In our recent study (11), we demonstrated that FVIIa/TF activated FVIII and then inactivated FVIII by limited proteolysis at Arg^{372} (and Arg^{740}) and Arg^{336} in the HCh, respectively. In contrast, this enzyme did not proteolyse at Arg^{1689} in the LCh. Due to the inhibition of LCh cleavage, the FVIII activation by FVIIa/TF appeared to result in ~10% of peak level relative to full activation of FVIII by thrombin. This was supported by an earlier report (12) by mutational analysis using R1689A FVIII mutant. Intriguingly, FVIIa/TF-catalysed activation of FVIII was more rapid than that by thrombin, however. In addition, FVIIa/TF could activate FVIII to some extent even in the presence of VWF. We proposed, therefore, that as well as interaction with FIX and/or FX, FVIII (complexed with VWF) was initially activated by FVIIa/TF on the surface of injured vessel walls, contributing to the initiation of haemostasis by enhancing the assembly of intrinsic tenase and by leading to the rapid local generation of thrombin in the early phases of coagulation (11).

Recombinant (r)FVIIa products have been used for the treatment of bleeding in haemophilia A patients with inhibitors, acquired haemophilia, and congenital FVII deficiency. The action of rFVIIa is mediated by TF-dependent and/or TF-independent mechanisms (13, 14). At pharmacological dosages (~100 µg/kg), rFVIIa binds to locally activated platelets independent of TF following vascular damage. FX is activated directly on the platelet surfaces, resulting in enhanced localised thrombin generation and the formation of stable fibrin clots (15). The initial platelet activation is mediated by FVIIa binding to TF exposed after tissue injury, and consequently the action of therapeutic rFVIIa is restricted to injured sites. More recently, a FVIIa analog NN1731, a variant of rFVIIa with three amino acid substitutions (V158D/E296V/M298Q) containing a thrombin/FIXa-mimicking catalytic domain, has been suggested to be more effective than rFVIIa products (16). Similar to rFVIIa, NN1731 binds to TF, generating similar amounts of TF-dependent FXa. In addition, NN1731 binds to activated platelets, but its enzymatic activity is much greater than rFVIIa (17). In a cell-based model of FX activation *in vitro*, NN1731 appeared to be ~30-fold more potent than rFVIIa on activated platelets in the absence of TF, and ~1.2-fold greater in the presence of TF-expressing monocytes (18). This property of NN1731 has been confirmed in animal (19) and *in vitro* human models (20). A clinical development program for NN1731 is ongoing for haemophilia A patients with inhibitors.

In the present study, we compared NN1731 with rFVIIa in mechanisms of FVIII activation. We demonstrated that NN1731 had a greater potential than rFVIIa as an activator for the up-regulation of FVIII activity. In addition, the enhanced catalytic activity of NN1731 was most evident in the absence of TF, and appeared to be less related to binding affinity. TF-independent FVIII activation might represent a potential extra mode of its enhanced haemostatic effect.

Materials and methods

Reagents

Purified rFVIII preparations were a generous gift from Bayer Corp. Japan (Osaka, Japan). rFVIIa and its analog (NN1731) were kindly provided by Dr. Mirella Ezban (Novo Nordisk, Bagsværd, Denmark). FVIIIa, LCh (^{1649}A3C1C2 and ^{1690}A3C1C2), HCh (A1-A2-B), A1, and A2 subunits of FVIII were isolated and purified from rFVIII (21). The rA3 and rC2 domains of FVIII were expressed and purified as previously described (22, 23). VWF was isolated and purified from FVIII/VWF concentrates using gel filtration and immune-beads coated with immobilised FVIII mAb (24). SDS-PAGE of the isolated subunits followed by staining with GelCode Blue Reagent (Pierce, Rockford, IL, USA) showed >95% purity. Protein concentrations were determined by the Bradford's method. The mAbC5 (25), recognising the acidic region of the A1 domain, was a generous gift from Dr. Carol Fulcher (Scripps Clinic, La Jolla, CA, USA). Human FIXa and FX (Hematologic Technologies, Essex Junction, VT, USA), FXa (Enzyme Research Laboratories, South Bend, IN, USA), FVIIa-specific inhibitor peptide E-76 (Bachem, Torrance, CA, USA), chromogenic Xa substrate S-2222 (Chromogenix, Milano, Italy), recombinant lipidated TF (Innovin®; Dade Behring, Marburg, Germany), and Glu-Gly-Arg-chloromethylketone (EGR-ck; Calbiochem, San Diego, CA, USA) were purchased from the indicated vendors. FVIII-deficient plasmas were purchased from George King Biomedical (Overland Park, KS, USA). All other coagulation factors in these plasmas were within normal activity levels. PL vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, and 30% phosphatidylethanolamine (Sigma, St Louis, MO, USA) were prepared using *N*-octylglucoside (26).

Active-site modified EGR-NN1731

NN1731 was inactivated by the addition of a 10-fold molar excess of EGR-ck in 50 mM HEPES, pH 7.2, and 0.1 M NaCl and incubation overnight at 4°C. Unbound EGR-ck was removed by extensive dialysis at 4°C in 20 mM HEPES, pH 7.2, 0.1 M NaCl, 5 mM $CaCl_2$, and 0.01% Tween 20 (HBS-buffer) (11). Inactivation of NN1731 was considered complete when residual FVIIa activity was <0.2% as measured in FVIIa-specific assay.

Clotting assay

FVIII activity was measured in a one-stage clotting assay using FVIII-deficient plasma. All reactions were performed at 37°C. FVIII products were incubated in HBS-buffer containing 0.1% bovine serum albumin (BSA) plus the indicated concentrations of NN1731, PL and TF. Samples were removed from mixtures at the indicated times, and FVIIa/TF reaction was immediately terminated by the addition of E-76 (2.5 µM) and 2,500-fold dilution. The presence of FVIIa/TF and E-76 in the diluted sample did not affect FVIII activity (<5%) in the coagulation assay.

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FXa generation assay

The rate of conversion of FX to FXa was monitored in a purified system. FXa was generated at 37°C in HBS-buffer containing 0.1% BSA. Various concentrations of FVIII and PL (80 µM) were incubated with FVIIa products (30 nM) without TF or with FVIIa products (0.1 nM) with TF (1 nM). The FVIIa reaction was terminated after 10 seconds (sec) by the addition of E-76 (2.5 µM). FXa generation was initiated by the addition of 2 nM FIXa and 200 nM FX. Aliquots were removed at the indicated time to assess initial rates of product formation, and added to tubes containing EDTA (100 mM) to stop the reaction. Rates of FXa generation were determined at 405 nm using a microtiter plate reader after the addition of chromogenic substrate, S-2222 (0.46 mM).

SDS-PAGE and Western blotting for FVIII cleavage

NN1731 (30 nM) in the presence or absence of 1 nM TF were added to 30 nM FVIII with 80 µM PL in HBS-buffer at 37°C. Aliquots were removed at the indicated times and the reactions were immediately terminated by adding SDS and boiling for 3 minutes (min). SDS-PAGE and Western blotting was performed using 8% gels at 150 V for 1 hour. Protein bands were probed using the indicated anti-A1 mAbC5 followed by goat anti-mouse IgG using an enhanced Chemifluorescence Western Blotting Kit (GE Healthcare, Little Chalfont, UK). Densitometric scans were quantitated using FLA3000 (Fuji Film, Tokyo, Japan)

Surface plasmon resonance (SPR)-based assay

The kinetics of FVIII and EGR-NN1731 interaction were determined by SPR-based assay using a BIAcore X instrument (Biacore AB, Uppsala, Sweden) (11). The reactions were run at 37°C. EGR-NN1731 was covalently coupled (0.4 ng/mm^2) to the CM5 chip. Association of the ligand was monitored at a flow rate of 30 µl/min for 2 min, and dissociation of bound ligand was recorded over a 2 min-period by replacing with buffer alone. Nonspecific binding, corresponding to ligand binding to the uncoated chip, was subtracted from the signal. Rate constants for association (k_{assoc}) and dissociation (k_{diss}) were determined using the commercial evaluation software (Biacore AB).

Data analysis

All experiments were performed at least four separate times, and means ± SD are shown. Nonlinear least squares regression analyses were performed using Kaleida Graph (Synergy Software, Reading, PA, USA). Kinetics parameters were determined in FXa generation assays. The K_m and V_{max} values were calculated by fitting the data using nonlinear least squares regression analysis in the Michaelis-Menten equation.

Results

FVIII activation by NN1731

Firstly, we examined whether NN1731 as well rFVIIa contributed to the activation and inactivation of FVIII. FVIII (30 nM) was incubated with the FVIIa product (30 nM) together with PL (80 µM) in the presence or absence of TF (1 nM), and FVIII activity was measured in a one-stage clotting assay. To eliminate the influence of FVIIa and TF/PL in this assay, 2,500-fold dilutions of reactant mixtures were utilised. The lowest level of FVIII activity detected in these assays was ~0.01 nM, and under these conditions, therefore, FVIII was used at ~30-fold higher than physiological concentrations. In addition, the pharmacological dose of rFVIIa is ~100 µg/kg (~25 nM in plasma), and the rFVIIa reactions *in vitro* in the absence of TF were very weak. As a consequence, FVIIa was utilised at 30 nM in these experiments. Time-course reactions of FVIII activation by NN1731 (panel a) and rFVIIa (panel b) are shown in ▶Figure 1A. In the presence of TF (1 nM; closed triangles), NN1731 enhanced FVIII activity very rapidly (<30 sec) by ~2.9-fold over the base level, and appeared to be slightly more potent than rFVIIa (~2.6-fold increase). This elevated FVIII activity rapidly decreased and fell below initial levels after ~10 min. In contrast in the absence of TF (open circles in ▶Fig. 1A), NN1731 promoted FVIII activity by 5~6-fold in a time-dependent manner over 5 min, and the plateau level remained constant for ~30 min after reaction. Furthermore, this peak level was ~2-fold greater than that in the presence of TF (1 nM). The increase in FVIII activity mediated by NN1731 was PL-dose dependent. The reaction was saturable at a concentration of 80 µM and less evident in the absence of PL (data not shown). Although rFVIIa alone increased FVIII activity very slowly, the peak activity was shown to be limited to ~1.7-fold of the initial level even after 30-min reaction. These results demonstrated that TF influenced NN1731 and rFVIIa reactions with FVIII in a similar manner, but NN1731 mediated significant activation of FVIII (as well as FX) in the presence of PL surface even in the absence of TF.

The role of TF in the pattern of FVIII activation by NN1731

Our results indicated that the mechanisms of NN1731– and rFVIIa-catalysed activation of FVIII were similar in the presence of TF (1 nM), but were significantly different in the absence of TF. Further experiments were designed, therefore, to compare FVIII activation by NN1731 and rFVIIa in the presence of various concentrations of TF. In the absence of TF, NN1731 induced an incremental increase in FVIII activity up to ~6-fold (▶Fig. 1A, panel a). The addition of TF depressed the plateau peak level of FVIII activity at >5 min in a dose-dependent manner, and FVIII activity decreased to below the initial level in the presence of TF (>1 nM). In contrast, FVIII activity in early-reaction phases with NN1731 (<30 sec) was increased dose-dependently by the addition of TF. In particular, FVIII activity with TF (>0.5 nM) reached maximum peak

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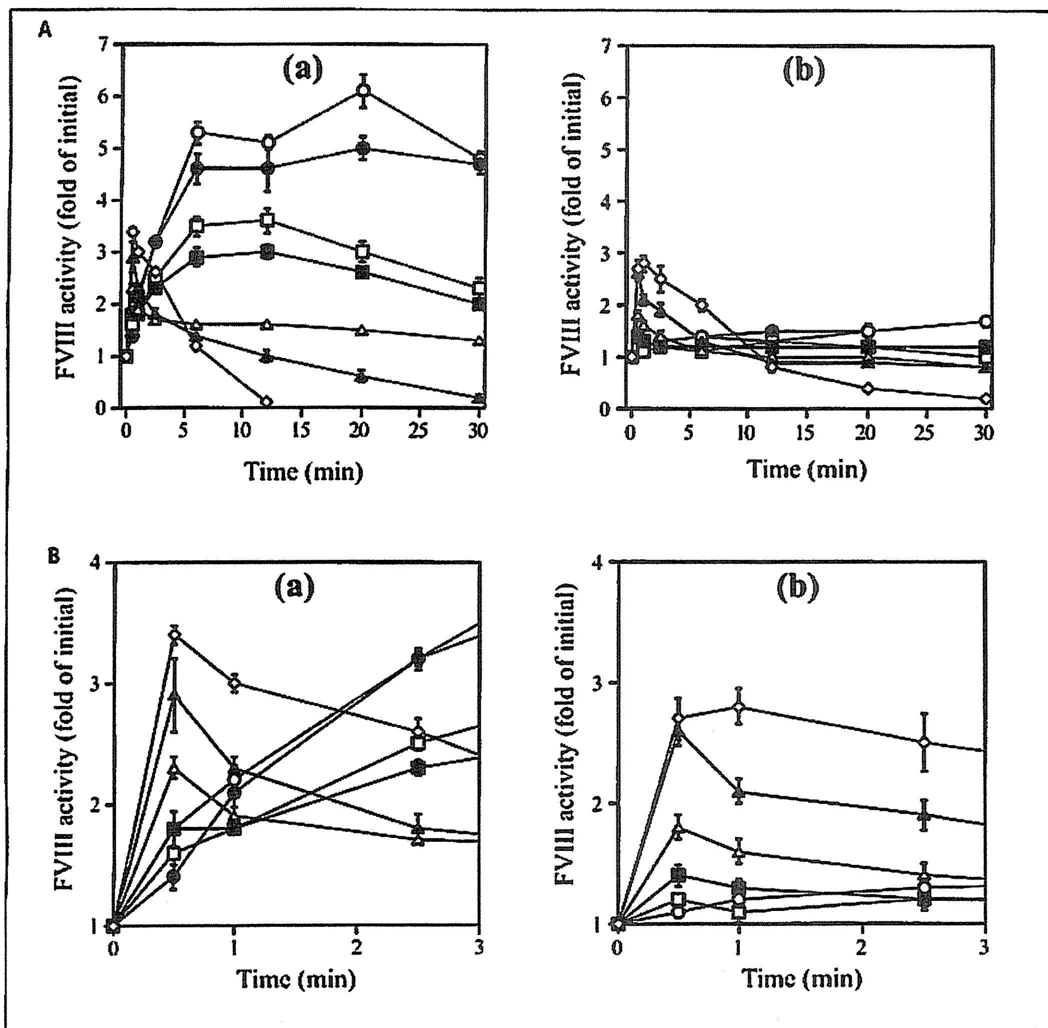

Figure 1: Activation of FVIII following reaction with NN1731 or rFVIIa in the presence of TF. A) FVIII (30 nM) was incubated with 30 nM NN1731 (panel a) or rFVIIa (panel b) with PL (80 μM) and various concentrations of TF (0 nM, ○; 0.01 nM, ●; 0.1 nM, □; 0.2 nM, ■; 0.5 nM, Δ; 1.0 nM, ▲; 2.0 nM, ◊) for the indicated times (0–30 min), after which the reaction was terminated by E-76 peptide. Each sample was tested immediately for FVIIIa activity in a one-stage clotting assay. B) Enlarged time course of NN1731 (panel a) or rFVIIa (panel b) reaction for 0–2.5 min in (A), respectively. The initial activity of FVIII was ~25 units/ml. Experiments were performed at least four separate times and means ± SD are shown.

levels during a 30 sec-reaction period (▶Fig. 1B, panel a). Conversely, rFVIIa in the absence of TF elevated FVIII activity very slowly (by ~1.7-fold), whilst rFVIIa with TF promoted FVIII activity rapidly within 30 sec in a dose-dependent manner (▶Fig. 1A, panel b). In the presence of TF (>1 nM), the pattern of rFVIIa-catalysed reaction was similar to that for NN1731 (▶Fig. 1B, panel b). These findings demonstrated that the biphasic reactions for activation and inactivation of FVIII appeared to be dramatically dependent of the presence of TF in both NN1731 and rFVIIa. Changes were more predominant for NN1731 in the presence of small amounts of TF (<0.2 nM).

FVIII proteolysis by NN1731

FVIII is activated very rapidly by rFVIIa/TF by proteolysis at Arg^{372} and Arg^{740} in the HCh, and subsequently inactivated following proteolysis at Arg^{336}. No cleavage of the LCh is evident in early-timed reactions (<30 min) (11). In the present study we noted that FVIII reactions mediated by NN1731 and rFVIIa in the presence of TF were different from those in its absence, and we further investigated, therefore, NN1731-catalysed proteolytic cleavage of FVIII using SDS-PAGE. Proteolysed fragments were visualised by Western blotting using anti-A1 mAbC5 recognising the A1 acidic region (▶Fig. 2A, B). Disappearance and/or appearance of the A1-A2-B, $A1^{1-372}$, and $A1^{337-372}$-A2, reflecting cleavage at Arg^{740}, Arg^{372}, and Arg^{336}, respectively, was quantitated by band densitometry (▶Fig. 2C). Cleavage sites of FVIII fragments derived from NN1731 proteolysis (with/without TF) were identified using automated NH_2-terminal sequence analysis. These cleavage sites were confirmed to be identical to those derived from rFVII/TF proteolysis (data not shown). Although HPLC-gel filtration was used to fractionate intact FVIII, $A1^{1-372}$ fragments remained evident even in the absence of FVIIa, indicating a possible high sensitivity of mAbC5. In the presence of TF (▶Fig. 2A), both NN1731 (panel a) and rFVIIa (panel b) rapidly (<0.5 min) proteolysed the HCh (A1-A2-B) into A1-A2 fragments by cleavage at Arg^{740}, and this was followed by sequential generation of $A1^{1-372}$ fragments by cleavage at Arg^{372}. Subsequently, $A1^{337-372}$-A2 fragments mediated

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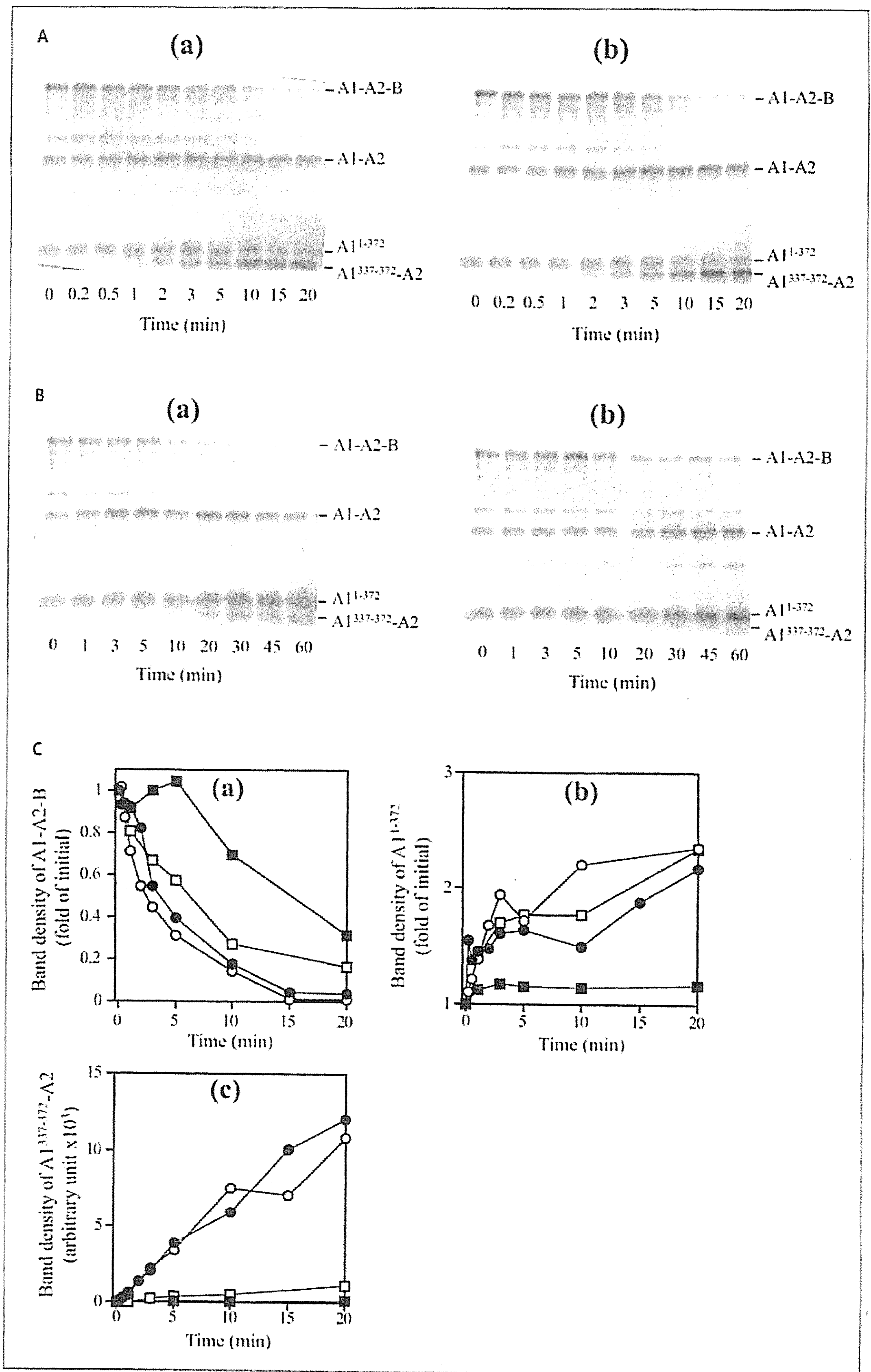

Figure 2: Comparison with FVIII cleavage by NN1731 and rFVIIa. FVIII (30 nM) was incubated with 30 nM NN1731 (panel a) or rFVIIa (panel b) in the presence (A) or absence (B) of TF (1 nM) with PL (80 µM) for the indicated times (plus TF; 0–20 min, minus TF; 0–60 min). Samples were run on 8% gels followed by Western blotting using anti-A1 mAbC5. C) Data (reaction for 0–20 min) obtained by quantitative densitometry of the intact A1-A2-B (panel a), $A1^{1-372}$ (panel b), and $A1^{337-372}$-A2 (panel c) in (A) and (B). The symbols used are: NN1731/TF, ○; rFVIIa/TF, ●; NN1731, □; rFVIIa, ■.

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by cleavage at Arg[336] were visualised at ~1 min. These findings were consistent with rapid up- and down-regulation of FVIII, although cleavage by NN1731/TF was somewhat faster than that by rFVIIa/TF. In the absence of TF (▶Fig. 2B), NN1731 (panel a) rapidly, and time-dependently proteolysed A1-A2-B into A1-A2 fragments by cleavage at Arg[740], followed by generation of A1[1-372] fragments by cleavage at Arg[372]. Subsequently, cleavage at Arg[336] led to the appearance of A1[337-372]-A2 fragments, ~10 min after reaction with NN1731. These data were in keeping with the peak level of FVIII activity observed during FVIII activation. In contrast, cleavage at Arg[740] and Arg[372] was markedly slower with rFVIIa (panel b) than with NN1731, and cleavage at Arg[336] appeared to be minimal within 60 min. These results were consistent with the differences in FVIII procoagulant activation mediated by NN1731 and rFVIIa in the absence of TF. Little cleavage of the LCh was observed in these experiments (data not shown), indicating that LCh reactions were not associated with FVIII activation by NN1731 or rFVIIa.

Kinetic analysis of FVIII activation by FVIIa products in FXa generation assays

To further investigate the functional mechanisms of NN1731 and rFVIIa with FVIII, kinetic parameters of FVIII activation by FVIIa products were determined in FXa generation assays. Various concentrations of FVIII and PL (80 μM) were incubated with FVIIa products (30 nM) without TF or with FVIIa products (0.1 nM) with TF (1 nM). Reactions were terminated at 10 sec, reflecting FVIII activation alone, by the addition of E-76. FXa generation was initiated by the addition of FIXa (2 nM) and FX (200 nM). Non-activated FVIII alone had little effect on FXa generation under these conditions (data not shown), and the rate of FXa generation was considered, therefore, to directly reflect the concentration of activated FVIII. Results are shown in ▶Figure 3 and summarised in ▶Table 1. In the presence of TF (▶Fig. 3A), the K_m obtained for FVIII activation by NN1731 was ~1.8-fold lower than for rFVIIa (27.3 and 49.2 nM, respectively), and the V_{max} obtained for FVIII activation by NN1731 was ~2.3-fold greater (70.0 and 30.4 nM·min^{-1}, respectively). In the absence of TF, however (▶Fig. 3B), although the K_m obtained by NN1731 was only ~1.3-fold lower than that seen with rFVIIa (50.5 and 68.1 nM, respectively), the V_{max} obtained by NN1731 was ~7.9-fold greater (92.5 and 11.7 nM·min^{-1}, respectively). Overall, in the absence of TF the catalytic efficiency of NN1731 for FVIII activation (V_{max}/K_m) was ~11-fold greater than that of rFVIIa, whilst in the presence of TF this measurement was ~4-fold greater for NN1731 than for rFVIIa. In this experiment, the concentrations of protease in the presence of TF were 300-fold lower than those in its absence, i.e. the apparent k_{cat} values of NN1731 and rFVIIa in the presence of TF were ~230- and ~780-fold higher, respectively, than those in its absence, and the affinities of NN1731 and rFVIIa for FVIII activation in the presence of TF were ~1.9- and ~1.4-fold higher than those in its absence.

Binding of FVIII(a) subunits to EGR-NN1731 in SPR-based assays

We have previously established a direct method for assessing interactions of FVIII with EGR-rFVIIa, in place of native rFVIIa, using SPR-based assays (11). Similar experiments were developed, there-

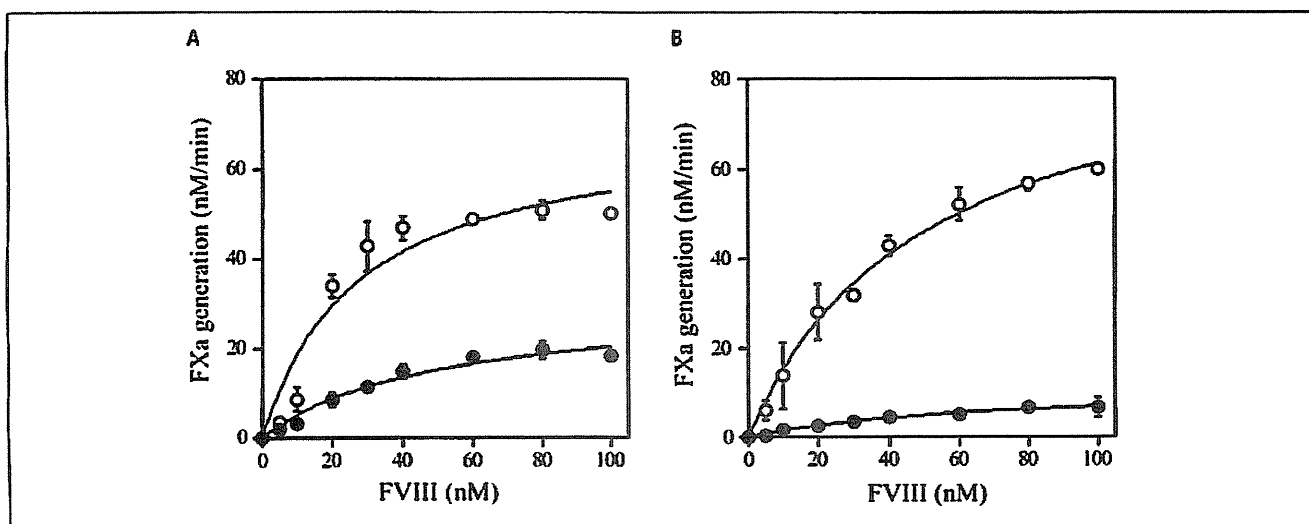

Figure 3: Kinetics analyses on FVIII activation caused by NN1731 and rFVIIa in FXa generation assay. Various concentrations of FVIII and PL (80 μM) were activated by 0.1 nM NN1731 (○) or rFVIIa (●) in the presence of TF (1 nM) (A) or activated by 30 nM NN1731 (○) or rFVIIa (●) in the absence of TF (B). The reaction was terminated at 10 sec by the addition of E-76. FXa generation was initiated by the addition of FIXa (2 nM) and FX (200 nM). Initial rates of FXa generation on FVIII activation by NN1731 and rFVIIa were plotted as a function of FVIII concentration and fitted to the Michaelis-Menten equation by non-linear least squares regression. Experiments were performed at least five separate times, and mean ± SEM values are shown.

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fore, to study interactions of FVIII and EGR-NN1731. Representative curves for FVIII and EGR-NN1731 binding are shown in ▶Figure 4. Binding parameters are summarised in ▶Table 2. FVIII and its active form (FVIIIa) bound to NN1731 with ~1.4-fold higher affinity (K_d; 0.62 and 5 nM) than to rFVIIa. In the competitive binding assay using EGR-FVIIa, we confirmed that the EGR-FVIIa inhibited the FVIII and EGR-FVIIa binding in similar dose-dependent manner (data not shown), supportive of validity of this assay. The HCh bound to NN1731 (K_d; 1.9 nM) with ~3.1-fold higher than to rFVIIa. These differences were more predominant than those with the LCh (by ~2.2-fold). In particular, the A2 fragment bound to NN1731 with ~2.1-fold higher affinity (K_d; 12.5 and 26.4 nM), indicating a likely significant contribution of this higher affinity for reactions of NN1731. No binding of the A1 domain was evident in these experiments. Thrombin-cleaved LCh (^{1690}A3C1C2) bound to NN1731 with a similar affinity to LCh, suggesting that the acidic region of the A3 domain was not an essential component of the NN1731-interactive site. The A3 and C2 fragments of LCh bound to NN1731 with a similar and less affinity than to rFVIIa. Overall, the findings suggested that the binding affinity of NN1731 for FVIII was somewhat greater than that of rFVIIa, and supported the results obtained in FXa generation assays, although the K_m values obtained in the enzyme assays were much greater than the K_d values in this binding assay. In addition, the A2 domain appeared to play a significant role in interactions with NN1731 as well as with rFVIIa.

Effect of VWF on FVIII activation by NN1731

Our previous study showed that the presence of VWF decreased maximum binding of FVIII to rFVIIa by ~50% and only modestly counteracted rFVIIa-catalysed FVIII activation, suggesting that VWF partially modulated FVIIa/TF-mediated activation of FVIII by direct competition with FVIII molecule (11). We have compared, therefore, the effects of VWF on NN1731- and rFVIIa-catalysed activation of FVIII using one-stage clotting assays. FVIII (30 nM) was preincubated with various concentrations of VWF prior to reaction with 30 nM NN1731 (panel a) or rFVIIa (panel b) in the presence of PL (80 μM) and TF (1 nM). In the presence of TF (▶Fig. 5A), VWF similarly and modestly moderated the spiked activation of FVIII by NN1731 and rFVIIa, and the subsequent inactivation reactions were also depressed in dose-dependent manners. In these experiments, VWF (20 μg/ml) inhibited FVIII activation by NN1731 and rFVIIa by only ~20%. In contrast, in the absence of TF (▶Fig. 5B), VWF inhibited FVIII activation by both products more significantly. VWF at physiological concentrations (10 μg/ml) decreased the peak activity of FVIII activation by NN1731 and rFVIIa by ~90% and ~85%, respectively. These findings were supported by data obtained from FVIII proteolysis by the FVIIa products in Western blotting. In the presence of TF, cleavage at Arg^{336} by both NN1731 and rFVIIa was inhibited by VWF dose-dependently, and this inhibition was more prominent than that at Arg^{372} and Arg^{740}. In the absence of TF, inhibition of NN1731-mediated cleavage at Arg^{372} and Arg^{740} appeared to be more significant, although inhibition of rFVIIa-induced cleavage at these sites was poorly observed (data not shown). The results indicated, therefore, that VWF inhibited NN1731-catalysed proteolysis of FVIII more potently in the absence of TF than in its presence.

Discussion

NN1731 is an analog of rFVIIa, in which three amino acid residues are substituted (V158D/E296V/M298Q), containing a thrombin/

Table 1: Kinetic parameters of FVIII activation by NN1731 and rFVIIa in FXa generation assays. Reactions were performed as described in *Methods*. Parameters were calculated by non-linear regression analysis using the Michaelis-Menten equation. Experiments were performed at least five separate times, and mean ± SEM values are shown.

	K_m	V_{max}	V_{max}/K_m
	nM	nM·min^{-1}	min^{-1}
NN1731/TF	27.3 ± 10.9	70.0 ± 10.3	2.56
rFVIIa/TF	49.2 13.5	30.4 ± 3.9	0.62
ratio	*(1.8-1)*	*(2.3)*	*(4.1)*
NN1731	50.5 ± 6.9	92.5 ± 6.0	1.83
rFVIIa	68.1 ± 17.4	11.7 ± 1.5	0.17
ratio	*(1.3-1)*	*(7.9)*	*(10.8)*

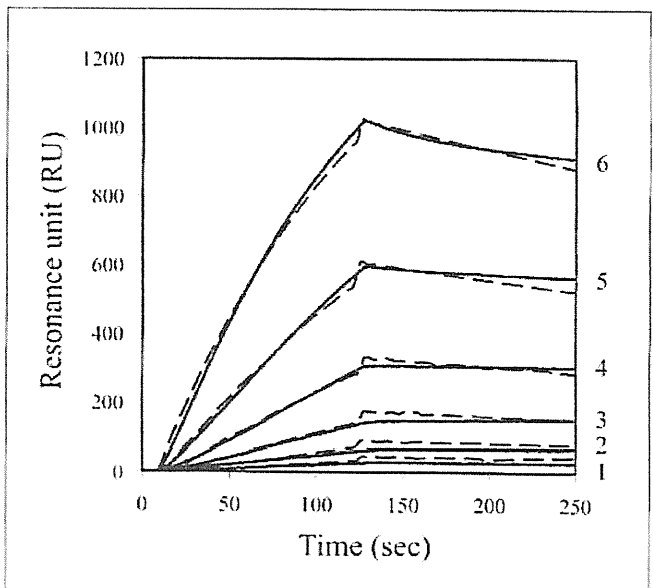

Figure 4: Direct binding of FVIII to EGR-NN1731. Various concentrations of the FVIII were injected to the EGR-FVIIa (0.4 ng/mm²) immobilised onto the sensor chip at a flow rate of 30 μl/min for 2 min, followed by the change of running buffer for 2 min. The solid and dashed lines 1–6 show representative response curves for the different concentrations of FVIII (0.315, 0.625, 1.25, 2.5, 5, 10 nM, respectively) and fitting curves.

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Table 2: Binding parameters between FVIII(a) subunit and EGR-FVIIa interaction in a SPR-based assay. Reactions were performed as described in *Methods*. Parameters were calculated by non-linear regression analysis using the evaluation software provided by Biacore AB. Experiments were performed at least five separate times, and mean ± SEM values are shown. The K_d values were calculated as k_{diss}/k_{ass}. *: Not determined.

Ligands	NN1731			rFVIIa	
	k_{ass}	k_{diss}	K_d	$K_d^{(11)}$	$K_d^{rFVIIa}/K_d^{NN1731}$
	$\times 10^4$ $M^{-1}s^{-1}$	$\times 10^{-3}$ s^{-1}	nM	nM	-fold
FVIII	297 ± 9	1.84 ± 0.03	0.62	0.82	1.3
FVIIIa	200 ± 11	9.93 ± 0.66	5.0	7.4	1.5
HCh	47.1 ± 1.2	0.89 ± 0.05	1.9	5.9	3.1
^{1649}A3C1C2	44.7 ± 1.0	3.82 ± 0.05	8.6	18.9	2.2
^{1690}A3C1C2	60.2 ± 4.3	7.10 ± 0.39	11.8	15.1	1.3
A1	n.d.*	n.d.*	-	-	-
A2	9.75 ± 0.60	1.22 ± 0.06	12.5	26.4	2.1
A3	4.35 ± 0.49	8.33 ± 0.28	191	273	1.4
C2	0.87 ± 0.03	6.32 ± 0.09	726	376	0.52

FIXa-mimicking catalytic domain. It is a more potent FX activator than rFVIIa in the presence of TF, and in particular activates FX strongly even in the absence of TF (17, 18). Recently we have further developed a previous study by Warren et al. that showed that FVIIa/TF catalysed proteolyses of FVIII (27), and suggested that FVIIa/TF activated not only FX and FIX, but also FVIII in physiological mechanisms of blood coagulation (11). In the present study, we compared the activation of FVIII mediated by rFVIIa and NN1731.

The rapid reaction of FVIII by NN1731 in the presence of TF appeared to be somewhat greater than that by rFVIIa. In its absence, however, NN1731 markedly increased FVIII activity, but rFVIIa did not significantly proteolyse the HCh. The peak level of NN1731-mediated FVIII activity was depressed by TF dose-dependently, although interestingly, even trace amounts of TF (<~0.2 nM) markedly shortened the time to peak of FVIII activation by NN1731. The peak activity mediated by NN1731/TF was lower by ~2-fold than that by NN1731 alone. FVIII inactivation associated with cleavage at Arg^{336} was evident only in the presence of TF, and TF dramatically altered not only cleavage velocity, but also the reaction pattern of NN1731-mediated FVIII activation and inactivation. Overall, we demonstrated that in the absence of TF, NN1731 likely revealed thrombin-like protease characteristics rather than those of native FVIIa on FVIII, whilst, in its presence, NN1731 behaved as more FVIIa-like than thrombin-like in these mixtures.

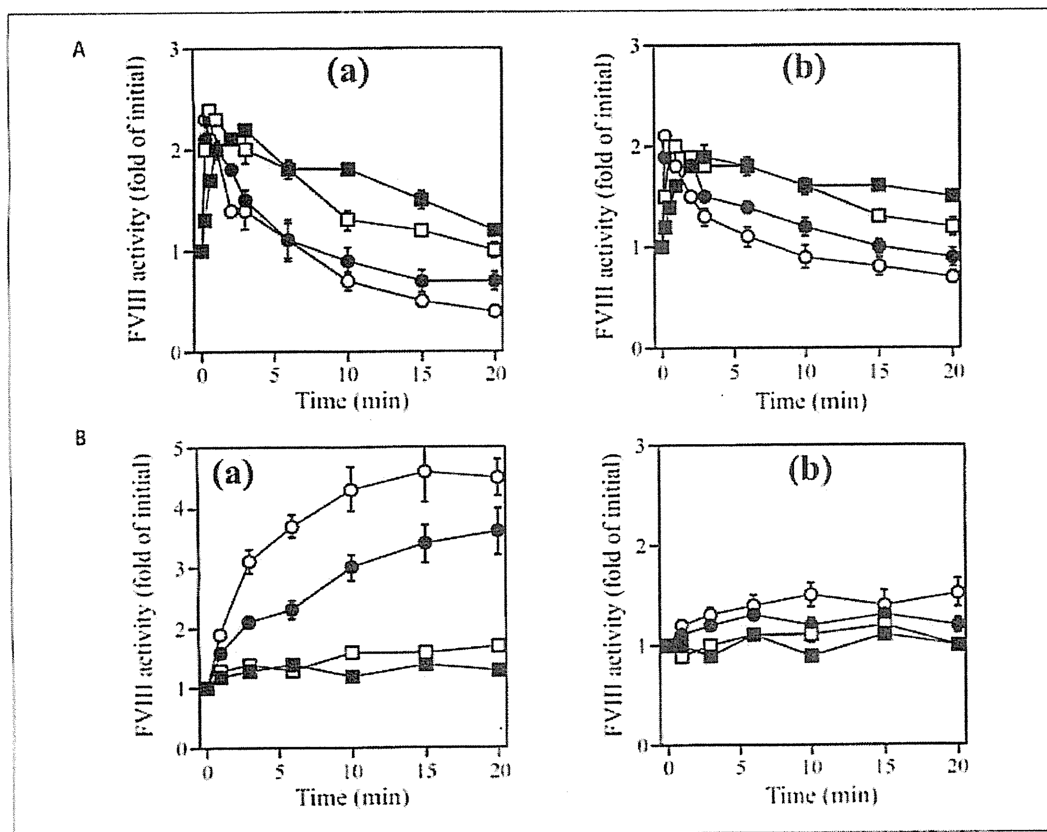

Figure 5: Effect of VWF on NN1731-catalysed reaction of FVIII. FVIII activation: FVIII (30 nM) was preincubated with various concentrations of VWF (0 µg/ml, ○; 5 µg/ml, ●; 10 µg/ml, □; 20 µg/ml, ■) prior to reaction with 30 nM NN1731 (panel a) or rFVIIa (panel b) in the presence (A) or absence (B) of TF (1 nM) with PL (80 µM). Each sample was tested immediately for FVIIIa activity in a one-stage clotting assay. The initial activity of FVIII was ~25 units/ml. Experiments were performed at least four separate times and means ± SD are shown.

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The V_{max} of NN1731-catalysed FVIII activation was significantly greater than that of rFVIIa in the absence of TF, but this was marginally greater in its presence. While, the K_m of FVIII and NN1731 interaction was marginally greater than that of rFVIIa, independently of TF. These findings supported that this potential of NN1731 without TF appeared likely to attribute to the catalytic activity of NN1731 rather than the binding affinity. Although SPR-based assays showed that NN1731 bound to FVIII slightly tightly compared to rFVIIa, the binding affinity observed in this method (K_d) was ~80-fold greater than that seen in FXa generation (K_m). This discrepancy may have been due to conformational changes in the presence of PL. An earlier report described that the increase for K_m on FX (or FIX) activation by FVIIa was dependent on the presence of PL (8). Alternatively, changes in binding affinity of EGR-NN1731 could have contributed to the findings. Some types of active-site-blocked FVIIa with soluble TF had been reported to possess ~5-fold higher affinity than native FVIIa (28), suggesting that the affinities of FVIII and EGR-FVIIa may be miscalculated.

Similar to rFVIIa/TF, NN1731/TF-mediated FVIII activation was partially moderated by the presence of VWF at the physiological concentration. Distinct from PL-dependent serine proteases that were regulated by VWF, however, rFVIIa and NN1731 in the presence of TF could activate FVIII complexed with VWF to a significant extent, although the FVII/TF-catalysed FVIII activation in the absence of VWF was reached at ~10% level of full activation of FVIII by thrombin due to the inhibition of LCh cleavage. From this finding, TF might compete with VWF for binding to FVIII, resulting in modest activation of FVIII by rFVIIa (and NN1731) with TF even in the presence of VWF. This potential interaction is currently under investigation. However, VWF at physiological concentrations significantly inhibited (by ~90%) rFVIIa- and NN1731-mediated FVIII activation in the absence of TF, might suggesting that under the conditions without exposed TF, NN1731 (and rFVIIa)-mediated FVIII activation would hardly occur, except for the presence of anti-FVIII inhibitors that prevent the FVIII-VWF interaction (as described below).

It is difficult to determine precisely whether FVIIIa was generated from FVIII by thrombin or FVIIa/TF in these complex mixtures, and our suggestion that FVIIa/TF activates FVIII as well as FX (and FIX) in the initiation phase of coagulation remains to be confirmed in circulating blood. Nevertheless, two of the current findings especially indicated that this reaction could be physiologically relevant. Firstly, SPR-based assays using high flow rates (30 µl/min) revealed high affinities for EGR-FVIIa products with FVIII (K_d; 0.6–0.8 nM), suggesting that FVIII at physiological concentrations (~1 nM) could interact with FVIIa under flow conditions. Although the binding affinities obtained using active-site modified FVIIa may be overestimated, the K_d value of EGR-FVIIa for FVIII was similar to that of active-site blocked FVIIa for soluble TF (28). Secondly, FVIII activation mediated by FVIIa/TF was more rapid (<30 sec) than that induced by thrombin (11). *In vivo*, the anti-coagulant protein tissue factor pathway inhibitor (TFPI) circulates in plasma. TFPI is complexed with generated FXa and abrogates the procoagulant activity of FVIIa/TF. Hence, significant inhibition of FVIIa/TF activity by TFPI/FXa complex was observed after 1 min *in vitro* (29). In a capillary model under flow conditions, adding TFPI completely blocked FXa generation induced by FVIIa/TF (30). In the presence of FVIII and FIX, however, FXa generation was observed at appreciable rates even at the same TFPI concentration. Taken together, FVIIa/TF reaction with FVIII under flow conditions could be presumed even in the presence of TFPI.

The clinical use of bypassing agents for haemophiliacs with inhibitor is well described (31). Furthermore, the presence of trace amounts of FVIII appears likely to govern the bleeding phenotype of haemophiliacs in the presence or absence of inhibitor (32). A number of inhibitory mechanisms of anti-FVIII inhibitors have been identified. Some inhibitors moderate FVIII activation by thrombin and FXa, and others limit FVIII-VWF interaction, FVIII-PL interaction, and FVIII-FIXa interaction (33). We have recently reported that FVIIa/TF-catalysed FVIII activation was not significantly affected by the various types of inhibitor (34). Thus,

What is known about this topic?

- The central role of factor (F)VIIa, a serine protease responsible for initiating blood coagulation in normal haemostasis, is the activation of FX and FIX.
- We have recently reported that FVIIa/tissue factor (TF) activates FVIII very rapidly by proteolysis at Arg^{372} and Arg^{740}, and it activates FVIII to some extent even in the presence of von Willebrand factor (VWF).
- As well as interaction with FIX and/or FX, FVIII (with VWF) is initially activated by FVIIa/TF on the injured vessel surface, may contributing to the initiation of haemostasis by enhancing the intrinsic tenase assembly and by leading to the rapid local generation of thrombin in the early phases of coagulation.
- A rFVIIa-analog, NN1731 (V158D/E296V/M298Q) containing a thrombin/FIXa-mimicking catalytic domain, is ~30-fold more effective on activated platelets without TF, but ~1.2-fold with TF, for FX activation, compared to rFVIIa. The action of NN1731 on FVIII activation remains unknown.

What does this paper add?

- In the presence of TF, NN1731 rapidly activated FVIII (~2.9-fold), followed by inactivation, and was slightly more active than rFVIIa. In its absence, NN1731-catalysed activation was enhanced ~6-fold at 5 min, and its peak level persisted for ~30 min.
- Both proteolysed the heavy chain at identical sites, independently of TF, but the cleavage by NN1731 alone was much slower at Arg^{336} than at Arg^{740} and Arg^{372}.
- The catalytic efficiency of NN1731-catalysed FVIII activation without and with TF was ~11- and ~4-fold greater, respectively, than equivalent reactions with rFVIIa.
- VWF moderated NN1731-catalysed activation more significantly than NN1731/TF.
- NN1731 is greater potential than rFVIIa in up-regulating FVIII activity in the absence of TF, and the TF-independent FVIII activation may represent a potential extra mode of its enhanced haemostatic effect.

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the ability of NN1731 to activate FX, FIX and FVIII even in the absence of TF could improve the therapeutic options for bleeding in haemophiliacs with inhibitor. The co-existence of FVIII and NN1731 in circulating blood could elicit improved responses in haemophiliacs with inhibitor. In particular, TF-independent NN1731 action to FVIII molecule might be very useful under the conditions of presence of anti-FVIII type 1 inhibitors, having the property of inhibition of FVIII-VWF association in circulating plasma (33).

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Conflict of interest
K.N. has received a grant from Bayer Hemophilia Award 2009.

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Effects of anti-factor VIII inhibitor antibodies on factor VIIa/tissue factor-catalysed activation and inactivation of factor VIII

Koji Yada; Keiji Nogami; Kenichi Ogiwara; Masaru Shibata; Midori Shima
Department of Pediatrics, Nara Medical University, Kashihara, Nara, Japan

Summary

Factor (F)VIIa/tissue factor (TF) rapidly activates FVIII activity by proteolysis at Arg^{372} and Arg^{740}, and subsequently inactivates FVIIIa activity by proteolysis at Arg^{336}, although this activation is weaker than that by thrombin. The effects of anti-FVIII inhibitor antibodies on these reactions remain unknown, however. In this study, 13 of anti-FVIII inhibitor antibodies recognising the A2 or C2 domain were prepared. None of them, irrespective of epitope specificity, significantly affected FVIIa/TF-catalysed FVIII activation in one-stage clotting assays. Anti-A2 and anti-C2 type 2 antibodies had little effect on the inactivation phase. Anti-C2 type 1 antibodies, however, modulated inactivation by 40–60% of that seen with control IgG, suggesting that the activity of FVIIIa generated by FVIIa/TF persisted in the presence of this specific type of inhibitor. SDS-PAGE analysis demonstrated that all antibodies had little effect on FVIIa/TF-catalyzed proteolysis at Arg^{372} and Arg^{740}. Anti-C2 type 1, however, significantly delayed cleavage at Arg^{336} in dose-dependent manners. Neither anti-A2 nor anti-C2 type 2 affected this reaction, and the findings were consistent with the results of the functional assays. In addition, anti-C2 monoclonal antibodies with type 1 and 2 demonstrated similar patterns of reaction as the anti-C2 polyclonal antibodies in FVIIa/TF-mediated FVIII mechanisms. We demonstrated that FVIIa/TF activated FVIII even in the presence of anti-FVIII antibodies, but inactivation patterns appeared to depend on inhibitor type. It could be important to determine the characteristic of these inhibitor antibodies for prediction of their effects on FVIIa-related FVIII reactions, and the results could have significant therapeutic implications.

Keywords

FVIII, FVIII inhibitor antibodies, FVIIa/TF, activation/inactivation, cleavage

Correspondence to:
Keiji Nogami, MD, PhD
Department of Pediatrics, Nara Medical University
840 Shijo-cho, Kashihara, Nara 634–8522, Japan
Tel.: +81 744 29 8881, Fax.: +81 744 24 9222
E-mail: roc-noga@naramed-u.ac.jp

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Introduction

Factor (F)VIII, a plasma protein deficient or defective in individuals with the severe congenital bleeding disorder, haemophilia A, functions as a cofactor in the tenase complex, responsible for phospholipid (PL)-dependent conversion of FX to FXa by FIXa (1). FVIII is synthesised as a single chain molecule consisting of 2,332 amino acid residues (molecular weight [MW]: ~300 kDa), and is arranged into three domains, A1-A2-B-A3-C1-C2, based on amino acid homology. FVIII is processed into a series of metal ion-dependent heterodimers by cleavage at B-A3 junction, generating a heavy chain (HCh) consisting of A1 and A2 domains together with heterogeneous fragments of proteolysed B domain linked to a light chain (LCh) consisting of A3, C1, and C2 domains (2). The catalytic efficiency of FVIII in the tenase complex is markedly enhanced by conversion into an active form, FVIIIa, by limited proteolysis by thrombin and FXa (3). Both enzymes proteolyse the FVIII HCh at Arg^{372} and Arg^{740}, and generate 50-kDa A1 and 40-kDa A2 subunits. The 80-kDa LCh is cleaved at Arg^{1689} producing a 70-kDa subunit. Proteolysis at Arg^{372} and Arg^{1689} is essential for generating FVIIIa cofactor activity (4). Cleavage at the latter site liberates FVIII from its carrier protein, von Willebrand factor (VWF) (5). FVIIIa activity is down-regulated by serine proteases including activated protein C (APC), following cleavage at Arg^{336} (3). This inactivation appears to reflect loss of a FX-interactive site, mediated by a modified interaction with the A2 subunit and an increased K_m of the truncated A1 for the substrate FX (6).

FVIIa is a potent serine protease responsible for initiating and propagating the blood coagulation cascade in normal haemostasis (7). Following injury to the blood vessel wall, tissue factor (TF) exposed to circulating blood, forms a complex with FVIIa, resulting in the initiation of haemostasis by the activation of FX (and FIX)

and the generation of minimal amounts of thrombin (8). These trace amounts of thrombin dissociate FVIII from VWF and promote platelet activation. Following these "priming" reactions, thrombin generation is accelerated by the propagation of tenase and prothrombinase enzymes on PL micelles exposed on platelet surfaces (9). In addition, a TF-independent cell-based FX activation mechanism has been identified involving direct binding to platelet membranes (10), and these concepts in particular have been applied to haemostatic therapy in haemophilia patients with inhibitors.

FVIII inhibitors develop as alloantibodies (alloAbs) in 20–30% of multi-transfused patients with haemophilia A and also arise as autoantibodies (autoAbs) in previously normal individuals resulting in acquired haemophilia A and severe bleeding tendency (11). The decrease or disappearance of FVIII(a) activity in the presence of inhibitors is associated with impairment of FVIII(a) cofactor function mediated by binding to functionally essential regions in the FVIII molecule. FVIII inhibitors can either inhibit FVIII completely or incompletely at saturating concentrations, corresponding to a classification of type 1 or type 2, respectively (12). Inhibitory epitopes have been localised to one or more of the A2, A3-C1, and C2 domains (13–15). In general, anti-C2 inhibitor antibodies with type 1 pattern prevent FVIII binding to PL, VWF, and FXa, whilst those classified as type 2 prevent FVIII binding to thrombin and FXa (16–18). On the other hand, anti-A2 antibodies prevent FVIII interaction with FIXa and thrombin (19, 20), and anti-A3-C1 antibodies prevent FVIII(a) binding to FIXa (15).

We have recently reported that the FVIIa/TF complex rapidly activated FVIII by limited proteolysis at Arg^{372} and Arg^{740} in the HCh (21). FVIIa/TF appeared to promote a "priming" mechanism to generate small amounts of FVIIIa in the early initiation phases of coagulation, although the degree of this activation was lower than that mediated by thrombin, due to the rapid cleavage at Arg^{336} and the much slower cleavage at Arg^{1689}. The effects of anti-FVIII inhibitor antibodies on this mechanism remain unknown, however. In the present study, we have examined the FVIIa/TF-catalysed activation and inactivation of FVIII in the presence of anti-FVIII antibodies. We demonstrated that FVIIa/TF activated FVIII even in the presence of anti-FVIII antibodies, but inactivation patterns appeared to depend on their epitope specificity and types. These findings supported that it could be important to determine the characteristic of these FVIII inhibitor antibodies to predict their physiological effects on FVIIa-related FVIII reactions. In addition, the results could have significant therapeutic implications.

Materials and methods

Reagents

Purified recombinant FVIII and FVIIa preparations were generously provided by Bayer Corp. Japan (Osaka, Japan) and NovoNordisk (Bagsværd, Denmark), respectively. The A1, A2, HCh, LCh, and thrombin-cleaved LCh fragments were isolated and purified from recombinant FVIII as previously reported (6, 22). A cDNA coding the C2 domain sequence of human FVIII was constructed, transformed into *Pichia pastoris* cells and expressed in a yeast secretion system (23). The C2 protein was purified by ammonium sulfate fractionation and cation-exchange HPLC. The recombinant A3 domain of FVIII was expressed in *Escherichia coli* using the pET expression system (Novagen, Madison, WI, USA) (24), and the protein was purified using His-Select affinity cartridges. SDS-PAGE of the isolated subunits followed by staining with GelCode Blue Stain Reagent (Pierce, Rockford, IL, USA) showed >95% purity. The monoclonal antibody (mAb), C5 (25), recognising the C-terminal end of the A1 domain, was a generous gift from Dr. Carol Fulcher. An anti-A2 mAb413 (epitope: 484–509) (26) was kindly provided by Dr. Evgueni Saenko. The mAbs ESH4 and ESH8, with different C2 epitopes (residues 2303–2332 and 2248–2285, respectively), were purchased from American Diagnostica Inc. (Greenwich, CT, USA). FVIIa-specific inhibitor peptide E-76 (Bachem, Torrance, CA, USA) and recombinant lipidated TF (Innovin®; Dade Behring, Marburg, Germany) were purchased from the indicated vendors. The biotinylation of mAbC5 was prepared using *N*-hydroxysuccinimido-biotin reagent (Pierce). PL vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, and 30% phosphatidylethanolamine (Sigma, St Louis, MO, USA) were prepared using *N*-octylglucoside (27). Protein concentrations were determined by the method of Bradford (28).

Anti-FVIII inhibitor antibodies

Thirteen anti-FVIII inhibitor alloAbs or autoAbs were obtained from Japanese patients with congenital severe haemophilia A or acquired haemophilia A, respectively. IgG fractions were prepared using protein A Sepharose (Amersham Biosciences, Uppsala, Sweden). $F(ab')_2$ fragments were prepared using immobilised pepsin-Sepharose (Pierce). The inhibitor titer of antibody $F(ab')_2$ was determined by the Bethesda assay by Kasper et al. (29). The kinetic patterns (type 1 and type 2 behaviours) of FVIII inactivation by these anti-FVIII antibodies were determined in one-stage clotting assays (12). Epitopes of these antibodies were performed by SDS-PAGE and Western blotting using isolated FVIII fragments (purified A1, A2, A3, C2, HCh and LCh, and thrombin-cleaved LCh fragments). Bound to FVIII fragments of anti-FVIII antibodies was detected by the addition of anti-human peroxidase-linked monoclonal antibody (The Binding Site Group Ltd, Birmingham, UK).

FVIII activation by FVIIa/TF

FVIII activity was measured in a one-stage clotting assay using FVIII-deficient plasma (Sysmex, Kobe, Japan) with an equipment of STart4 Hemostasis Analyzer® (Diagnostica Stago, Asnieres,

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France). All reactions were performed at 37°C. FVIII activation by FVIIa/TF was performed as previously reported (21). FVIII (10 nM) was preincubated with the indicated concentrations of anti-FVIII antibodies or mAbs IgG $F(ab')_2$ for 1 hour (h), and then reacted with FVIIa/TF (1 nM) and PL (20 μM) in 20 mM HEPES, 150 mM NaCl, and 0.01% Tween 20 (HBS-buffer) containing 5 mM $CaCl_2$ and 0.1% bovine serum albumin. The final concentrations (f.c.) of inhibitors were adjusted to 2 BU/ml. Control $F(ab')_2$ was performed at the concentration of 500 nM. Samples were removed from the mixtures at the indicated times, and FVIIa/TF reaction was immediately terminated by the addition of the FVIIa-inhibitor E-76 (2.5 μM) and 200-fold dilution. The presence of FVIIa/TF, E-76, and anti-FVIII antibodies, in the diluted samples did not affect FVIII activity (<0.5%) in the coagulation assays (data not shown).

Cleavage of FVIII by FVIIa/TF

FVIII (10 nM) was preincubated with the indicated concentrations of anti-FVIII antibodies or mAbs IgG $F(ab')_2$ for 1 h, and then mixed with FVIIa/TF (1 nM) and PL (20 μM) in HBS-buffer containing 5 mM $CaCl_2$ (21). Aliquots were removed at the indicated times and the reactions were immediately terminated and prepared for SDS-PAGE by adding E-76 and SDS and by boiling for 3 minutes (min).

Electrophoresis and Western blotting

SDS-PAGE was performed using 8% gels at 150 V for 1 h. For Western blotting, protein samples were transferred to a polyvinylidene difluoride membrane using a Bio-Rad mini-transblot apparatus at 50 V for 2 h in buffer containing 10 mM CAPS, pH 11 and 10% (v/v) methanol. Protein bands were probed using unlabelled or labelled anti-A1 mAbC5, followed by a goat anti-mouse peroxidase-linked secondary mAb (MP Biomedicals, Aurora, OH, USA) or horseradish peroxidase-labelled streptavidin (Chemicon, Australia), respectively. Signals were detected using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA, USA). Densitometric scans were quantitated using Image J 1.38 (National Institute of Health, Bethesda, MD, USA).

Results

Properties of anti-FVIII inhibitor alloAbs or autoAbs

Our goal in this study was to determine the effects of anti-FVIII inhibitor antibodies, in particular, the effects of antibodies with different recognising epitopes, on FVIIa/TF-catalysed activation and inactivation of FVIII. We chose anti-FVIII antibodies, therefore, based on the recognition of either the A2 or C2 domain of major inhibitor epitopes. Determination of the domain specificity of anti-FVIII antibodies was performed by Western blotting using isolated FVIII fragments (data not shown). Two cases bound to the HCh and A2, but any little reacted with either the A1 or LCh. Eleven cases bound to the (thrombin-cleaved) LCh and C2, but failed to react with the A1, A2, and A3. To confirm the specificity of these reactions, the antibodies of the former or the latter were preincubated with excess amounts of A2 or C2 in a fluid phase, prior to reaction with the HCh or LCh, respectively, followed by immunoblotting. The reactivities to the HCh of two cases were completely lost by the A2 domain, suggestive of the recognition of A2 epitope(s). Similarly, the reactivities to the LCh of 11 cases were lost competitively by the C2 domain, suggestive of the recognition of C2 epitope(s), although the contamination of other epitope could be completely excluded. The inhibitor titres of all cases, determined by the Bethesda assay, ranged from 6.3 to 440 BU/mg. The kinetic patterns of FVIII inactivation for 13 cases were also investigated (12). Two anti-A2 cases showed the type 1 pattern (named by cases 12 and 13). Seven of 11 anti-C2 cases showed the type 1 pattern (named by cases 1–7), and the other 4 cases showed the type 2 pattern (named by cases 8–11). Taken together, the properties of 13 anti-FVIII allo- or autoAbs and 3 anti-FVIII mAbs used in the present study are summarised in ▶Table 1.

Table 1: Characterisation of anti-FVIII inhibitor antibodies obtained from congenital or acquired haemophilia A patients and anti-FVIII mAbs.

Case	Type of anti-FVIII Ab	Epitope	Type of kinetics	Inhibitor titer (BU/mg)
1	alloAb	C2	1	151 ± 3
2	alloAb	C2	1	300 ± 7
3	autoAb	C2	1	13.6 ± 0.8
4	autoAb	C2	1	14.4 ± 1.0
5	autoAb	C2	1	440 ± 10
6	autoAb	C2	1	21.8 ± 1.8
7	autoAb	C2	1	42.3 ± 2.4
8	alloAb	C2	2	73.5 ± 3.2
9	autoAb	C2	2	18.3 ± 1.5
10	autoAb	C2	2	524 ± 18
11	autoAb	C2	2	6.3 ± 0.8
12	alloAb	A2	1	141 ± 6
13	alloAb	A2	1	29.8 ± 1.3
ESH4	mAb	C2	1	39 ± 2
ESH8	mAb	C2	2	10,000 ± 34
413	mAb	A2	1	59,500 ± 69

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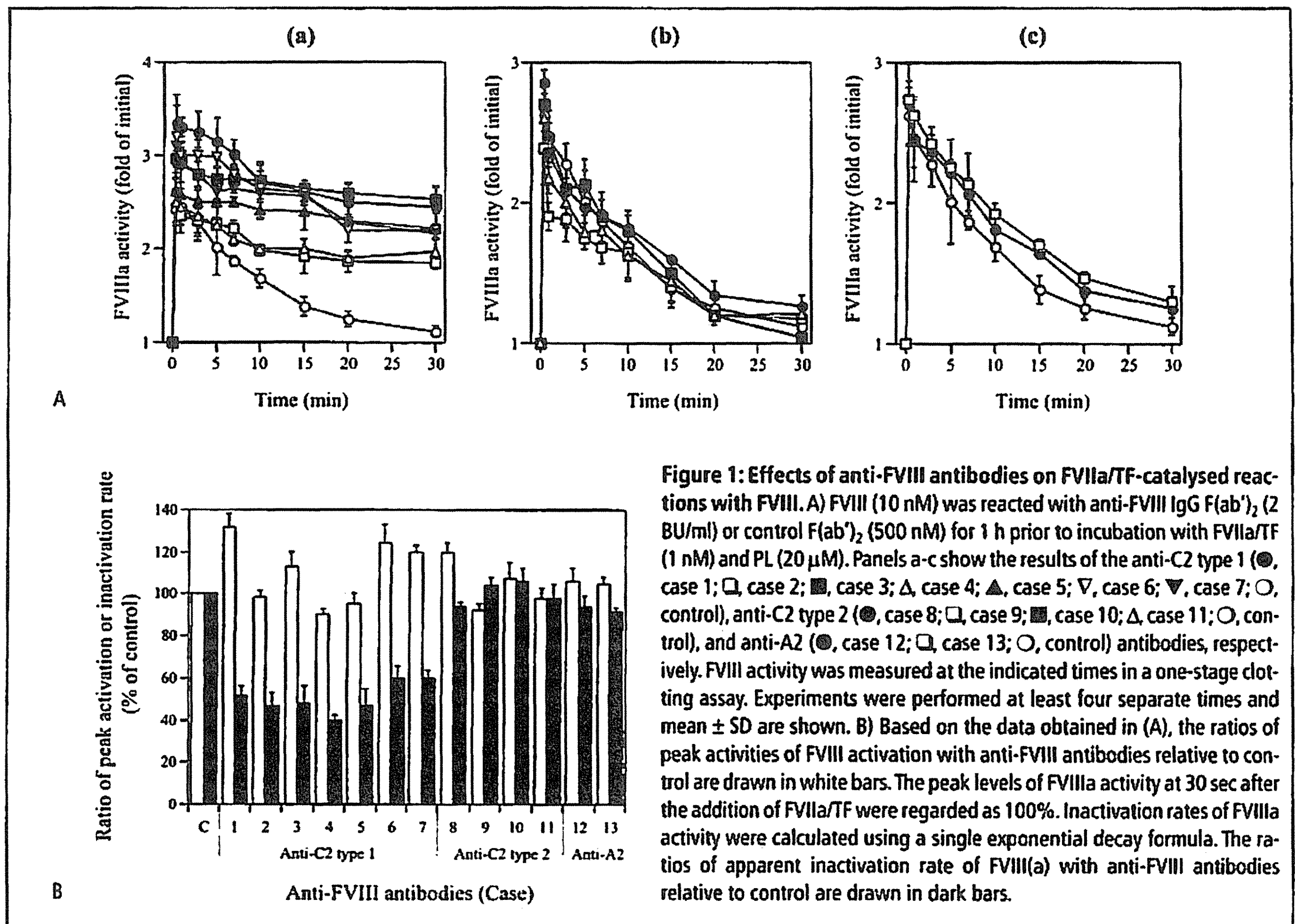

Figure 1: Effects of anti-FVIII antibodies on FVIIa/TF-catalysed reactions with FVIII. A) FVIII (10 nM) was reacted with anti-FVIII IgG F(ab')$_2$ (2 BU/ml) or control F(ab')$_2$ (500 nM) for 1 h prior to incubation with FVIIa/TF (1 nM) and PL (20 μM). Panels a-c show the results of the anti-C2 type 1 (●, case 1; □, case 2; ■, case 3; △, case 4; ▲, case 5; ▽, case 6; ▼, case 7; ○, control), anti-C2 type 2 (●, case 8; □, case 9; ■, case 10; △, case 11; ○, control), and anti-A2 (●, case 12; □, case 13; ○, control) antibodies, respectively. FVIII activity was measured at the indicated times in a one-stage clotting assay. Experiments were performed at least four separate times and mean ± SD are shown. B) Based on the data obtained in (A), the ratios of peak activities of FVIII activation with anti-FVIII antibodies relative to control are drawn in white bars. The peak levels of FVIIIa activity at 30 sec after the addition of FVIIa/TF were regarded as 100%. Inactivation rates of FVIIIa activity were calculated using a single exponential decay formula. The ratios of apparent inactivation rate of FVIII(a) with anti-FVIII antibodies relative to control are drawn in dark bars.

Effects of anti-FVIII antibodies on FVIIa/TF-catalysed FVIII activation

FVIIa/TF-catalysed activation of FVIII in the presence of anti-FVIII antibodies was assessed in one-stage clotting assays. Reaction mixtures were diluted 200-fold to exclude the direct influence of FVIIa, TF/PL, FVIIa-inhibitor, and anti-FVIII antibodies in these assays. FVIII (10 nM) was used at a concentration of ~10-fold higher than the physiological level, since the minimum level for measurement of FVIII activity was ~0.01 nM. Control experiments showed that in mixtures of FVIII (10 nM) and FVIIa/TF (1 nM) with PL, FVIII activity rapidly reached a peak of ~2.6-fold of the initial level after 30 seconds (sec). Subsequently, its procoagulant activity diminished to the initial level at ~20 min. FVIII was preincubated with anti-FVIII antibodies: seven of anti-C2 type 1 (▶Fig. 1A, panel a), four of anti-C2 type 2 (▶Fig. 1A, panel b), and two of anti-A2 (▶Fig. 1A, panel c) prior to mixing with FVIIa/TF, followed by measuring the FVIII activity. These inhibitor titres were adjusted to 2 BU/ml. Representative time-course reactions for each group are shown in ▶Figure 1A, and the ratio of peak FVIII activity in the presence of these antibodies relative to control is illustrated in ▶Figure 1B. The FVIII activity (t=0) with anti-FVIII antibodies before the addition of FVIIa/TF was ~3 U/dl. The FVIIa/TF-catalysed activation of FVIII was not inhibited by the antibodies but rather slightly enhanced, compared to control IgG F(ab')$_2$ (500 nM). The results were independent of the epitope specificity and inhibitor type of anti-FVIII antibodies. We further evaluated the effect of FVIIa/TF-mediated inactivation in the presence of these antibodies relative to control, peak levels of FVIII activity at 30 sec after the addition of its protease were regarded as 100%, and the timed-dependent decrease of peak levels of FVIIIa activity was evaluated using the formula of single exponential decay. The spontaneous A2 dissociation from FVIIIa might somewhat affect the obtained rate constants on the inactivation phases, however. Therefore, these rates represented the restricted values only in this analysis used for the comparison with that of control, and were expressed as apparent. The apparent rate for control F(ab')$_2$ was 0.15 ± 0.04 min^{-1}. The ratios of these inactivation rates with the different anti-FVIII antibodies relative to control are illustrated in ▶Figure 1B. All cases of anti-C2 type 2 (cases 8–11) and anti-A2 (cases 12 and 13) had little effect on the inactivation phase. Each of the anti-C2 type 1, however, depressed inactivation by 40–60% of that seen with control. These results suggested that the coagulant activity of FVIII, activated by FVIIa/TF, appeared to persist in the presence of anti-C2 type 1 antibodies.

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Effects of anti-FVIII antibodies on FVIIa/TF-catalysed cleavage of FVIII HCh

FVIIa/TF rapidly activates FVIII by proteolysis of the HCh at Arg^{372} (and Arg^{740}), and inactivates FVIIIa by proteolysis at Arg^{336} (21). Little cleavage of the LCh was observed in the initial phase of blood coagulation. ▶Figure 2 represents a diagrammatic illustration of the pathways for FVIIa/TF-catalysed cleavage of the HCh, and it is proposed that pathway I is more rapid than pathway II. On this basis, therefore, we hypothesised that the effects of the anti-C2 type 1 antibodies on FVIIa/TF-catalysed FVIII inactivation might be due to modified cleavage of the HCh at Arg^{336}. To examine this, SDS-PAGE using same samples obtained in time-coursed reaction (in ▶Fig. 1) was utilised to investigate the effects of anti-FVIII inhibitors on FVIIa/TF-catalysed cleavage of the HCh. FVIII (10 nM) was preincubated with anti-C2 type 1 (case 1) or anti-A2 (case 12) (2 BU/ml) for 1 h prior to reaction with FVIIa/TF (1 nM). ▶Figure 3A shows time-course patterns of HCh cleavage, analysed by Western blotting using anti-A1 mAbC5. The appearance of A1-A2 represents cleavage at Arg^{740}, and the appearance and disappearance of $A1^{1-372}$ represent the cleavage at Arg^{372} and the subsequent cleavage at Arg^{336} (pathway I). The appearance of $A1^{337-372}$-A2 represents cleavage at Arg^{336} (pathway II). Although HPLC-gel filtration was used to fractionate intact FVIII, $A1^{1-372}$ fragments remained evident in the absence of FVIIa/TF, suggesting that mAbC5 was highly sensitive in these circumstances (21). Similar to HCh cleavage in the presence of control IgG $F(ab')_2$ (500 nM, ▶Fig. 3A, panel a), cleavage at Arg^{740}, Arg^{372}, and Arg^{336} was not affected by anti-A2 for case 12 (▶Fig. 3A, panel c). These results were consistent with those obtained in FVIIa/TF-catalysed FVIII coagulation activation. In addition, other anti-A2 (case 13) and all anti-C2 type 2 (cases 8–11) had little effect on HCh cleavage (data not shown). In contrast, anti-C2 type 1 for case 1 markedly delayed both the disappearance of $A1^{1-372}$ and the appearance of $A1^{337-372}$-A2 (▶Fig. 3A, panel b), indicative of a delay in cleavage at Arg^{336}. These findings were supported by analysis of the ratio of the $A1^{337-372}$-A2/$A1^{1-372}$ substrate using scanning densitometry (▶Fig. 3A, panel d). Cleavage at Arg^{740} and Arg^{372} was not significantly affected. Similar patterns of cleavage were observed in the presence of the other anti-C2 type 1 (cases 2–7, data not shown). To confirm the specificity of these reactions, FVIII (10 nM) was mixed with increasing amounts of anti-C2 type 1 for case 1 for 1 h prior to incubation with FVIIa/TF (1 nM) for 15 min (▶Fig. 3B). Western blotting (▶Fig. 3B, panel a) and band densitometry (▶Fig. 3B, panel b) revealed that the presence of anti-C2 for case 1 increased the appearance of $A1^{1-372}$ and decreased the appearance of $A1^{337-372}$-A2 fragments in dose-dependent manners.

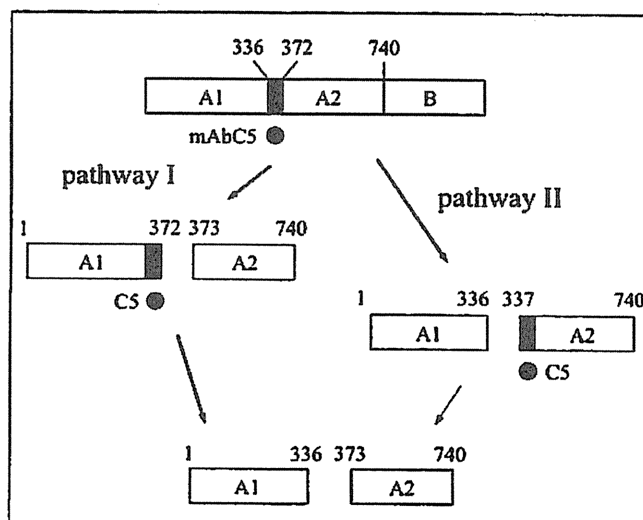

Figure 2: A diagram of the proposed pathways for FVIIa/TF-catalysed cleavage of the FVIII HCh.

Effects of distinct kinetic types of anti-C2 antibodies on FVIIa/TF-catalysed cleavage at Arg^{336}

The observations above suggested that anti-C2 type 1 antibodies moderated the inactivation phase of FVIIa/TF-catalysed FVIII reactions by moderating cleavage at Arg^{336} in the HCh, whilst anti-C2 type 2 did not affect this cleavage. To further compare the different types of these antibodies, therefore, FVIII (10 nM) was mixed with anti-C2 type 1 or type 2 (2 BU/ml) for 1 h prior to incubation with FVIIa/TF (1 nM) for 15 min. As above, the time-course of pathway I (cleavage at Arg^{336} leading to the disappearance of $A1^{1-372}$) and pathway II (the appearance of $A1^{337-372}$-A2) was monitored by SDS-PAGE and Western blotting using anti-A1 mAb (▶Fig. 4A). Again, the ratios of $A1^{337-372}$-A2/$A1^{1-372}$ were determined using quantitative densitometry (▶Fig. 4B). In the presence of 500 nM control IgG $F(ab')_2$, this ratio was 5.3 ± 0.6, and the ratios (5.0–7.5) were similar in the presence of anti-C2 type 2 antibodies. In the presence of anti-C2 type 1, however, these ratios (0.48–1.7) were significantly lower by three- to 11-fold than that of control. These findings were broadly similar to the ratio seen with anti-C2 type 1 for case 1 shown in ▶Figure 3B (~20-fold lower than control), confirming that the anti-C2 type 1 antibodies delayed FVIIa/TF-catalysed cleavage of the HCh at Arg^{336}, in a mechanism that was different from that of anti-C2 type 2.

Effects of anti-C2 mAbs on FVIIa/TF-catalysed reaction of FVIII

Polyclonal type 1 and type 2 anti-C2 antibodies are believed to reflect distinct epitopes. Two anti-C2 mAbs, ESH4 and ESH8, have been shown to represent the epitopes of anti-C2 type 1 and type 2 antibodies, respectively (16). We examined, therefore, FVIIa/TF-catalysed FVIII coagulation activity in the presence of these anti-C2 mAbs (▶Fig. 5A). Clotting assays were performed with FVIII (10 nM) and FVIIa/TF (1 nM) in the presence of each of the anti-C2 mAbs (2 BU/ml). ESH8 had little effect on FVIIa/TF-catalysed FVIII reaction. ESH4, however, enhanced FVIII activation

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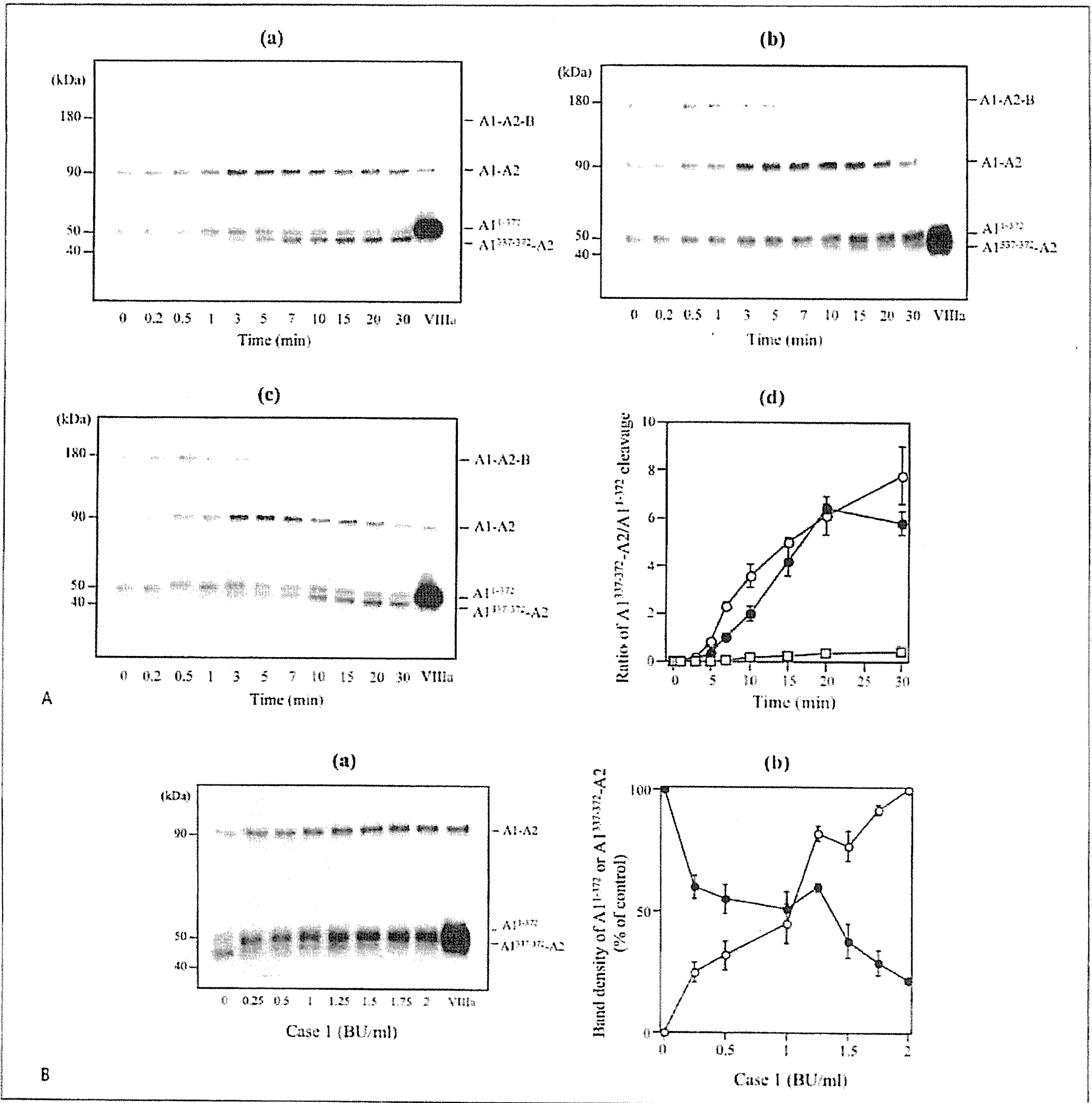

Figure 3: Effects of anti-A2 and anti-C2 antibodies on FVIIa/TF-catalysed cleavage of the FVIII HCh. A) FVIII (10 nM) was reacted with anti-FVIII IgG F(ab')$_2$ (2 BU/ml, panels a-c; control, anti-C2 for case 1, anti-A2 for case 12, respectively) for 1 h prior to incubation with FVIIa/TF (1 nM) and PL (20 µM) for the indicated times. Same samples obtained in Figure 1 were analysed for FVIIa/TF-catalysed cleavage of HCh. Samples were run on 8% gels followed by Western blotting using anti-A1 mAb. The right lane, VIIIa, shows thrombin-cleaved FVIII. Panel d shows the data obtained by quantitative densitometry of the $A1^{337-372}$-A2/$A1^{1-372}$ ratio in panels a-c. Experiments were performed at least three separate times and mean ± SD are shown. The symbols used are: ○, control; □, case 1; ●, case 12. B) Dose-dependent inhibition of anti-C2 type 1 for case 1. FVIII (10 nM) was preincubated with increasing amounts of anti-C2 IgG F(ab')$_2$ (case 1) for 1 h, followed by reaction with FVIIa/TF (1 nM) and PL (20 µM) for 15 min. Samples were run on an 8% gel followed by Western blotting using anti-A1 mAb (panel a). Panel b shows the data obtained by quantitative densitometry of the $A1^{1-372}$ and $A1^{337-372}$-A2 bands in panel a. Band densities of $A1^{1-372}$ without or with (2 BU/ml) anti-C2 were regarded as 0 or 100%, respectively. Band densities of $A1^{337-372}$-A2 without anti-C2 were regarded as 100%. Experiments were performed at least three separate times and mean ± SD are shown. The symbols used are: ○, $A1^{1-372}$; ●, $A1^{337-372}$-A2.

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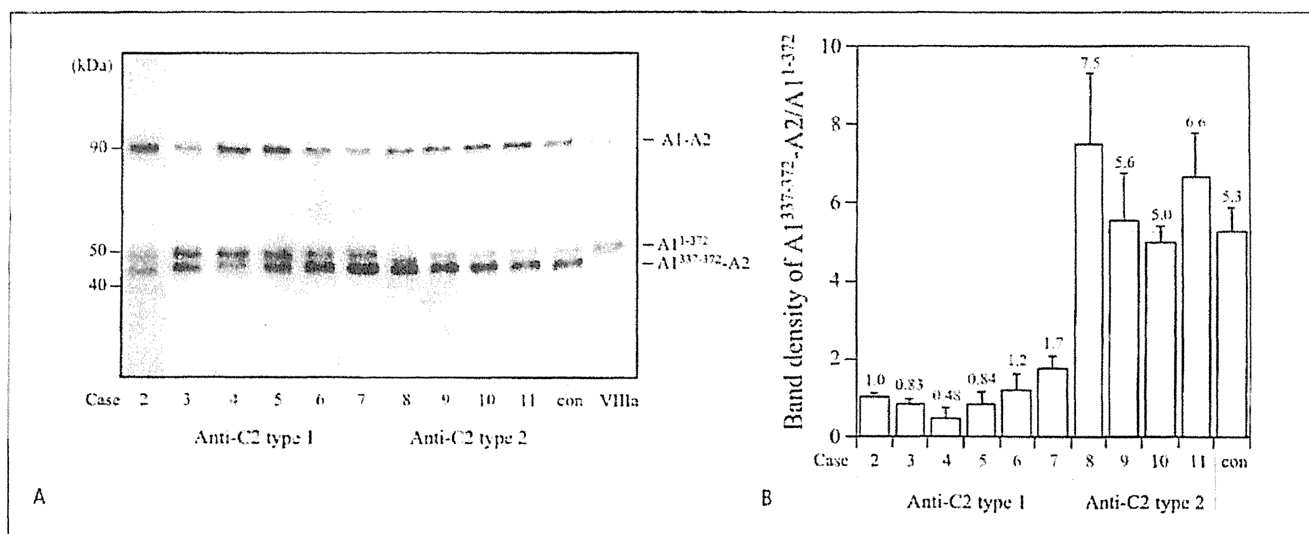

Figure 4: FVIIa/TF-catalysed cleavage of the HCh at Arg^{372} and Arg^{336} in the presence of different types of anti-C2 antibodies. A) FVIII (10 nM) was reacted with anti-C2 IgG F(ab')$_2$ (2 BU/ml) or control F(ab')$_2$ (500 nM) for 1 h prior to incubation with FVIIa/TF (1 nM) and PL (20 μM) for 15 min. Samples were run on an 8% gel followed by Western blotting using anti-A1 mAb. The VIIIa lane shows thrombin-cleaved FVIII. B) Data obtained by quantitative densitometry on ratio of A1$^{337-372}$-A2/A1$^{1-372}$ band in A. Experiments were performed at least three separate times and mean ± SD are shown. The dashed line shows the value obtained in control.

and moderated subsequent FVIIIa inactivation. Using the analysis principles described in ▶Figure 1, ESH4 inhibited the inactivation phase of FVIIIa with ~20% of the rate observed with control (0.14 ± 0.03 min^{-1}). The elevation of peak FVIII activation with ESH4 (by ~1.5-fold of control) was possibly due to the relative enhancement of activated FVIIIa as a consequence of delayed FVIIIa inactivation. These findings were consistent with those in the presence of polyclonal anti-C2 type 1 and type 2. In addition, an anti-A2 mAb 413, containing the epitope of anti-A2 alloAb, did not affect the FVIIa/TF-catalysed reaction of FVIII, similar to the results in ▶Figure 1 obtained in the presence of anti-A2 alloAbs.

These effects of anti-C2 mAbs were further examined by SDS-PAGE. ▶Figure 5B shows the sequential cleavage of HCh analysed by Western blotting using biotinylated mAbC5 and by band densitometry (▶Fig. 5B, panel c). Preincubation with ESH8 did not significantly affect HCh cleavage (▶Fig. 5B, panel b), whilst ESH4 (▶Fig. 5B, panel a) markedly delayed both the disappearance of A1$^{1-372}$ and the appearance of A1$^{337-372}$-A2, indicative of slower cleavage at Arg^{336}. Cleavage at Arg^{372} was not significantly affected. These observations were in keeping with those of polyclonal anti-C2 type 1 and type 2.

Discussion

We have recently demonstrated that FVIIa/TF, known to be a pivotal initiator of blood coagulation, activated FVIII very rapidly in the initial phases of blood coagulation (21). It could be expected, therefore, that anti-FVIII inhibitor alloAbs and autoAbs might affect FVIIa/FVIII-related clotting mechanisms. In the present study, we have demonstrated for the first time that anti-FVIII C2 antibodies with type 1 inhibitor pattern moderated FVIIa/TF-catalysed FVIIIa inactivation by delaying cleavage of the FVIII HCh at Arg^{336}.

Major epitopes for anti-FVIII inhibitor antibodies recognise the A2 and/or C2 domains of FVIII (13, 14). In the present study, to examine the effects of anti-FVIII antibodies with different epitopes, on FVIIa/TF-catalysed reaction of FVIII, we have chosen IgG F(ab')$_2$ fractions from the 2 anti-A2, 7 anti-C2 type 1, and 4 anti-C2 type 2 (▶Table 1). Since FVIIa/TF rapidly induced FVIII activation and inactivation, it was difficult to analyse precisely the effects of antibodies with different inactivation types. Consequently, the percentage peak activity relative to control was evaluated as the effects of antibodies in the activation phase. Furthermore, peak FVIII activity that reached at 30 sec was regarded as 100%, and the timed-dependent reduction in peak activity was expressed as apparent inactivation rate. In addition, the inactivation phase appeared to be partly governed, however, by the spontaneous dissociation of the A2 domain from FVIIIa through the cleavage at Arg^{372}, and thus, the obtained values were likely governed by both effects of the cleavage at Arg^{336} and spontaneous A2 dissociation. The analyses were restricted, therefore, to an estimate of the differences in the inactivation pattern with the different anti-FVIII antibodies. Nevertheless, our findings showed that only anti-C2 type 1 moderated the inactivation phase, and the validity of the results were strongly supported by demonstrating inhibition of cleavage of the FVIII HCh at Arg^{336}.

Anti-C2 type 1 and type 2 antibodies showed different effects on FVIIa/TF-catalysed FVIII reactions. Polyclonal anti-FVIII anti-

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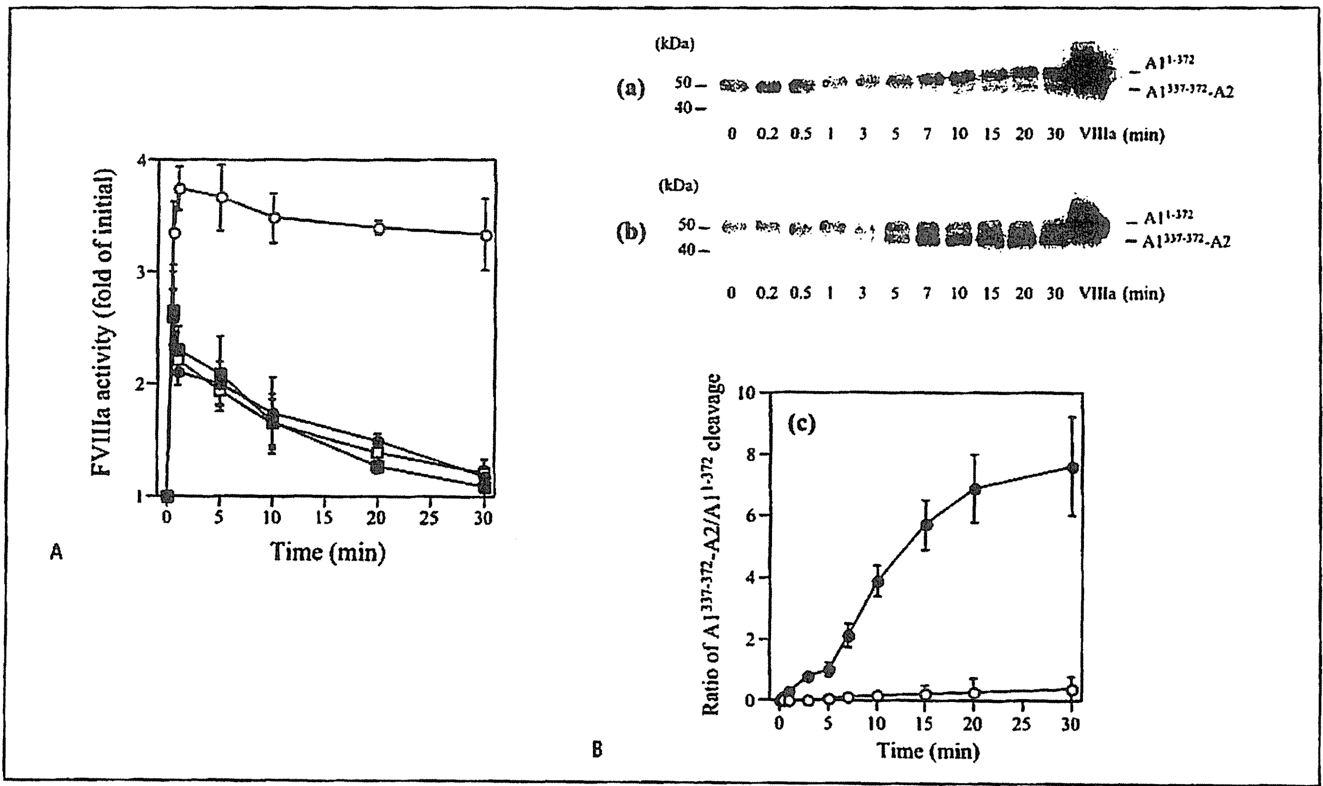

Figure 5: Effects of anti-C2 mAbs on FVIIa/TF-catalysed reactions with FVIII. A) FVIII activation: FVIII (10 nM) was reacted with anti-C2 or A2 mAb F(ab')$_2$ (2 BU/ml) or control F(ab')$_2$ (500 nM) for 1 h prior to incubation with FVIIa/TF (1 nM) and PL (20 μM). FVIII activity was measured at the indicated times in a one-stage clotting assay. Experiments were performed at least four separate times and mean ± SD are shown. The symbols used are: ○, ESH4; ●, ESH8; ■, 413; □, control. B) HCh cleavage: FVIII (10 nM) was reacted with anti-C2 mAbs (2 BU/ml, panels a and b; ESH4 and ESH8, respectively) for 1 h prior to incubation with FVIIa/TF (1 nM) and PL (20 μM) for the indicated times. Same samples obtained at time-coursed reaction in A were analysed. Samples were run on 8% gels followed by Western blotting using biotinylated anti-A1 mAb. The VIIIa lane shows thrombin-cleaved FVIII. Panel c shows the data obtained by quantitative densitometry of the $A1^{337-372}$-$A2/A1^{1-372}$ ratio in B. Experiments were performed at least three separate times and mean ± SD are shown. The symbols used are: ○, ESH4; ●, ESH8.

bodies could contain epitopes other than C2, but we demonstrated that the murine anti-C2 mAbs, ESH4 (type 1 behavior) and ESH8 (type 2 behaviour), known to contain the specific epitopes recognised in anti-C2 antibodies (16), gave similar results to the human antibodies. On the basis of inhibitory mechanisms, anti-C2 antibodies are classified into two categories. Type 1 antibodies block the interaction of FVIII with VWF, PL, and FXa, and type 2 block the interaction of FVIII with thrombin and FXa (15–17). We have recently reported that the C2-FIXa interaction was not affected by either type of anti-C2 (30). In addition, since the C2 domain contains the interactive-region essential for FVIII activation by thrombin and FXa (31, 32), anti-C2 antibodies inhibit thrombin (and FXa)-induced FVIII activation. Our present data showed that anti-C2 type 1, but not anti-C2 type 2 moderated FVIIa/TF-catalysed FVIIIa inactivation by moderating cleavage of the FVIII HCh at Arg^{336}, suggesting the different effects of these antibodies on thrombin, FXa, and FVIIa/TF reactions. The possibility that anti-C2 type 1 might affect the FVIII-PL interactions involved in FVIIa/TF-catalysed mechanisms could not be completely excluded. However, FVIIa/TF-catalysed activation and cleavage of FVIII are little observed in the absence of PL surfaces (21), supporting that FVIIa/TF-catalysed FVIII reaction would be little observed if anti-FVIII antibodies significantly blocked the FVIII-PL interactions. In the present study, furthermore, anti-C2 type 1 antibodies did not affect FVIII activation mediated by cleavage at Arg^{372} and Arg^{740}, and these at the concentrations employed did not significantly block the FVIII-PL interactions in ELISA-binding assays (data not shown). However, at higher concentrations than those employed, anti-C2 type 1 inhibited the FVIII-PL interaction (data not shown). Taken together, we believe that these antibodies did not directly affect FVIIa/TF-catalysed activation of FVIII under the current conditions.

Although the cleavage at Arg^{336} by FVIIa/TF was significantly delayed in the presence of all cases for anti-C2 type 1 antibodies, compared to in the presence of anti-A2 or anti-C2 type 2 as well as control IgG, these inhibitory effects were shown to a greater or lesser extent (in ▶Fig. 4). Anti-FVIII antibodies are polyclonal in nature and possess the differed specific activity (BU/mg). In addition, we have often experienced the different inhibitory effects of anti-C2 antibodies, for example, on FVIII-VWF binding or FVIII-

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What is known about this topic?

- FVIIa/TF that activates FX and FIX is a potent serine protease responsible for initiating and propagating the blood coagulation cascade in normal haemostasis.
- We have recently reported that FVIIa/TF rapidly activated FVIII due to the limited proteolysis at Arg^{372} and Arg^{740} in the HCh, and appeared to promote a "priming" mechanism to generate small amounts of FVIIIa in the early phase of coagulation, although this activation is weaker than that mediated by thrombin.
- Anti-FVIII inhibitor antibodies either inhibit FVIII activity completely or incompletely at saturating concentrations, corresponding to type 1 or type 2, respectively, through some inhibitory mechanisms on the FVIII association with several coagulation proteins.
- The effects of anti-FVIII antibodies on FVIIa/TF-catalysed reaction of FVIII remain unknown at present, however.

What does this paper add?

- FVIIa/TF activated FVIII by proteolysis at Arg^{372} and Arg^{740} even in the presence of anti-FVIII antibodies, irrespective of epitope specificity.
- However, anti-C2 type 1 antibodies blocked the FVIIa/TF-catalysed FVIIIa inactivation through the significantly delayed cleavage at Arg^{336} in the HCh.
- Neither anti-A2 antibodies nor anti-C2 type 2 antibodies affected the FVIIa/TF-related FVIII reaction, however.
- It could be important to determine the characteristic of these anti-FVIII inhibitor antibodies for prediction of their physiological effects on FVIIa/TF-related FVIII reactions, and the results could have significant therapeutic implications.

PL binding, etc. It would not be so terrible, therefore, that anti-C2 type 1 inhibited the cleavage at Arg^{336} to a greater or lesser extent, and we suppose that these antibodies represent a homogeneous group (function) of inhibitors on FVIIa/TF-mediated reaction of FVIII.

Two possible mechanisms are implicated in the inhibition of cleavage at Arg^{336} by anti-C2 type 1. It could be that the epitope (residues 2181–2243 [33] and/or 2315–2330 [34]) might contain the FVIIa-interactive site(s) responsible for regulating the cleavage at Arg^{336}. In this context, we previously demonstrated using direct binding assays with active-site modified EGR-FVIIa that the C2 domain contained FVIIa-interactive site(s) (21). Alternatively, conformational changes in FVIII or steric hindrance mediated by the binding of anti-C2 type 1 might affect FVIIa/TF-induced cleavage at Arg^{336}. Of interest, the epitope(s) recognised by anti-C2 type 2 does not seem likely to contain FVIIa-interactive site(s), and residues 484–509 within the A2 domain associated with some coagulation proteins (35–37) do not appear to be associated with FVIIa/TF reactions. Investigations on FVIIa-interactive site(s) responsible for cleavage at Arg^{336} are in progress.

Our findings have demonstrated that even in the presence of anti-FVIII inhibitor antibodies, FVIIa/TF could activate FVIII to a similar extent in their absence. Also, the activation of FVIII in FVIIa/TF-catalysed reactions could persist longer in the presence of anti-C2 type 1 than other antibodies. It could be important for therapeutic purposes, therefore, to determine the characteristics of these anti-FVIII inhibitor antibodies for prediction of their effects on FVIIa-FVIII association in individual patients, especially those with acquired haemophilia A with anti-C2 type 1 inhibitors. The results could provide insights into the use of combination therapy, to activate FVIII by FVIIa complexed with TF on PL surfaces, by administrating mixtures of FVIII and FVIIa in patients with congenital haemophilia A with inhibitors as well as those with acquired haemophilia A. Furthermore, the findings would provide the challenging possibility of selective therapy using FVIII and FVIIa concentrates, supporting a recent report by Berntorp's group on the efficacy for combination therapy of FVIII and bypassing agent for haemophilia A with inhibitors (38).

Conflict of interest

None declared.

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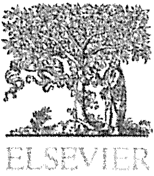

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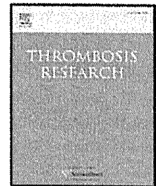

Regular Article

Regulation of coagulation factors during liver regeneration in mice: Mechanism of factor VIII elevation in plasma

Kohei Tatsumi [a,b], Kazuo Ohashi [b,*], Sanae Taminishi [a], Yoshihiko Sakurai [a], Kenichi Ogiwara [a], Akira Yoshioka [a], Teruo Okano [b], Midori Shima [a]

[a] *Department of Pediatrics, Nara Medical University, Nara, Japan*
[b] *Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan*

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ABSTRACT

Introductions: The profiles of coagulation factor production during liver regeneration process remains to be fully elucidated. The present study was aimed to perform a comprehensive analysis whether hepatic gene expression was differentially regulated relative to the secretion of biologically active coagulation factors using a mouse model of liver regeneration.
Materials and Methods: Liver regeneration was induced by performing a 2/3 partial hepatectomy (PHx). Plasma samples were assessed for coagulation factor activities (fibrinogen, prothrombin, V, VII, VIII, IX, X, XI, XII, and XIII) and the liver mRNA levels of coagulation, anti-coagulation, and fibrinolytic factors were quantified by real-time RT-PCR during the phase of liver regeneration.
Results: At the peak of liver regeneration, the expression levels for all of the genes analyzed were found to be reduced in a time-dependent manner. Consistent with the gene expression levels, plasma activities of all coagulation factors, except for FVIII, were temporally declined during the same time frame. FVIII paradoxically demonstrated a significant increase ($P<0.05$) in plasma activities concomitant with the decrease of liver mRNA expression levels. We found that the increase in plasma FVIII activities might be associated with (1) a delay in the inactivation of plasma FVIII caused by increased VWF in plasma and decreased FVIII clearance in the liver, (2) the rapid release of FVIII from the storage sites, and (3) the alteration of intracellular trafficking pathway of FVIII.
Conclusions: The present study demonstrated that the process of liver regeneration involves a general reduction for many of the coagulation cascade proteins, but there are paradoxical increases in plasma levels of FVIII and VWF.

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Introduction

Liver regeneration is an intrinsic event by which the liver is able to recover from an injurious loss of functional hepatic mass due to either surgical resection, or toxic, chemical or viral-based challenges [1,2]. The molecular events involved in the liver regenerative process are very complex, and the altered gene expression ultimately orchestrates the integration of these distinct pathways to promote the regenerative biological response [1–3]. Multiple studies have elucidated potential mechanistic pathways that may be involved in the process of liver regeneration, but many aspects of this phenomenon in terms of its gene expression profiles and its associated functional phenotypes remain to be fully elucidated [1,2].

The process of liver regeneration has been shown to produce variable effects on blood clotting. In patients with thrombotic events after partial hepatectomy, plasma samples demonstrated normal or hypocoagulability as determined by measuring prothrombin time (PT) and activated partial thromboplastin time (aPTT) [4]. However, whole blood analysis using thrombelastography showed hypercoagulability [5]. This apparent hemostatic conundrum may be explained by a general reduction in the production of many factors related to the clotting cascade, which are known to be largely generated in the hepatocytes [6–9], concomitant with a paradoxical increase in the plasma level of FVIII. FVIII is an essential blood clotting factor and mutations in this specific gene results in an X-linked coagulation disorder known as hemophilia A. After FVIII is produced and secreted into the circulation, FVIII associates with von Willebrand factor in a noncovalent complex. Low plasma levels of FVIII leads to a tendency towards clotting inefficiencies, whereas high plasma levels of FVIII can result in various thrombotic diathesis, such as deep vein thrombosis or extra-hepatic portal vein obstruction [10,11]. Furthermore, elevated plasma levels of FVIII are detected in pathological conditions with abnormal inflammatory states or other physiological stresses [12,13]. For

* Corresponding author. Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo, 162-8666, Japan. Tel.: +81 3 3353 8111x30234; fax: +81 3 3359 6046.
E-mail address: ohashi@abmes.twmu.ac.jp (K. Ohashi).

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