louis, MO, USA) were obtained from the indicated vendors. Phospholipid (PL) vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, 30% phosphatidylethanolamine (Sigma-Aldrich) were prepared as previously described (13).

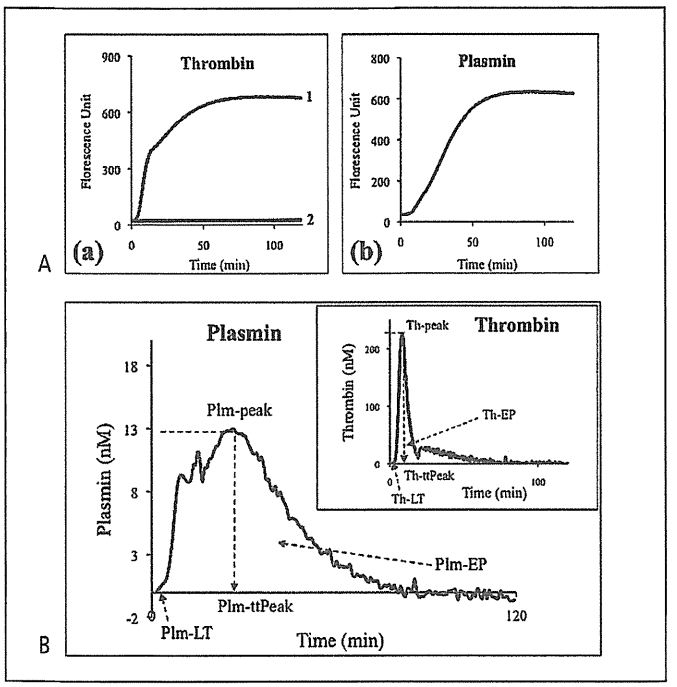

Figure 1: Simultaneous T/P-G assay. A) Mixtures of TF, tPA, and PL (f.c. 1 pM, 3.3 nM, and 4 □M) were added to normal plasma prior to evaluating TG and PG as described in *Methods*. Representative curves of TG (a) and PG (b) are illustrated. Each curve of TG obtained by trigger reagent or by EDTA alone shows line 1 or 2 in panel (a), respectively. B) First derivative (velocity) of TG (inset) and PG obtained in (A). LT: lag time, Peak: peak thrombin or peak plasmin, ttPeak: time to peak, EP: endogenous potential of thrombin or plasmin.

Fresh plasma samples

Normal plasma was prepared from 20 normal healthy individuals. Blood was drawn into evacuated anticoagulant tubes (blood: 3.8% (w/v) trisodium citrate; 9:1). After centrifugation for 15 minutes (min) at 1,500 g, the plasmas were stored at -80°C, and thawed at 37°C immediately prior to the assays. Patients' plasmas were obtained from 20 haemophilia A patients. FVIII:C levels in 12 patients were <0.2 IU/dl (severe type), and in eight patients were 1.0-5.0 IU/dl (moderate type). FVIII:C levels were measured by conventional one-stage clotting assay. All blood samples were obtained after informed consent following local ethical guidelines during routine follow-up of patients in the Nara Medical University Haemophilia Program.

Simultaneous thrombin and plasmin generation (T/P-G) assay

The T/P-G assay was established using a modification of the method reported by Simpson et al. (11). Briefly, trigger reagents (TF, PL, and tPA) were mixed in 20 mM HEPES, pH 7.2, 150 mM NaCl and 0.01% Tween 20, at concentrations of 6.5 pM TF, 26 μM PL, and 21 nM tPA. Final concentrations of reactants in plasma samples were 1 pM TF, 4 μM PL, and 3.3 nM tPA. The assay blank contained 77 mM EDTA, to evaluate the non-specific effects of thrombin that did not play a role in coagulation reactions in this assay (e.g. complexed with α2-macroglobulin). Two fluorometric specific substrates, Z-Gly-Gly-Arg-AMC and Boc-Glu-Lys-Lys-MCA, were used for the detection of the key enzymes, thrombin and plasmin, respectively. The final concentration of each substrate was 100 μM. Plasma samples (80 μl) were added to wells in flat-bottom, black polystyrene (Nunc; Thermo Scientific, Waltham, MA, USA), 96-well plates, and six wells were dedicated to each sample. Twenty μl of the trigger reagent (TF/PL/tPA) were added to four plasma wells, and equivalent volume of EDTA, instead of TF/PL/tPA, was added to the other two wells. And then two duplicated plasma wells were used to measure thrombin generation (TG) using thrombin substrate and plasmin generation

Table 1: Parameters of simultaneous T/P-G assay in normal plasmas (n=20).

Parameters	TG				PG			
	25%	Median	75%	Min-max	25%	Median	75%	Min-max
LT (min)	2.1	3.0	4.1	1.7–5.2	3.0	3.7	4.1	2.3–6.7
Peak (nM)	149	193	245	84–284	12.2	14.1	15.2	9.5–16.6
ttPeak (min)	7.5	8.2	9.0	6.8–11.2	21.0	22.5	23.2	12.8–29.2
EP (nM*min)	3,071	3,207	3,420	2,705–3,560	568	590	608	515–688

(PG) using plasmin substrate. The other two wells were used as EDTA blank wells. The thrombin and plasmin assay wells were kept separate to avoid concerns of potential interference and interactions with signal detection. The microtitre plates were placed in the fluorometer and allowed to warm to 37°C for 10 min. Following the addition of reactant solution, 10 µl of appropriate fluorometric substrate was added to each well, and thrombin substrate (10 µl) was added to EDTA blank wells. The dispenser of the fluorometer was flushed with 20 µl warmed 100 mM $CaCl_2$, and then 20 µl of $CaCl_2$ was added to all assay wells. The development of the fluorescent signal was monitored at 45-second intervals over a period of 2 hours using a Fluoroskan Ascent microplate reader (Thermo Electron Co., Waltham, MA) with a 390 nm (excitation) and 460 nm (emission) filter set. Standard curves were prepared using serially diluted purified α-thrombin and plasmin as previously described by Hemker et al. (14). Since the curve of TG obtained from EDTA blank wells (*line* 2) was regarded as non-specific reaction of thrombin, these data was subtracted from those of TG obtained from trigger-induced coagulation (*line* 1 in ▶Figure 1A-a). Data analyses were performed using excel software. From the first derivative (velocity) of thrombin and plasmin generation obtained above, the parameters; lagtime (LT), endogenous potential (EP), peak levels (Peak), and time to peak (ttPeak), were recorded.

Data analyses

Measurements were obtained in several separate assays as indicated, and the mean ± standard deviation are shown.

Results

The modified T/P-G assay with normal plasmas

The aim of the present study was to establish a simultaneous T/P-G assay in separate microtitre wells that would permit assessment of the interplay between coagulation and fibrinolytic mechanisms. For this purpose, we modified the STP methodology that

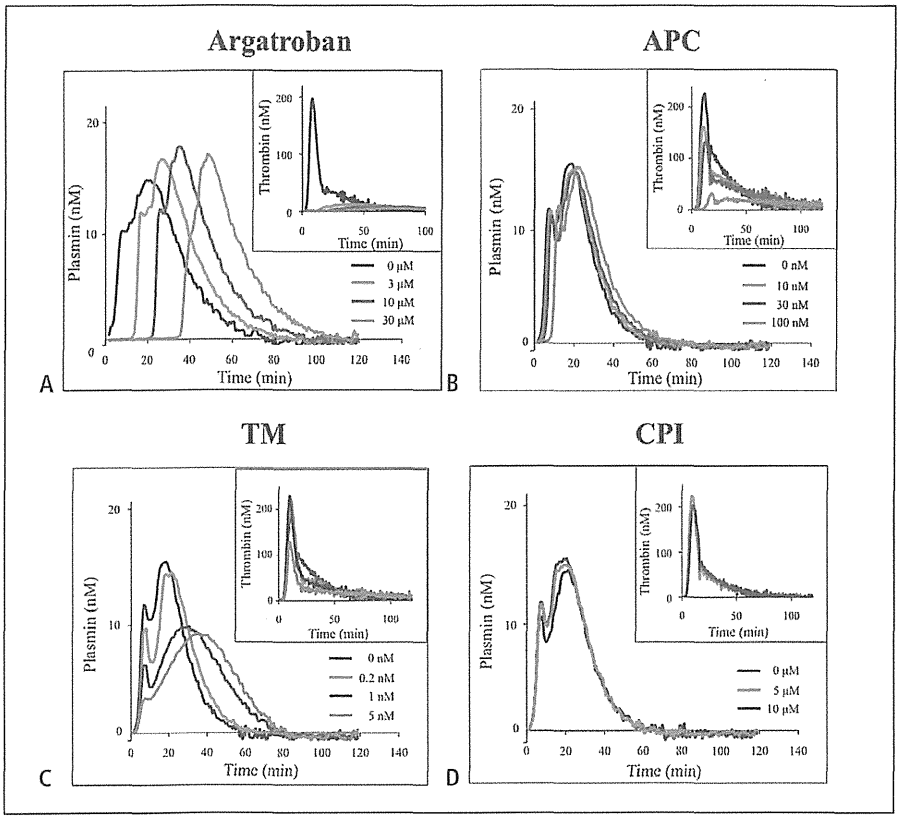

Figure 2: Effects of various coagulant inhibitors on simultaneous T/P-G. Normal plasma was incubated with various amounts of argatroban (panel A; 0–30 □M), APC (panel B; 0–100 nM), TM (panel C; 0–5 nM), and CPI (panel D; 0–10 □M), prior to measurements of TG (inset) and PG as described in *Methods*. Representative curves of TG and PG are illustrated.

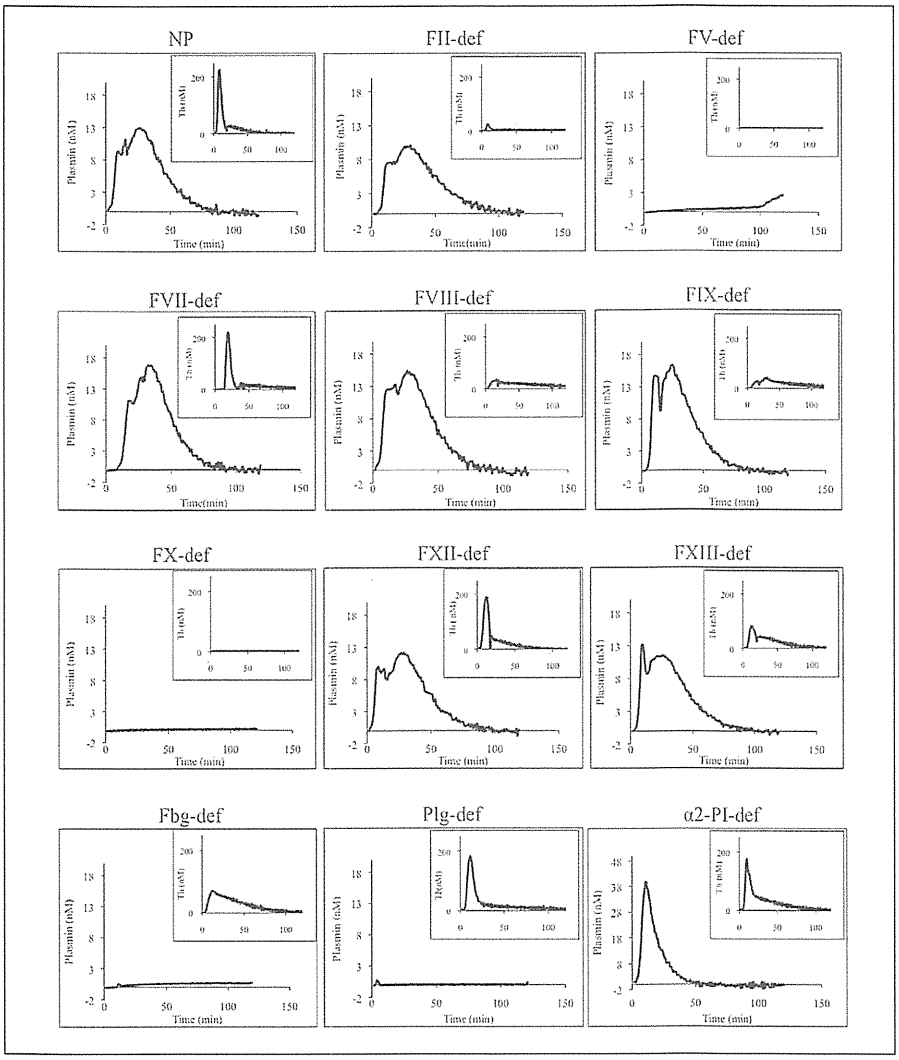

Figure 3: T/P-G with individual factor-deficient plasmas. Individual coagulant factor-deficient plasmas were incubated with trigger reagents, prior to measurements of TG (inset) and PG as described in *Methods*. Normal plasma (NP) was used as control. Representative curves for TG and PG are illustrated.

had been previously reported by Simpson et al. (11). A major difference between the STP and NHA (12) was the concentrations of reagents in the trigger mixture (TF and tPA), and it appeared that this might be critical for this assay. The effects of various concentrations of TF and tPA were examined, therefore, as described in *Methods*. Optimal conditions for low concentrations of trigger reagents similar to those used in the NHA were determined. TF and tPA increased both the TG and PG in dose-dependent manners, and the optimal concentrations determined by LT, Peak, and ttPeak of thrombin and plasmin generation were 1 pM for TF and 3.3 nM for tPA, respectively (data not shown). The concentration of TF and tPA was intermediate and less, respectively, compared to those used in the NHA (TF/tPA: 0.28 pM/5.4 nM [12]) and in the STP (TF/tPA: 5 pM/7.5 nM [11]). The individual curves for TG and PG obtained using normal plasma under these conditions are shown in ▶ Figure 1A. The first derivative data from the raw curves demonstrating thrombin and plasmin generation (T/P-G) are illustrated in ▶ Figure 1B. The four parameters derived from the TG and PG are summarised in ▶ Table 1.

Intra-assay variation was determined from eight separate assays, and demonstrated a similar coefficient of variation (CV) for the TG (0.75-7.8%) and the PG parameters (1.1-7.0%). In addition, inter-assay variation was examined using a single sample per day measured over seven different days. The CV in these experiments ranged between 3.7-17.6% (TG) and 6.6-15.7% (PG), respectively (▶ Table 2). These results were similar to those reported previously (11, 12).

Table 2: Intra- and inter-assay coefficient variation (CV).

	Intra-assay CV		Inter-assay CV	
	TG	PG	TG	PG
	%		%	
LT (min)	7.8	7.0	17.6	15.7
Peak (nM)	6.0	1.9	9.7	10.2
ttPeak (min)	3.9	4.8	7.2	6.6
EP (nM*min)	0.75	1.1	3.7	9.1

The intra-assay variation experiments were evaluated from eight times samples. The inter-assay was calculated using a single sample per day measured over 7 different days.

Interplay between coagulation and fibrinolysis in the modified T/P-G

To investigate whether the modified T/P-G assay reflected the sequential interplay of coagulation (represented by the TG) and fibrinolysis (represented by the PG), generation of the key enzymes was evaluated in the presence of argatroban (a thrombin-specific inhibitor), activated protein C (APC), and thrombomodulin (TM). It was anticipated that these agents could induce different responses in coagulation and fibrinolysis. Normal plasma was incubated with increasing concentrations of argatroban, APC, and TM, prior to the T/P-G measurements. The addition of argatroban resulted in a significant impairment in TG in a dose-dependent manner, and demonstrated a >90% decrease in peak thrombin at a concentration of 10 μM (therapeutic blood level). In contrast, the lagtime in PG as well as in TG was prolonged, but the peak level of plasmin was elevated in the presence of up to 10 μM argatroban and reached a plateau at >10 μM (▶Figure 2A). The effects of APC and TM, known to be central in anti-coagulant mechanisms, were different from those of argatroban. The addition of APC (0-100 nM, ▶Figure 2B) and TM (0-5 nM, ▶Figure 2C) decreased the coagulation capacity in TG in similar dose-dependent manners. Moreover, the addition of TM also decreased the PG dose-dependently, with an ~50% level of peak plasmin at a concentration of 1 nM. The addition of APC, however, resulted in little decrease in PG. The findings showed that TG was depressed by all three agents. PG was delayed by TM but was enhanced by argatroban, the thrombin inhibitor. Our assay seemed to be particularly sensitive, therefore, to thrombin activatable fibrinolysis inhibitor (TAFI)-mediated effects (15). Furthermore, the addition of CPI, a competitive inhibitor of TAFI, mediated a slight dose-dependent increase in PG (▶Figure 2D).

Evaluation of individual factor-deficient plasma samples in the modified T/P-G assay

To investigate the role of individual coagulation factors in the interaction between coagulation and fibrinolysis, we examined individual, specific factor-deficient plasmas (-def) in the modified T/P-G assay. ▶Figure 3 illustrates representative curves from TG (*inset*) and PG with these plasma samples. In the TG assays with FVII-def, the lag-time alone was prolonged. With FVIII-def and FIX-def (components in the tenase assembly), the levels of peak thrombin were significantly diminished as previously reported (16). A mild reduction in peak thrombin was observed with FXIII-def and fibrinogen-def. With common pathway-related components, however, especially FV-def and FX-def (relevant to prothrombinase assembly), and with FII-def very little TG was evident. Similarly, there appeared to be very little PG with FV-def and FX-def. PG was modestly decreased with FII-def compared to normal plasma, but it was of interest that the capacity for PG was much greater than TG with this specific deficient plasma. PG with fibrinogen-def was markedly impaired, confirming the importance of fibrin formation as a scaffold to drive PG. With the other coagulant deficient-plasmas PG was equal to that with normal plasma. There was no PG with plasminogen-def, whereas with α2-PI-def, PG was enhanced by ~3-fold compared with normal plasma.

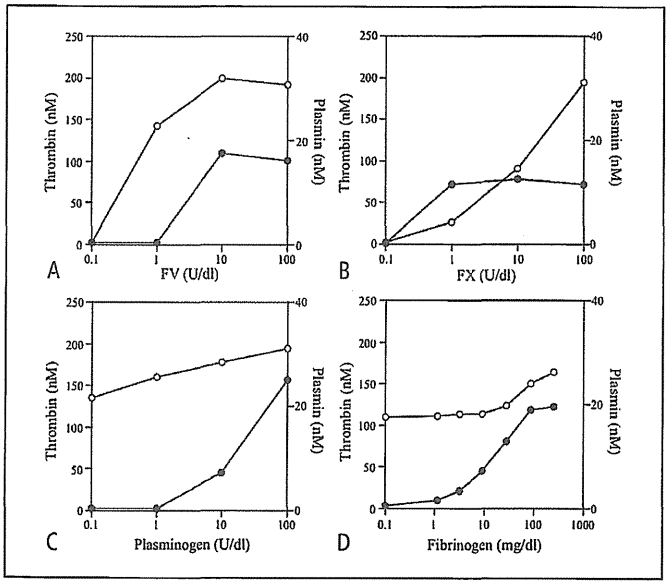

Figure 4: Effects of purified individual factors on T/P-G assays. Various amounts of purified coagulant proteins FV (panel A), FX (panel B), plasminogen (panel C), or fibrinogen (panel D) were added to the respective factor-deficient plasma, prior to measurements of TG (□) and PG (□) as described in *Methods*. The parameters (peak thrombin and peak plasmin) were plotted as functions of protein concentrations.

Effects of individual coagulant factors in the modified T/P-G assay

The experiments described above indicated that the common pathway-related factors (FV and FX), fibrinogen, and plasminogen uniquely influenced TG and PG. These effects were further examined, therefore, by the addition of purified exogenous coagulation factors to the respective deficient plasma. The addition of various amounts of FV and FX to FV-def and FX-def respectively, resulted in significant improvements in both TG and PG (▶Figure 4A, B), suggesting that these factors were essential to both mechanisms. However, 1 U/dl of FX possessed the same potential capacity for PG as 100 U/dl, whilst FV at 1 U/dl level did not mediate PG. It seemed possible, therefore, that the influence of FV on PG was greater than that of FX. With plasminogen-def, the addition of purified plasminogen increased the TG as well as PG dose-dependently, and it appeared that PG exerted a somewhat positive effect on TG (▶Figure 4C). This finding tended to confirm the close inter-relationship between coagulation and fibrinolysis. Interestingly, the addition of small amounts of fibrinogen (10 mg/dl) induced little change in TG (▶Figure 4D) but significant improvements in PG, demonstrating that small amounts of generated fibrin are likely to be sufficient for PG.

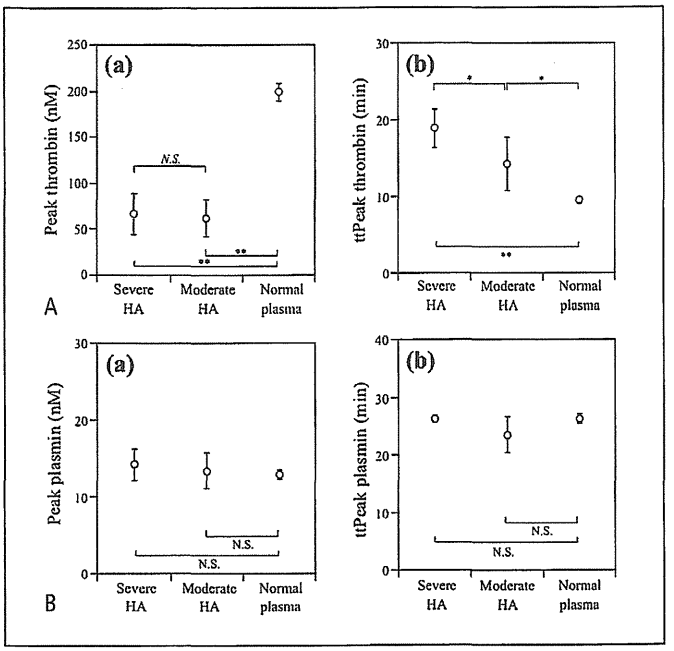

Figure 5: T/P-G in plasmas from patients with various phenotypes of haemophilia A. The trigger reagents were added to haemophilia A plasma (severe or moderate type) or normal plasma prior to evaluating TG (A) and PG (B) as described in *Methods*. The peak level (a) and ttPeak (b) of thrombin or plasmin were derived from the T/P-G data obtained. In all instances, results are shown as mean ± SD. HA: haemophilia A. *; p<0.05, **; p<0.01, N.S.; not significant.

Simultaneous T/P-G in patients with haemophilia A

The balance between coagulation and fibrinolysis in relation to levels of FVIII activity was examined in haemophilia A patients with various phenotypes. The ttPeak parameter in TG was significantly different among severe type (<0.2 U/dl), moderate type (1-5 U/dl), and normal plasma, but the peak thrombin in severe type was similar to that in moderate type (▶Figure 5Aa-b), confirming that TF-trigger TG assays are poorly sensitive to very low levels of FVIII activity (17). The peak plasmin parameter in PG with both severe type and moderate type in haemophilia A appeared to be slightly higher than normal, but the differences were not significantly different. The ttPeak parameter in PG with haemophilia A was the same as with normal plasma (▶Figure 5Ba-b). These results were in keeping with the relationship between bleeding symptoms and impairment of coagulation in patients with haemophilia A, and emphasised the imbalance between coagulation and fibrinolysis in these patients.

Discussion

The present study was designed to establish a modified T/P-G technique, based on the STP assay (11) that could provide an enhanced understanding of the relationship between coagulation and fibrinolysis. The previous assay did not appear to be sufficiently sensitive for the assessment of physiological TG and PG interactions. A particularly important point of TG assays is the precise measurement endogenous, generated active-thrombin, but it is difficult to explore normal control mechanism(s) - i.e. dilution of activated factors in flow states, etc. - are not in play in these assays. In addition, the binding of free thrombin and α2-macroglobulin (α2M), a representative thrombin inhibitor in plasma, creates a thrombin-α2M complex that is biologically inactive (18). This complex, however, together with free thrombin reacts with thrombin substrates in fluorometric TG assays, resulting in difficulties in interpretation. Hemker et al. estimated that 30% of thrombin formed was ultimately complexed with α2M (1). The simultaneous STP-assay and NHA reported previously (11, 12), therefore, appeared to highlight an issue of concern regarding the presence of thrombin-α2M complexes. In our studies, although it was difficult to preclude non-specific effects in the coagulation reactions, we utilised EDTA solution in our control buffers, and could preclude the influence of the α2M-thrombin complex that did not play a role in the coagulation reactions during this assay.

The original STP-assay and NHA offered advantages for evaluating individual TG and PG simultaneously in separate microtitre wells and in single wells, respectively (11, 12). The NHA provided data that identified the tight interplay between coagulation and fibrinolysis, but the STP assay did not. The single well system of the NHA, however, could be affected by untoward interference and interactions with signal detection. A major difference between these assays was the concentrations of reagents (TF and tPA) used in trigger mixtures, and we focused on the low concentrations of TF and tPA used in the NHA in an attempt to advance the STP assay using separate wells. We confirmed the potential capability of our T/P-G assay to identify the interplay between coagulation and fibrinolysis by demonstrating increases in TG caused by the addition of plasminogen, together with unique and opposite responses in TG and PG caused by argatroban, APC and TM (see ▶Figure 2). In particular, similar to the NHA, our modified assay was also sensitive to TAFI-mediated effects on fibrinolysis (15). Fundamentally, it appeared that the decrease in TG, mediated by argatroban and APC, reduced TAFI activation, and resulted in an increase in profibrinolytic activity (and no decrease in APC). In contrast, in keeping with current concepts, the addition of TM appeared to result in the formation of thrombin-TM complexes that significantly catalysed both the PC and TAFI activation, resulting in the impairment of TG and PG, respectively. Furthermore, enhanced fibrinolysis was observed in association with CPI-mediated inhibition of TAFI activation. From these data, our modified simultaneous T/P-G assay reflected the interplay between coagulation and fibrinolysis, and appeared to offer a relatively straightforward means to assess the important these balances.

In this respect, our assay revealed a significant role for individual coagulation factors in interacting mechanisms of coagulation and fibrinolysis. In particular, i) TG appeared to be responsible for the initiation of PG, and even trace amounts of TG promoted sufficient PG. ii) The absence of PG impaired TG (e.g. in fibrinogen-def), and PG was governed by the concentration of fibrinogen. iii) FV and FX, components of the prothrombinase assembly, were absolutely essential for the expression of both TG and PG, and FVIII

(and FIX), components of the tenase assembly, enhanced TG but did not affect PG. iv) The deficiency of anti-fibrinolytic factor, α2-PI, enhanced PG but had little effect on TG. These findings confirm that haemostasis is governed by a controlled balance between coagulation and fibrinolysis. In particular, unlike FVIII and FIX-def (haemophilia A and B), FV-def rarely results in major bleeding. It may be that both TG and PG are markedly compromised in FV-def, but TG alone is severely impaired in FVIII-def. Likewise, in α2-PI-def and Plg-def, enhanced PG and depressed PG, respectively, confer haemorrhagic or thrombotic tendencies.

We further demonstrated that haemorrhagic symptoms in patients with haemophilia A depended on significant impairment of coagulation and were not likely to be associated with modified fibrinolysis. Previous reports (19, 20) have suggested that patients with severe haemophilia A have enhanced fibrinolytic capacity, mediated by reduced TG and the subsequently decreased TAFI activation. We clearly demonstrated the opposite effects of APC and TM on PG but there were no differences in fibrinolytic capacity in patients with severe haemophilia A. It may be that our assay lacks sensitivity in this group of patients, and the relationship between bleeding tendency and phenotype is often not consistent, but nevertheless it seems unlikely that the haemophilia phenotype can be attributed to the imbalance between coagulation and fibrinolysis.

Our current findings provide challenging data for further studies. i) Although the modified T/P-G assay was designed to examine haemostasis *in vivo*, other factors that are likely to contribute to physiological haemostasis, including the vascular endothelium, platelets, and leucocytes, must be considered. ii) Our assay was performed using platelet-poor plasma, and it may be that the use of platelet-rich plasma or whole blood would provide important additional information. iii) A single assay condition was utilised for all variable coagulation deficiencies. Broader consideration of anti-coagulant pathways might be useful. iv) The local effects of TG and PG at sites of vascular injury could be pivotal for complete assessment of clinical disorders of haemostasis.

In conclusion, the control of haemorrhage and thrombosis is based on a sensitive balance between coagulation and fibrinolysis. Our assay would appear to be clinically helpful for the screening of patients with defective haemostasis in congenital disorders. Wider application of techniques of this nature could help to advance knowledge of the complex, functional interacting mechanisms of coagulation and fibrinolysis.

What is known about this topic?

- Simultaneous evaluation of thrombin and plasmin facilitates an overall understanding of normal and pathological haemostasis.
- Combined thrombin and plasmin generation assays have become to be adapted as the technique to evaluate the haemostatic state in patients with various haemostatic disorders.

What does this paper add?

- We established a simultaneous thrombin and plasmin generation (T/P-G) measurement using separate microtitre wells to retain sensitivity with the interplay between coagulation and fibrinolysis.
- The most evident impairments in TG were observed with deficiencies of coagulation factors participating in the prothrombinase complex. Defects in PG were observed with deficiencies of factor (F)V, FX, fibrinogen, and plasminogen.
- TG appeared to be a prerequisite for the initiation of PG, and overall PG was governed by fibrinogen concentration.
- TG, but not PG, in haemophilia A correlated with levels of FVIII:C, confirming that the abnormal haemostasis in haemophilia A results in a severe imbalance between coagulation and fibrinolysis.
- The modified T/P-G assay could provide an enhanced understanding of haemorrhage and thrombosis in clinical practice.

Conflicts of interest

None declared.

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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 neutralised 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 1 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.

Keywords

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

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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 immobilisation, 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 (FVIII) 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 recognised 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 microtitre plates coated with anti-rabbit VWF antibody and incubated at 37°C for 2 hours (h). The contents of each well were removed, the plates were incubated with 100 µl of horseradish peroxidase (HRP)-conjugated anti-human FVIII antibody for 1 h at room temperature, washed and then perborate/3, 3', 5, 5'-tetramethylbenzidine substrate (100 µl) was added. After 20 minutes (min) 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 min at room temperature, and incubated with rFVIII (final concentrations: 0, 0.025, 0.25, 2.5 IU/ml) for 10 min. 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 min 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 min 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 haemostatic parameters before, 30 and 60 min after rFVIII infusion were measured using a ROTEM analyser (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 min and at 2,580 g for 10 min 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 min (16). Platelets sedimented from PRP by centrifugation at 1,010 g for 10 min 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 (min). Thereafter, collagen (Nycomed Austria GmbH, A-4020 Linz, Austria), adenosine 5'-diphosphate (ADP, Sigma-Aldrich Corp.), botrocetin (American Diagnostica Inc., Stamford, CT, USA) or thrombin (Sigma-Aldrich Corp.) was added as agonists. Platelet aggregation was measured using a PA-20 aggregation analyser (Kowa, Aichi, Japan). Changes in light transmittance caused by each agonist were recorded for 10 min (5 min 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 (TF) 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 homogenised 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 min, the supernatant (vessel sample; 100 µl containing 100 µg protein) was incubated with rabbit plasma (100 µl) for 1 min. Clotting assays were then started by adding 20 mM CaCl2 (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 neutralising 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 immunised 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 neutralising activity against TF/factor VII-catalysed 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 neutralising 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) was diluted 100-fold with 25 mM $CaCl_2$ and R37 was incubated with the dilute PT reagent or the rabbit plasma for 10 min at 37°C prior to the clotting experiments. R37 dose-dependently prolonged PT under both conditions (see Suppl. Figure 1, available online at www.thrombosis-online.com).

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 min 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 (▶Figure 6A). The signal intensity was measured in all images from all animals. The vessels were washed with saline 1 min after ICG infusion and the average fluorescence intensity in each ROI was analysed 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

At 1 h after thrombus induction, jugular veins were fixed in 4% paraformaldehyde for 24 h 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 visualised 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 analysed 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, USA). 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 (▶Figure 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 (▶Figure 2A). We infused rabbits

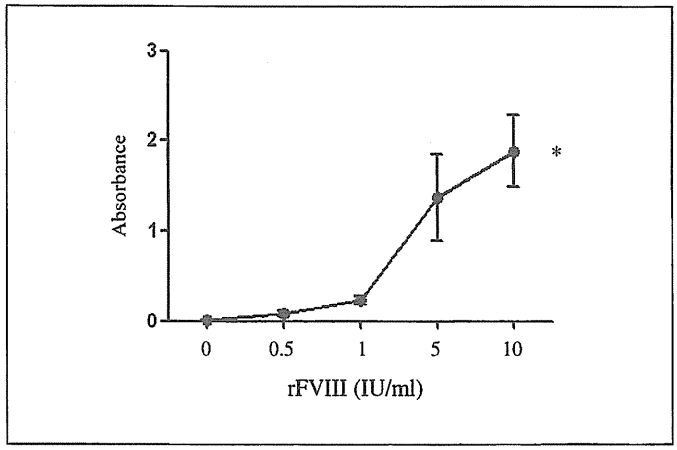

Figure 1: Affinity of rFVIII for rabbit VWF. Affinity between rFVIII and rabbit VWF was measured by ELISA. Diluted rabbit plasma with or without rFVIII was added to microtitre plates coated with anti-rabbit VWF antibody. The rFVIII dose-dependently bound to rabbit VWF and was detected using anti-human FVIII antibodies. (*$p < 0.0001$; $n = 5$ each).

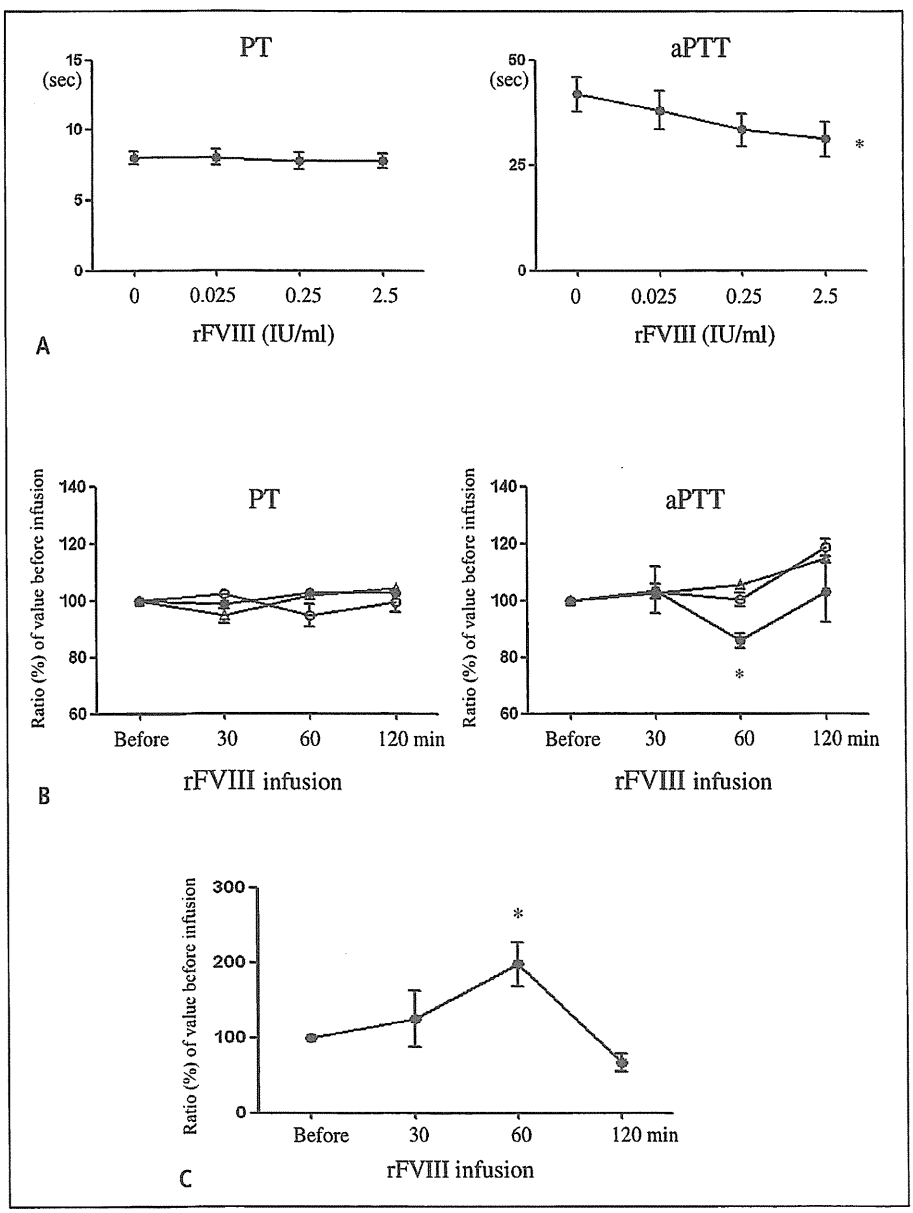

Figure 2: Effects of rFVIII on PT and aPTT *in vitro* and *ex vivo*, and plasma FVIII activity. A) Recombinant human FVIII shortened aPTT *in vitro* (*$p < 0.0001$), but did not affect PT ($p = 0.37$; $n = 4$ each). B) Three doses of rFVIII (20, 40 or 100 IU/kg) were infused into rabbits. Blood samples 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 1 h (*$p < 0.05$ 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 min ($n = 4$ each; *$p < 0.05$ vs before rFVIII infusion).

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 x 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.

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 (▶Figure 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 min after rFVIII (100 IU/kg) increased about two-fold for up to 1 h after, compared with that before rFVIII infusion (198 ± 58 %, p < 0.05; ▶Figure 2C).

Recombinant human FVIII enhances thrombin generation and whole blood coagulation

▶Table 1 and ▶Table 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.

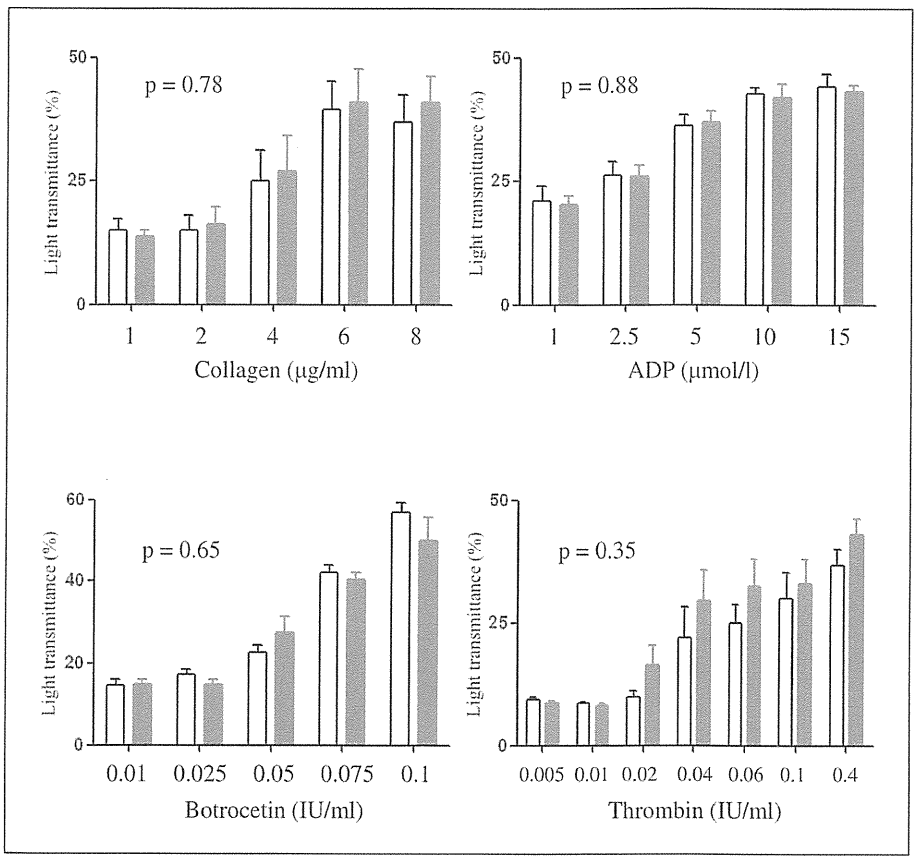

Figure 3: Platelet aggregation with or without rFVIII *in vitro*. Rabbit PRP and washed platelets with (filled columns) or without (opened columns) rFVIII were prepared as described in *Materials and methods*. Platelet aggregation was initiated by adding collagen, ADP, botrocetin or thrombin. Infused rFVIII did not affect platelet function (n = 4 each).

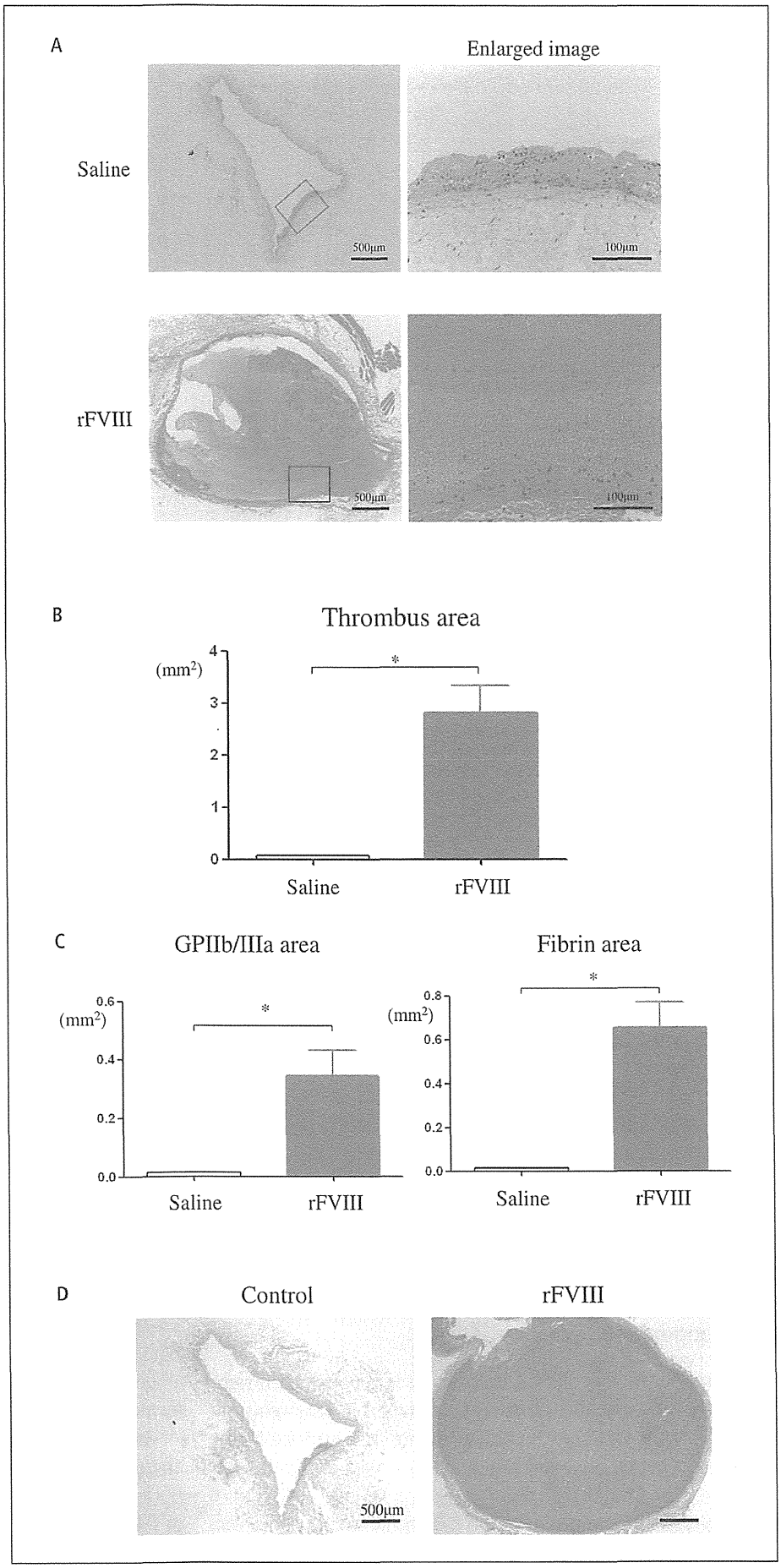

Figure 4: Infused rFVIII enhances thrombus formation in rabbit jugular vein. A) Representative light microphotographs of venous thrombi 1 h after endothelial denudation with or without rFVIII infusion. B) Area with thrombi formed in rabbit jugular veins 1 h after endothelial denudation. Recombinant human 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 localised by staining with anti-human FVIII antibody.

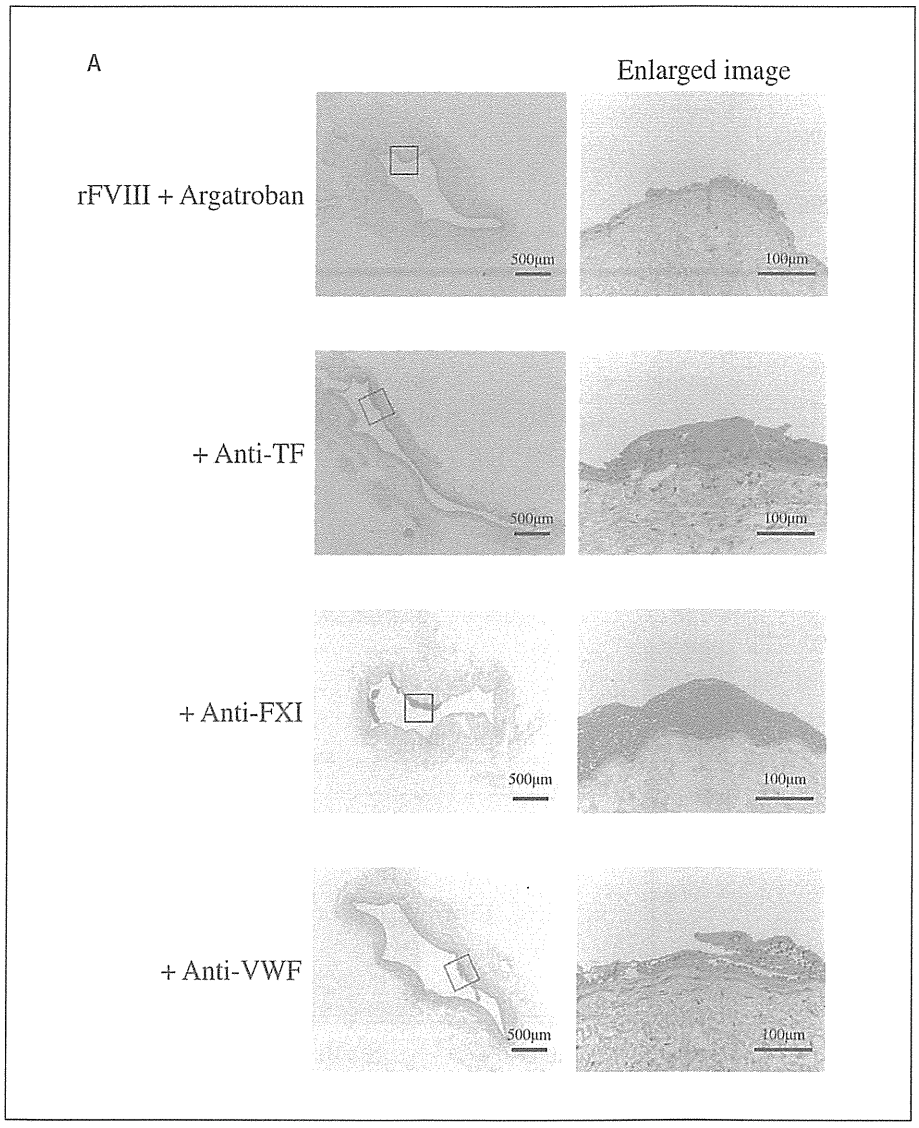

Figure 5: Thrombin, TF, FXI, and VWF are required for thrombus formation enhanced by rFVIII in rabbit jugular veins. A) Representative light microphotographs of venous thrombi 1 h after endothelial denudation that proceeded immediately after infusions of rFVIII and inhibitor of thrombin, TF, FXI and VWF.

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 (▶Figure 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 1 h 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 1 h 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 (▶Figure 4A) and 11 of 24 jugular veins (46%) became occluded within 1 h. The mean thrombus areas was about 200-fold larger in the rFVIII, than in the control group (▶Figure 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 (▶Figure 4C). The ratio of GPIIb/IIIa and fibrin immunopositive to thrombus areas did not differ between the rFVIII-infused and control groups. Throm-

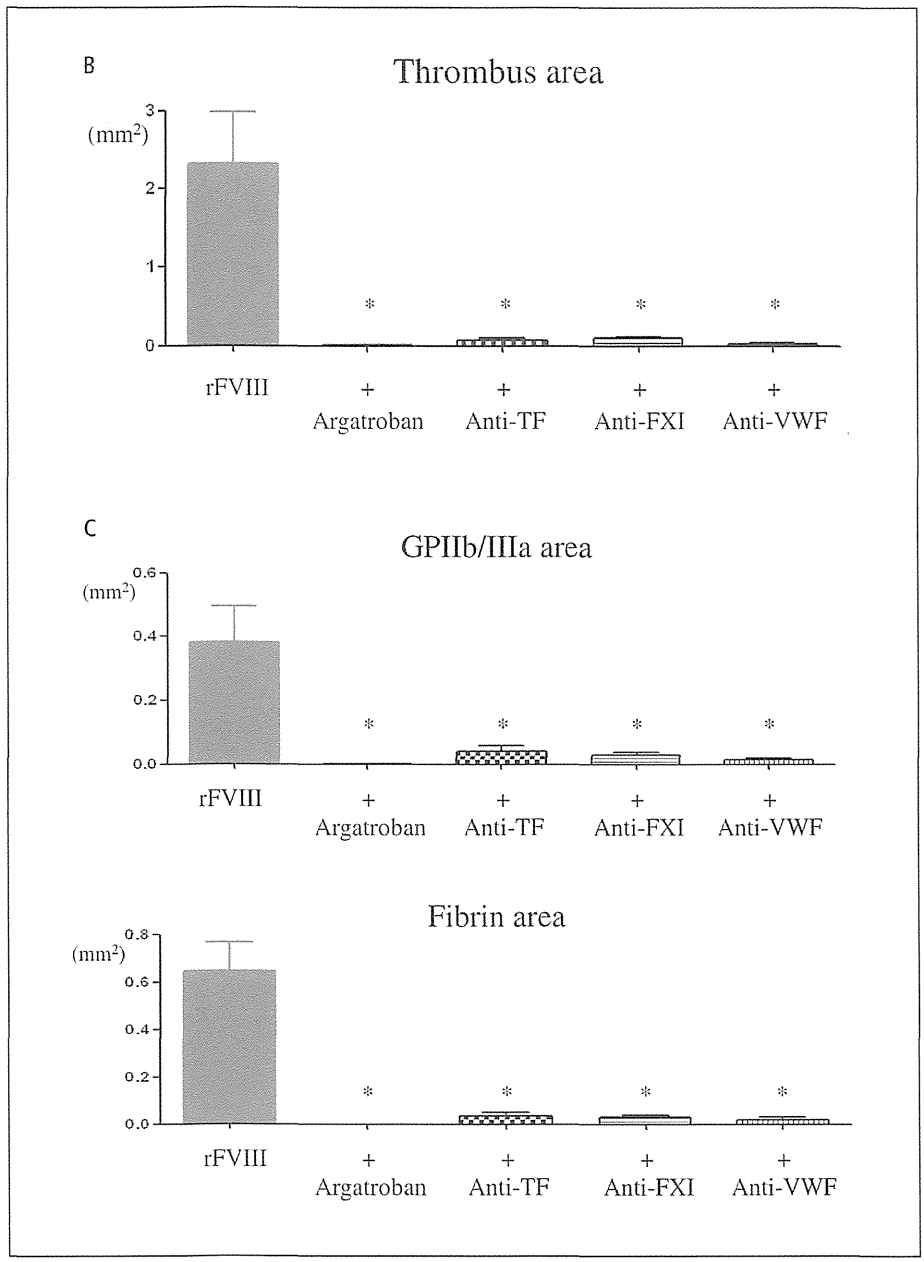

Figure 5 continued: B) Area with thrombi formed in rabbit jugular veins 1 h after endothelial denudation (*p < 0.0001 vs rFVIII group, n = 16 sections each). C) GPIIb/IIIa and fibrin immunopositive areas in thrombi of jugular veins (*p < 0.0001 vs rFVIII group, n = 16 sections each).

bi were immunopositive for human FVIII in the rFVIII-infused, but not in the control group (▶Figure 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 (▶Figure 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 min 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 (▶Figure 6B and C; Suppl. Movie 1, available online at www.thrombosis-online.com), 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 (▶Figure 6B and C, Suppl. Movie 2, available online at www.thrombosis-online.

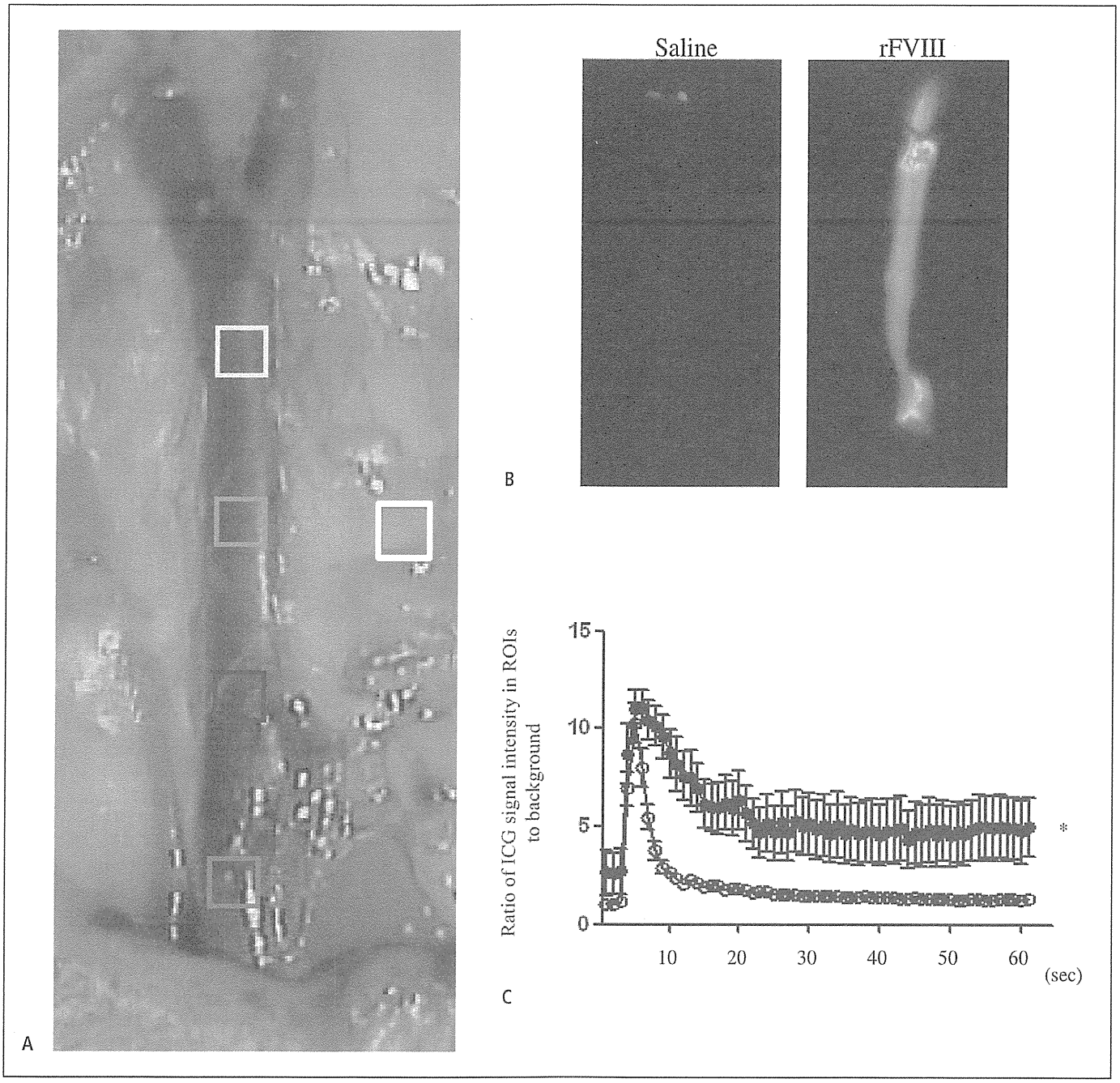

Figure 6: Fluorescence intensity of ICG at 15 min after endothelial denudation. A) Four ROIs (yellow, green, blue, and pink frames) were set at regular intervals on rabbit jugular vein before rFVIII infusion. One adjacent ROI (white frame) was set beside ROIs as background. B) Fluorescent images at 15 min after endothelial denudation and at 15 sec 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).

com). ▶ 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 three-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 min, respectively). An infusion of argatroban or anti-FXI antibody initially

prevented the increase in the fluorescence intensity, and then gradually reduced the intensity (Suppl. Movies 3 and 4, available online at www.thrombosis-online.com). Anti-VWF antibody also suppressed the increase in intensity, but to a lesser extent than argatroban or anti-FXI antibody (▶Figure 8A), whereas anti-TF antibody did not affect fluorescence intensity (▶Figure 8A, Suppl. Movies 5 and 6, available online at www.thrombosis-online.com). 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 1 h after endothelial denudation. Anti-TF antibody slightly suppressed thrombus and fibrin areas, but the difference did not reach significance (▶Figure 8B and C).

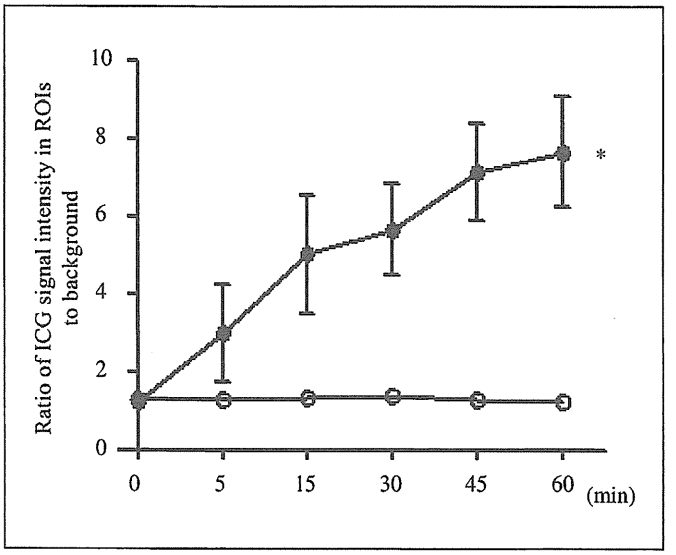

Figure 7: Ratio of ICG to background fluorescence intensity before and after endothelial denudation. Saline (open circles) or 100 IU/kg of rFVIII (closed circles) was infused immediately before endothelial denudation. This graph shows average fluorescence intensity before, and 5, 15, 30, 45 and 60 min after endothelial denudation (*p < 0.01 vs control, n = 4 each).

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 five-fold increase in the risk of DVT (3). The aPTT of the rabbit decreased by 60 min, and normalised 120 min 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 h 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* (▶Figure 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.

FVIII is recruited by binding to VWF on surfaces comprising collagen and thrombus (28). We previously reported that FVIII colocalises 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 (▶Figure 5A-C). As reported (32), a blockade of VWF and GPIbα interaction abolished platelet adhesion on a collagen sur-