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Original Articles: Blood coagulation, fibrinolysis and cellular haemostasis

Coagulation potentials of immobilized factor VIII in flow-dependent fibrin generation on platelet surfaces

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Summary

Coagulation factor VIII (FVIII) plays an essential role in haemostasis. To date, physiologic activity of FVIII circulating in the bloodstream (S-FVIII) is evaluated by classic coagulation assays. However, the functional relevance of FVIII (-von Willebrand factor complex) immobilized on thrombogenic surfaces (I-FVIII) remains unclear. We used an in vitro perfusion chamber system to evaluate the function of I-FVIII in the process of mural thrombus formation under whole blood flow conditions. In perfusion of either control or synthetic haemophilic blood, the intra-thrombus fibrin generation on platelet surfaces significantly increased as a function of I-FVIII, independent of S-FVIII, under high shear rate conditions. This I-FVIII effect was unvarying regardless of anti-FVIII inhibitor levels in synthetic haemophilic blood. Thus, our results illustrate coagulation potentials of immobilized clotting factors, distinct from those in the bloodstream, under physiologic flow conditions and may give a clue for novel therapeutic approaches for haemophilic patients with anti-FVIII inhibitors.

Key Words: factor VIII, von Willebrand factor, flow, fibrin generation, hemophilia

Introduction

Haemostatic plug formation at sites of injured vascular walls is a critical human defense response that ensures blood flow to vital organs.[1] Following platelet adhesion and aggregation, blood coagulation mechanisms lead to the fibrin network formation within platelet thrombi to stabilize the haemostatic plug.[1-3] Coagulation factor VIII (FVIII) plays a pivotal role as a cofactor in factor X activation by activated factor IX, thus drastically amplifying thrombin generation in the coagulation process.[4-6] Indeed, patients with congenital deficiency in this factor, known as haemophiliacs, exhibit serious bleedings throughout their life.[5, 6]

To date, physiologic activity of FVIII is evaluated mostly by plasma coagulation assays that determine the capability of fibrin clot formation in closed stirring systems in vitro. However, the experimental conditions of such soluble-phase assays differ considerably from the in vivo haemostatic conditions, in which solid-phase blood coagulation occurs on platelet surfaces under whole blood flow[3]. In this regard, we have focused on FVIII-von Willebrand factor (VWF) complex immobilized to thrombogenic surfaces as a solid-phase source of FVIII (immobilized FVIII; I-FVIII). We were able to discriminate between I-FVIII and those circulating in the bloodstream (soluble FVIII; S-FVIII).

Using a perfusion chamber system, we show that I-FVIII, independent of S-FVIII, plays a role in the intra-thrombus fibrin-network formation in mural thrombus generation under high shear rate conditions. In the absence of S-FVIII, I-FVIII normalized in a concentration-dependent manner the reduced fibrin deposition in synthetic hemophilic blood regardless of the circulating anti-FVIII inhibitor titre. Our results may imply the alternative therapeutic potentials of targeting I-FVIII for patients with haemophilia and high titre anti-FVIII inhibitors.

Materials and Methods

Blood collection

This work was approved by the institutional review board of Nara Medical University. Blood was collected from 5 non-smoking healthy volunteers, who had not taken any medications in the previous 2 weeks. Two different ways of blood collection (anticoagulation by 1/10th volume of 3.8% sodium citrate or 20 μM of argatroban; Tanabe-Mitsubishi Co.,Tokyo, Japan) were employed for evaluation of intra-thrombus fibrin generation under flow. For the citrated blood, 50 μg/ml of corn trypsin inhibitor (CTI; Haematologic Technologies Inc, VT., USA) was added into the blood sample to minimize the contact activation of blood, then 8 mM of $CaCl_2$ was added to initiate blood coagulation just prior to perfusion. With regards to the argatroban-treated blood, this relatively low argatroban concentration was determined to allow gradual thrombin generation without the flow path occlusion by gross clot formation during blood perfusion, which makes it suitable for the evaluation of fibrin generation, as well as platelet adhesion and aggregation, when the time lag from blood drawing to the perfusion start was strictly adjusted among experiments, as described.[7, 8]

Preparation of VWF-coated glass surfaces containing varying concentration of FVIII.

Human native VWF (FVIII-VWF complex) was purified from cryoprecipitate as previously described.[9] FVIII-free VWF was obtained by the rechromatography of purified FVIII-VWF complex with Separose-CL6B in the presence of 0.35 M $CaCl_2$ as described,[10] and the complete depletion of FVIII was confirmed by ELISA assay for FVIII as previously described.[11] Glass plates which had been coated with purified FVIII-free VWF as described[8, 12] were reacted with recombinant FVIII (Kogenate FS provided by Bayer Pharmaceutical Co., Osaka, Japan) at varying concentrations (0 as a

negative control, 0.1, 0.5, 1, 2.5, 5, and 10 units (IU)/mL) for 2 h at room temperature. After non-adherent proteins were washed out, the amount of FVIII immobilized to the glass-bound VWF was quantified by ELISA-based assay. Briefly, a rubber ring (diameter: 8 mm) was placed on a VWF-coated glass on which various amounts of FVIII was immobilized. A peroxidase-conjugated anti-FVIII human polyclonal antibody previously described[11] was then reacted to a glass surface inside the rubber ring, followed by the routine ELISA assay procedures. The final reactant with enzyme activity inside the ring was collected, transferred to an ELISA-plate, and the enzyme intensity was determined at the wave length of 492 nm, reflecting the amount of surface-immobilized FVIII.

In vitro perfusion studies

In perfusion studies for the evaluation of platelet adhesion and aggregation, whole blood anticoagulated with argatroban was immediately incubated with the fluorescent dye DiOC6 (1 μM; Molecular Probes Inc., Eugene, Oregon) for 10 min at 37°C to label platelets, allowing visualization of platelet-surface interactions by confocal laser scanning microscopy (CLSM, FV300; Olympus Co., Tokyo, Japan), as described.[7, 13-15] DiOC6-labeled platelets was aspirated through the chamber by a syringe pump (Model CFV-3200, Nihon Kohden Co., Ltd., Tokyo, Japan), producing a 250 (typical low) or 1500 (typical high) shear rate at the 37°C situation, as described.[7, 13-16] Fluorescent images were viewed by CLSM at 1-μm intervals from the VWF surface to a height of 60 μm from the surface, and used to calculate the percentage of the area covered by adhering platelets (surface coverage) and each thrombus volume in a defined area at the indicated time points as described.[7,15] Briefly, surface coverage of platelet thrombi was evaluated based on sliced images at 2-μm height from the VWF surface, and total volume of platelet thrombi in a defined area was calculated by summing all sliced images of identical portions using the image-analyzing computer software (Image Pro

Plus version 4.5; Planetron, Tokyo, Japan).

In experiments for the intra-thrombus fibrin generation, whole blood without DiOC6-labelling of platelets was perfused. Intra-thrombus fibrin generation was evaluated by image analysis of thrombi immunostained with an anti-fibrin specific antibody.[7, 8] In brief, thrombi generated on a coverslip were fixed, reacted with a mixture of mouse anti-fibrin antibody (15 μg/mL; NYB-T2G1, which does not recognize fibrinogen, from Accurate Chemical., Westbury, NY) and rabbit anti-fibrinogen antibody (15 μg/mL; DAKO Cytomation; Kyoto, Japan) for 90 min at 37°C, stained with a mixture of Cy3-conjugated anti-mouse IgG (5 μg/mL; Sigma-Aldrich Co., Tokyo, Japan) and FITC-conjugated anti-rabbit IgG (5.7 μg/mL; BIOSOURCE, Camarillo, CA), and viewed by CLSM. The extent of intra-thrombus fibrin increase was evaluated as a "fibrin/fibrinogen" ratio of intensity of fibrin-fluorescence relative to that of fibrinogen-fluorescence. Three-dimensional (3D) images of thrombi was constructed by the image-analyzing system of CLSM based on successive horizontal slices as previously described.[7,15]

Preparation of synthetic "acquired" hemophilic blood

Under the informed consent, plasma samples were obtained from a Japanese patient with severe haemophilia A and a high-titre inhibitor, and the anti-FVIII inhibitor IgG (human alloantibody) was purified from the patient's plasma by Protein A-Sepharose chromatography as previously described.[11] Synthetic "acquired" haemophilic blood were prepared by incubating control whole blood with varying concentrations of purified inhibitor IgG, at final inhibitor IgG titres of 5 (#1), 10 (#2), and 20 (#3) Bethesda U/mL (Table 1). The remaining FVIII clotting activities and inhibitor titres in corresponding plasma samples, measured by activated partial thromboplastin time (aPTT)-based assay, are also included in the Table 1.

Statistical analysis

Statistical differences between two groups of data were evaluated by Student's t-test. In case of multiple comparisons, two-way factorial ANOVA was employed. P values < 0.05 were considered to denote statistical significance.

Results

Preparation of VWF-coated glass surfaces containing varying concentrations of FVIII

Various concentrations of recombinant FVIII were incubated with a glass plate which had been coated with FVIII-free VWF. After non-adherent proteins were extensively washed out, amounts of FVIII immobilized onto VWF-coated glass surface (I-FVIII) were determined by the ELISA-based assay. Thus, I-FVIII increased as a function of recombinant FVIII added to a VWF-coated surface, reaching plateau at the FVIII concentrations greater than 5 U/mL (Figure 1). As a result, various VWF-coated glass plates with different I-FVIII density (del-FVIII as a control, #A, #B, #C and #D as indicated in the Figure 1) were successfully prepared.

Effects of I-FVIII on platelet adhesion and aggregation on VWF-coated surface under high or low shear rate condition

To evaluate the effects of I-FVIII on basic platelet functions under flow conditions, whole blood was perfused over a VWF-coated glass surface in the presence (#D-plate; see Figure 1) or absence (del-FVIII) of I-FVIII under a high (1500 s^{-1}) or low (250 s^{-1}) shear rate condition. The process of platelet adhesion and aggregation was evaluated by the time-course of surface coverage or volume of platelet thrombi generated on a VWF-coated glass surface. No significant differences in thrombus size were confirmed in those two groups (with or without I-FVIII) under both high and low shear rate conditions (Figure 2). Thus, I-FVIII does not seem critically involved in the platelet adhesion and aggregation under flow conditions.

Effects of I-FVIII on fibrin generation within thrombi generated on VWF-coated surface under high or low shear rate condition

Intra-thrombus fibrin generation was evaluated under flow conditions. In contrast to the basic platelet functions, the fluorescent 3D images indicate that I-FVIII enhances the intra-thrombus fibrin deposition under high shear rate condition (Figure 3A). Statistical analysis also confirmed that the intra-thrombus fibrin generation, as a function of I-FVIII, significantly increased as compared to those generated in the absence of I-FVIII (del-FVIII) under high shear rate, while no effects of I-FVIII were observed under low shear rate (Figure 3B).

Effects of I-FVIII or S-FVIII on intra-thrombus fibrin generation in perfusion of synthetic "acquired" haemophilic blood under high shear rate condition

Synthetic "acquired" haemophilic blood (see Table 1) was perfused over a VWF-surface in the presence or absence of I-FVIII under high shear rate condition (1500 s^{-1}). In some experiments to evaluate S-FVIII, recombinant FVIII was added in sample synthetic haemophilic blood 30 min prior to perfusion. As shown in the Figure 4, I-FVIII significantly increased fibrin generation within synthetic haemophilic thrombi in the absence of S-FVIII. The fibrin/fibrinogen ratios of haemophilic thrombi in the presence of I-FVIII are nearly equal to that of control thrombi in the absence of I-FVIII (Figure 4). Note also that these I-FVIII effects are unvarying regardless of anti-FVIII inhibitor levels in synthetic haemophilic blood, while the effects of S-FVIII was totally abolished at the higher inhibitor levels.

Discussion

The blood coagulation process, essential for thrombosis and hemostasis, is a solid-phase event that occurs on cell surfaces of activated platelets or endothelium.[3, 17] Under rapid blood flow in vivo, immobilization of clotting factors on a thrombogenic surface could be crucial for the proper coagulation responses in such solid-phase blood coagulation. In this context, we here proposed a novel concept of "I-FVIII" (FVIII-VWF complex immobilized to a surface), and evaluated the functional relevance of I-FVIII, discriminating from S-FVIII, under experimental whole blood flow conditions.

To evaluate physiologic relevance of I-FVIII, we first compared the overall process of mural thrombus formation on FVIII-free VWF immobilized to a glass surface with that on native FVIII-VWF complex under flow conditions. In terms of the size of platelet thrombi as well as intra-thrombus fibrin generation, no significant differences were confirmed among those two surfaces under both high and low shear rate conditions (results not shown), suggesting the limited contribution of native I-FVIII in this regard. This consequence is not surprising because only 1 out of over 50 VWF subunits is presumably occupied by FVIII molecule in native FVIII-VWF complex.[6, 18,19] In order to further explore the functional relevance of I-FVIII, we therefore exploited this FVIII-binding capacity of native VWF immobilized to the surface. We added an excess amount of exogenous FVIII to immobilized VWF and successfully prepared several VWF-surfaces with different I-FVIII density (Figure 1).

Despite sufficient levels of S-FVIII inherently present in normal control blood, I-FVIII significantly enhanced blood coagulation in a concentration-dependent manner within platelet thrombi under high shear rate conditions (Figure 3), albeit with no appreciable effects on basic platelet functions (Figure 2). In general, the increase of fibrin

generation is associated with the increase of final thrombus volume under such high shear rate conditions.[7] These discrepant results may be due at least in part to the different anticoagulation of sample blood; i.e., a small amount of thrombin inhibitor argatroban was used for the evaluation of platelet adhesion and aggregation, while the intra-thrombus fibrin generation was evaluated with recalcified citrated blood.

Unlike classic coagulation assays such as aPTT that evaluate fibrin clot formation in soluble phase, the intra-thrombus fibrin generation in our experimental approach reflects solid-phase blood coagulation on platelet surfaces and may be more representative of in vivo haemostasis.[3,17] Thus, it is assumed that local concentrations of several clotting factors must be sustained for the proper protease-substrate reactions under blood rheological situations in vivo.[20-22] In this regard, when clotting factors are tightly immobilized on local thrombogenic sites, they may work better under blood flow conditions than those flowing in the bloodstream. Indeed, this scenario may be consistent with our observations that the effects of I-FVIII on solid-phase blood coagulation are very profound under high shear rate conditions (Figure 3B), where blood flow is so rapid that soluble blood clotting proteins could be easily washed out from the local thrombogenic sites. In contrast, S-FVIII may be able to contribute more efficiently to flow-dependent fibrin generation in the absence of I-FVIII under low shear rate conditions where blood flow is relatively slow.

In light of recent modeling studies incorporating the coagulation cascade and platelet deposition under flow, thrombus growth is assumed to be limited by the transport of clotting factor zymogens into the interior of thrombus.[20, 22] I-FVIII fixed at the central core of generating thrombi could be apparently advantageous for such coagulation responses under flow, as compared to S-FVIII which must bind first to platelet surfaces and penetrate into thrombus against blood flow. Thus, an unusually high density of I-FVIII bound to VWF on the basal layer of a thrombogenic surface can sufficiently compensate for the complete lack of S-FVIII in the bloodstream, as seen in the synthetic

"acquired" haemophilic blood (Table 1, Figure 4). Interestingly, the effects of I-FVIII on synthetic haemophilic blood, unlike S-FVIII, were unvarying regardless of the anti-FVIII inhibitor titre in the blood under high shear flow conditions (Figure 4). Presumably, anti-FVIII IgGs in the bloodstream cannot easily interact with and neutralize I-FVIII when blood flow is quite fast as is the case under high shear rate conditions.

Taken together, these findings may give a clue for a novel therapeutic approach against patients with haemophilia and high titer of anti-FVIII inhibitors. Since I-FVIII bound to VWF at sites of vessel injury is more resistant to inhibitor attack compared to S-FVIII, I-FVIII could effectively enhance the coagulation potentials of blood from such haemophilic patients.

Conflicts of interest

None declared.

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Table 1. Synthetic "acquired" haemophilic blood prepared by incubating control whole blood with varying concentrations of purified anti-FVIII inhibitor IgG

Synthetic haemophilic blood	Inhibitor titre in whole blood (BU/mL)	Plsma FVIII:C (%)	Remaining inhibitor titre in plasma (BU/mL)	aPTT (sec)	aPTT with CTI (sec)
#1	**5**	**1.1**	**12.2**	**108. 2±3.2**	**166.2±5.2**
#2	**10**	**< 1.0**	**22.0**	**111.7±4.3**	**178.4±7.1**
#3	**20**	**< 1.0**	**44.0**	**112.5±4.5**	**172.4±4.8**
Control	**0**	**100**	**0**	**38.5±2.3**	**84.2±2.5**

Figure legends

Figure 1.

Preparation of VWF-coated glass surfaces containing varying concentrations of FVIII. A glass plate was coated with FVIII-free VWF and recombinant FVIII (0, 0.1, 0.5, 1, 2.5, 5, or 10 U/mL). Each data point represents mean ± standard deviation (SD) in 3 independent experiments. Note that I-FVIII as determined by the enzyme activity at optical density 492 nm increased as a function of recombinant FVIII added to a VWF-coated surface, reaching plateau at the FVIII concentrations greater than 5 U/mL. Thus, various VWF-coated glass plates with different I-FVIII density (del-FVIII as a control, #A, #B, #C and #D as indicated in the figure) were prepared.

Figure 2.

Time course of platelet adhesion and aggregation on a VWF-coated surface in the presence or absence of I-FVIII under high or low shear rate condition. Whole blood from healthy volunteers containing DiOC6 (1 μM)-labeled platelets, mildly anticoagulated with argatroban, was perfused over a VWF-coated glass surface with (#D) or without (del-FVIII) I-FVIII under high (1500 s^{-1}) or low (250 s^{-1}) shear rate. The process of platelet adhesion and aggregation was evaluated by the surface coverage of thrombi generated at the time points indicated in the figure. Each data point represents mean ± SD in 3 independent perfusions using blood from 3 individual donors.

Figure 3. Functional evaluation of FVIII bound to VWF immobilized on a glass surface (I-FVIII). (A) Visual evaluation of I-FVIII on fibrin generation within thrombi generated on VWF-coated surface under high shear rate condition. Citrated whole blood from healthy volunteers was perfused over a VWF-coated glass surface with or without I-FVIII under high (1500 s^{-1}) shear rate. Just prior to perfusion, $CaCl_2$ was added to the sample blood (8 mM) to initiate blood coagulation responses. Thrombi generated on VWF-coated glass surface at 7 min-perfusion in the presence (#D) or absence (del-FVIII) of I-FVIII under 1500 s^{-1} shear were fixed, double-stained (FITC-fibrinogen: green and Cy3-fibrin: red) and viewed by CLSM. The 3D images of

thrombi were representative of 5 independent flow experiments (original magnifications: X 600). Merged 3D images, obtained by superimposing two images of the identical portion, indicate that I-FVIII enhances the intra-thrombus fibrin deposition under high shear rate condition. **(B)** Effects of I-FVIII on fibrin generation within thrombi generated on VWF-coated surface under high or low shear rate condition. Experimental conditions were as described in the panel (A) legend. Thrombi generated on various VWF-coated glass surfaces at 7 min after perfusion were fixed, double-stained and viewed by CLSM. Bars represent mean (+ SD) fibrin/fibrinogen ratio in 25 defined areas (each 133 x 100 mm) examined (5 areas randomly selected in 5 independent perfusions of blood from 5 individual donors). Note that the intra-thrombus fibrin generation, as a function of I-FVIII, significantly ($*P < 0.01$) increased as compared to those generated in the absence of I-FVIII (del-FVIII) under high shear rate, while no effects of I-FVIII were observed under low shear rate.

Figure 4. Effects of I-FVIII or S-FVIII on intra-thrombus fibrin generation in perfusion of synthetic "acquired" haemophilic blood under high shear rate condition. Experimental conditions are as described in the Fig. 3 legend. Citrated whole blood from healthy donors rendered haemophilic by anti-factor VIII human antibody was perfused over a VWF-surface in the presence (#D; indicated as "+ (plus)" in the I-FVIII column) or absence (del-FVIII; indicated as " – (minus) ") of I-FVIII under high shear rate condition (1500 s^{-1}). Such synthetic "acquired" haemophilic blood was prepared by incubating control whole blood with varying concentrations of purified inhibitor IgG (see Table 1). In some experiments to evaluate S-FVIII, recombinant FVIII (3 U/ml) was added in sample synthetic haemophilic blood 30 min prior to perfusion (indicated as "+" in the S-FVIII column; the "+" symbol in parenthesis represents inherent FVIII present in normal blood). Bars represent mean (+ SD) fibrin/fibrinogen ratio of thrombi generated at 7-min perfusion in 15 defined areas (each 133 x 100 μm) examined (5 areas randomly selected in 3 independent sets of experiment using blood from 3 individual donors). Note that I-FVIII significantly ($*P < 0.01$) increased fibrin generation within synthetic haemophilic thrombi in the absence of S-FVIII. The fibrin/fibrinogen ratios of haemophilic thrombi in the presence of I-FVIII are nearly equal (n.s.; not significant) to that of control thrombi in the absence of I-FVIII (see the right end and left end bars). Note also that these I-FVIII effects are unvarying (n.s.; not significant) regardless of anti-FVIII inhibitor levels in synthetic haemophilic blood, while the effects of S-FVIII

was totally abolished at the higher inhibitor levels.

Extra table

1. 'What is known on this topic'

- Coagulation factor VIII (FVIII) plays a pivotal role as a cofactor in factor X activation by activated factor IX, thus drastically amplifying thrombin generation in the coagulation process.
- Physiologic activity of FVIII is so far evaluated mostly by plasma coagulation assays that determine the capability of fibrin clot formation in closed stirring systems in vitro.
- However, the experimental conditions of such soluble-phase assays differ considerably from the in vivo haemostatic conditions, in which solid-phase blood coagulation occurs on platelet surfaces under whole blood flow.

2. 'What this paper adds'

- We have focused on FVIII-von Willebrand factor (VWF) complex immobilized to thrombogenic surfaces as a solid-phase source of FVIII (immobilized FVIII; I-FVIII), and were able to discriminate between I-FVIII and those circulating in the bloodstream (soluble FVIII; S-FVIII).
- Using a perfusion chamber system, we show that I-FVIII, independent of S-FVIII, plays a role in the intra-thrombus fibrin-network formation in mural thrombus generation under high shear rate conditions. In the absence of S-FVIII, I-FVIII normalized in a dose-dependent manner the reduced fibrin deposition in synthetic haemophilic blood regardless of the circulating anti-FVIII inhibitor titre.
- Our results may imply the alternative therapeutic potentials of targeting I-FVIII for patients with haemophilia and high titre anti-FVIII inhibitors.

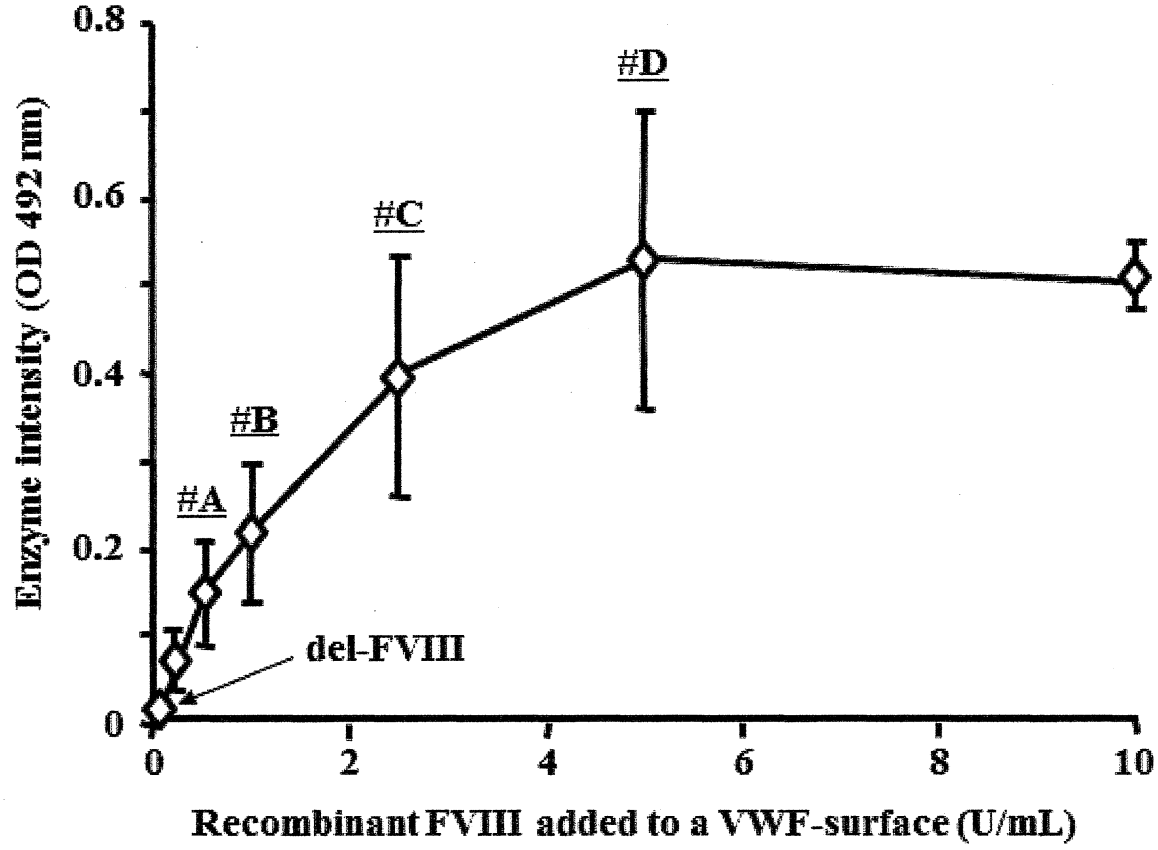

0.8
0.6
0.4
0.2
0
Enzyme intensity (OD 492 nm)
#A
#B
#C
#D
del-FVIII
0
2
4
6
8
10
Recombinant FVIII added to a VWF-surface (U/mL)

Fig. 1

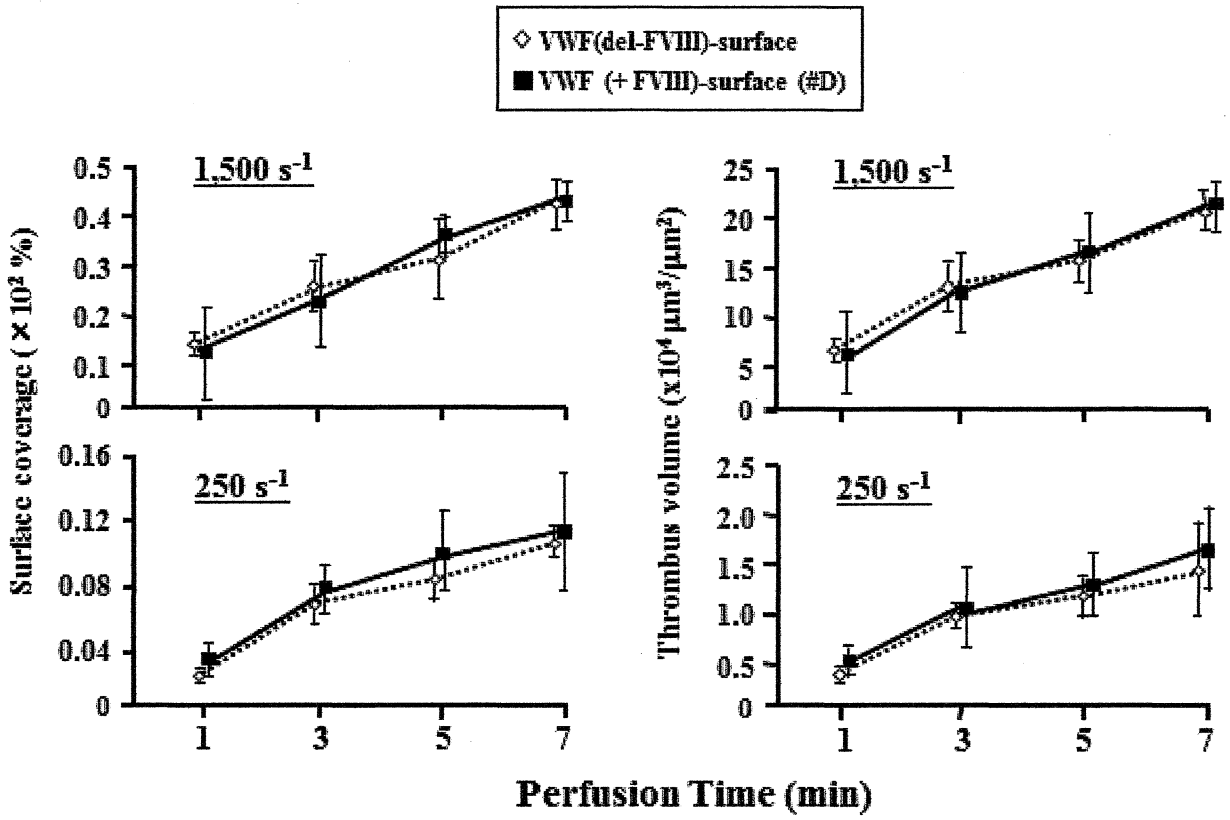
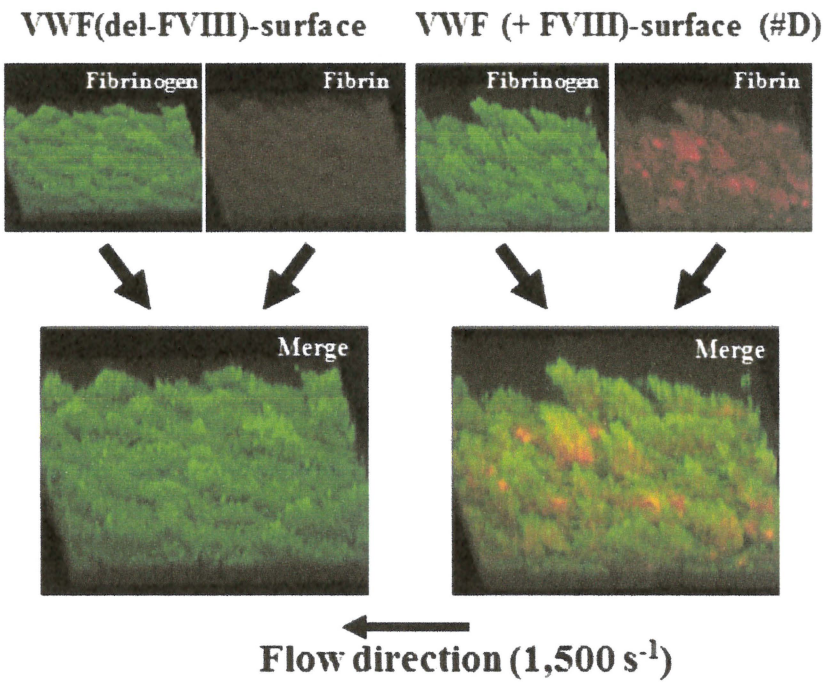

◇ VWF(del-FVIII)-surface
■ VWF (+ FVIII)-surface (#D)
1,500 s-1
250 s-1
Surface coverage (×10² %)
Thrombus volume (x10⁴ μm³/μm²)
Perfusion Time (min)

Fig. 2

A


VWF(del-FVIII)-surface
VWF (+ FVIII)-surface (#D)
Fibrinogen
Fibrin
Fibrinogen
Fibrin
Merge
Merge
Flow direction (1,500 s-1)

B

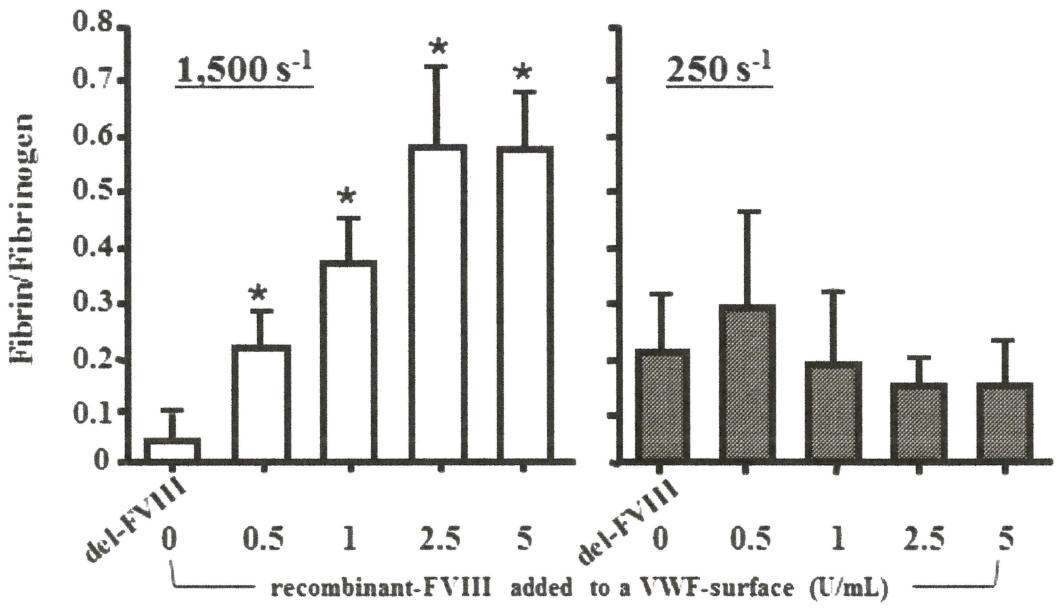
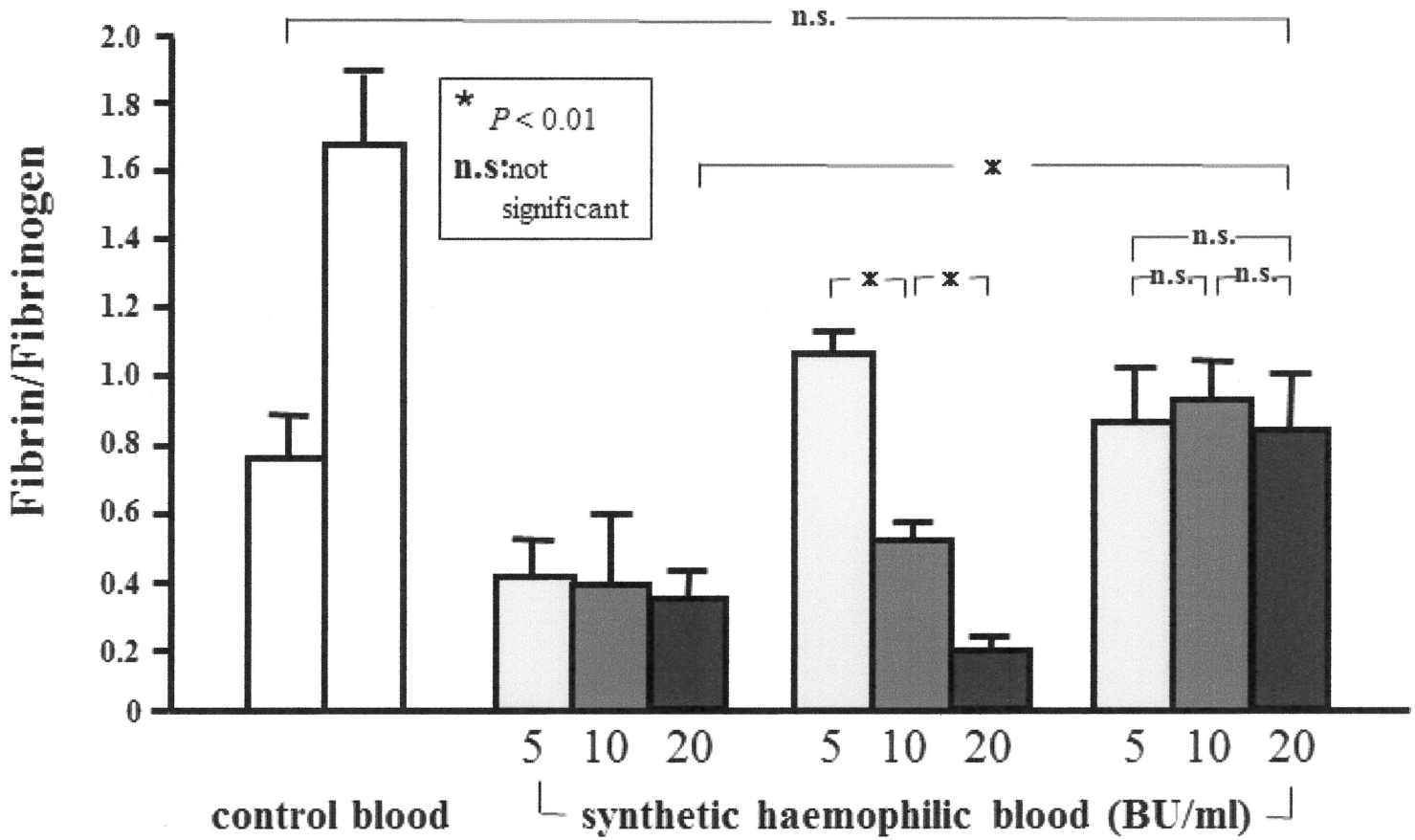

Fibrin/Fibrinogen
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1,500 s-1
250 s-1
*
*
*
*
del-FVIII
0
0.5
1
2.5
5
del-FVIII
0
0.5
1
2.5
5
recombinant-FVIII added to a VWF-surface (U/mL)

Fig. 3



S-FVIII in sample blood	(+)	(+)	-	-	-	+	+	+	-	-	-
I-FVIII on VWF-surface	-	+	-	-	-	-	-	-	+	+	+

Fig. 4