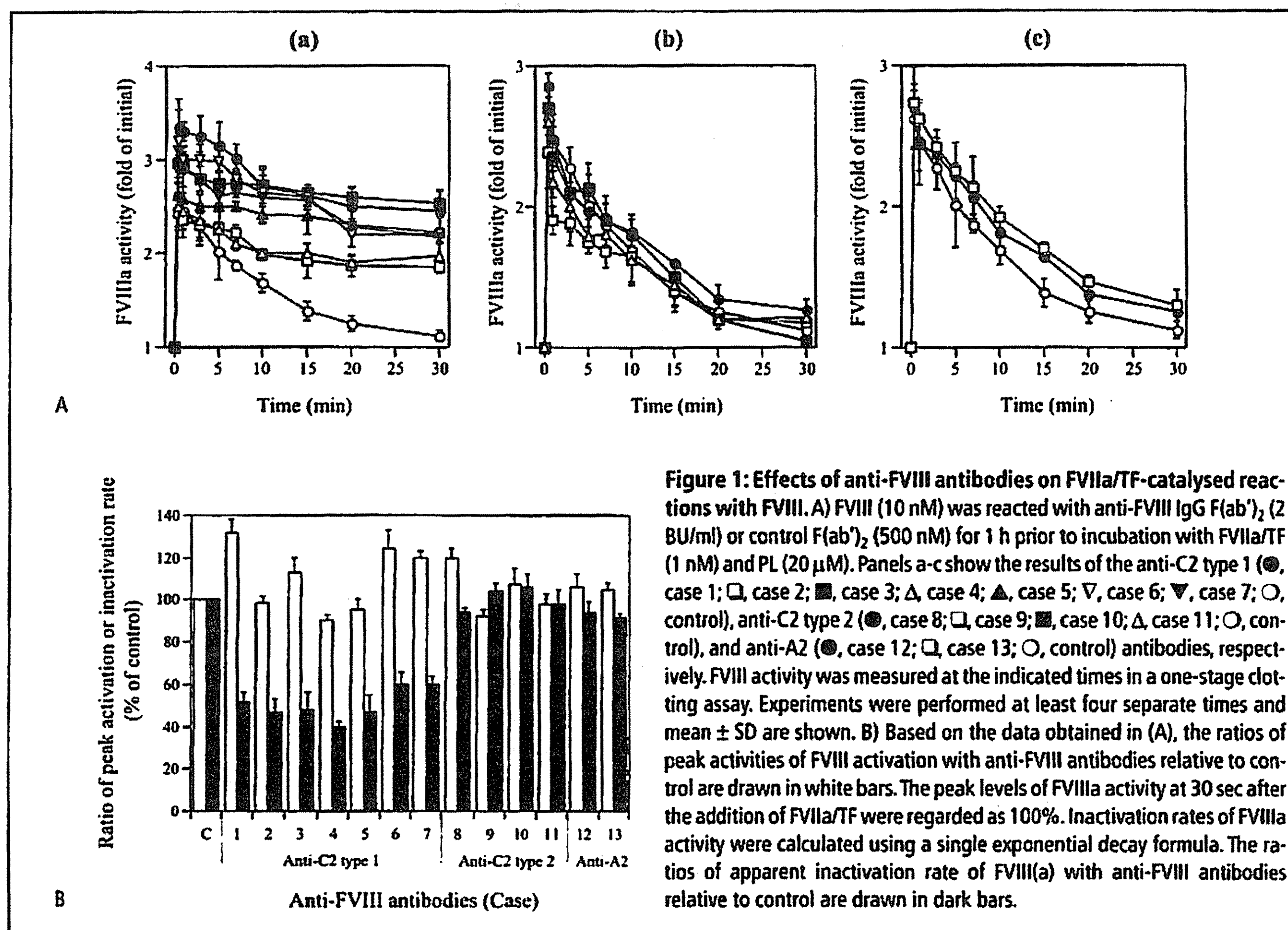

Figure 1: Effects of anti-FVIII antibodies on FVIIa/TF-catalysed reactions with FVIII. A) FVIII (10 nM) was reacted with anti-FVIII IgG F(ab')$_2$ (2 BU/ml) or control F(ab')$_2$ (500 nM) for 1 h prior to incubation with FVIIa/TF (1 nM) and PL (20 μM). Panels a-c show the results of the anti-C2 type 1 (●, case 1; ❑, case 2; ■, case 3; Δ, case 4; ▲, case 5; ∇, case 6; ▼, case 7; ○, control), anti-C2 type 2 (●, case 8; ❑, case 9; ■, case 10; Δ, 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.

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.

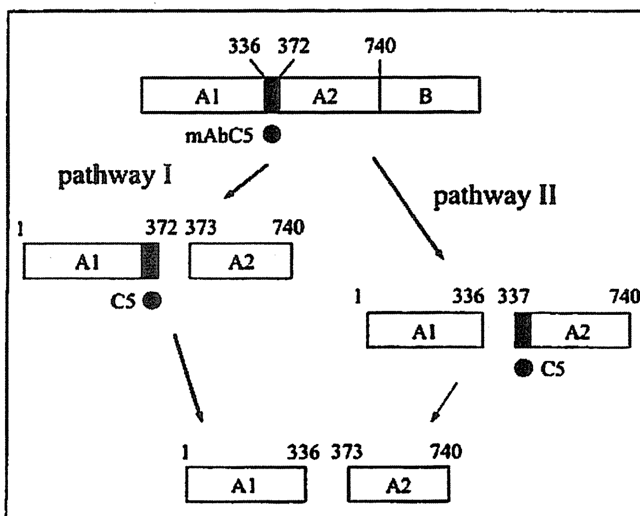

Figure 2: A diagram of the proposed pathways for FVIIa/TF-catalysed cleavage of the FVIII HCh.

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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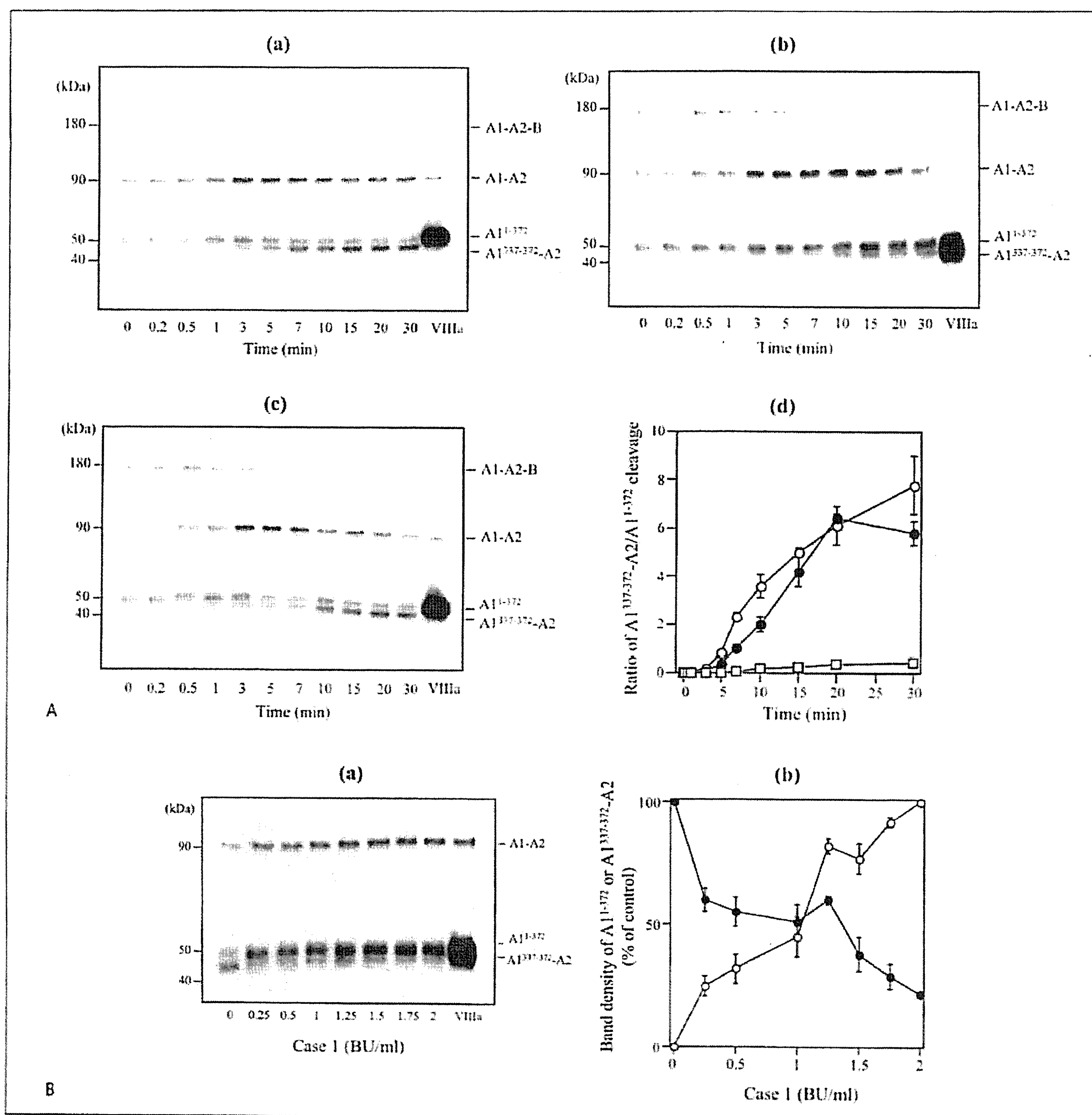

Figure 3: Effects of anti-A2 and anti-C2 antibodies on FVIIa/TF-catalysed cleavage of the FVIII HCh. A) FVIII (10 nM) was reacted with anti-FVIII IgG F(ab')$_2$ (2 BU/ml, panels a-c; control, anti-C2 for case 1, anti-A2 for case 12, respectively) for 1 h prior to incubation with FVIIa/TF (1 nM) and PL (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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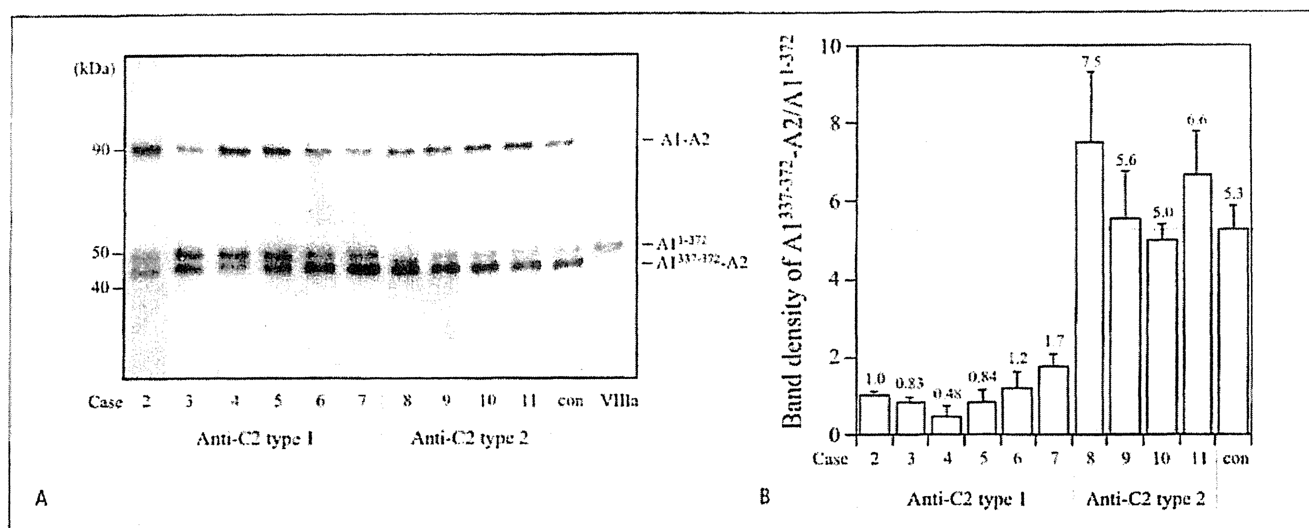

Figure 4: FVIIa/TF-catalysed cleavage of the HCh at Arg^{372} and Arg^{336} in the presence of different types of anti-C2 antibodies. A) FVIII (10 nM) was reacted with anti-C2 IgG F(ab')$_2$ (2 BU/ml) or control F(ab')$_2$ (500 nM) for 1 h prior to incubation with FVIIa/TF (1 nM) and PL (20 μM) for 15 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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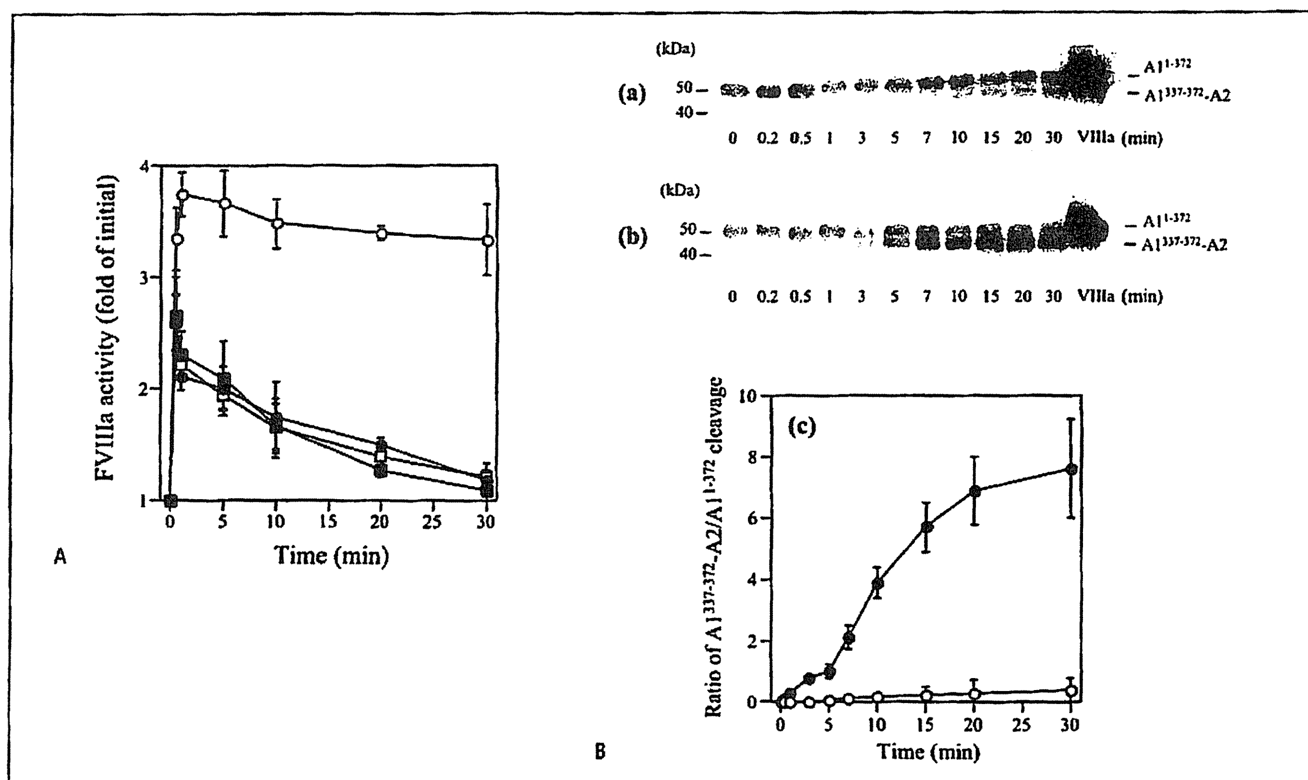

Figure 5: Effects of anti-C2 mAbs on FVIIa/TF-catalysed reactions with FVIII. A) FVIII activation: FVIII (10 nM) was reacted with anti-C2 or A2 mAb F(ab')$_2$ (2 BU/ml) or control F(ab')$_2$ (500 nM) for 1 h prior to incubation with FVIIa/TF (1 nM) and PL (20 μM). FVIII activity was measured at the indicated 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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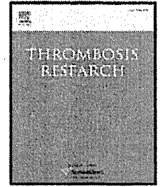

Regular Article

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

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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

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these reasons, FVIII plays an important role in hemostasis regulation, and the increased FVIII activity during liver regeneration may indicate a pivotal role in mediating the hypercoagulability of the blood promoting abnormal thrombotic events. At the present time, however, the differential gene expression and protein activity between FVIII and all other clotting factors during liver regeneration remains to be fully elucidated.

In the present study, we induced liver regeneration in mice by performing a 2/3 partial hepatectomy, the most commonly-used experimental procedure to induce compensatory liver regeneration mode [14]. During this liver regeneration process, we investigated the gene expression profile of coagulation factors and fibrinolytic factors as well as plasma activities of these factors at different time points following hepatectomy.

Materials and methods

Animals

Wild-type C57BL/6 female mice, 10-12 weeks old, were used in this study. Experimental protocols were developed in accordance with the guidelines outlined by our institutional animal committee at Nara Medical University. Mice were placed in cages within a temperature-controlled room having a 12-h light / dark cycle (8:00 a.m. lights on / 8:00 p.m. lights off).

Two-thirds partial hepatectomy (PHx)

The liver regeneration was induced by performing a 2/3 partial hepatectomy (PHx) under the general anesthesia with isofluorane as previously described [3,14–16]. In brief, a small upper midline incision was made, and the liver was exposed allowing for the removal of the median and left lobes. This surgical procedure was performed at within a specified time window (between 8:00-10:00 a.m.) to minimize the circadian rhythm variations that may influence the speed and peak of the regenerative activity as previously reported [17]. We performed two sets of PHx experiments for the purpose of (i) evaluating liver gene expression profiles with the corresponding plasma activity of secreted coagulation factors, (ii) specific investigation of factor VIII mRNA expression changes in several mouse tissues after PHx.

Experiment 1: Comparison of liver mRNA expression levels and plasma levels of coagulation factors

C57BL/6 mice received 2/3 PHx (n = 25), and the liver lobes that were removed from each mouse at the time of PHx were treated as quiescent control liver samples. The mice were sacrificed at 1, 2, and 5 days after PHx (n = 9, 8, and 8, respectively), and the livers were harvested immediately snap-frozen in liquid nitrogen. The livers were kept at -80 °C until the extraction of RNA was performed to analyze gene expression profiling by quantitative RT-PCR. For this experiment, we examined the following gene groups: i) coagulation factors, including fibrinogen (*Fgb*), prothrombin (*F2*), factor V (*F5*), factor VII (*F7*), factor FVIII (*F8*), factor IX (*F9*), factor X (*F10*), factor XI (*F11*), factor XII (*F12*), and factor XIIIβ subunit (*F13b*); ii) coagulation-related or fibrinolytic factors, including antithrombin (*Serpinc1*), protein C (*Prosc*), protein S (*Pros1*), plasminogen (*Plg*), von Willebrand factor (*VWF*), and a disintegrin-like and metalloproteinase with thrombospondin type 1 motif 13 (*Adamts13*), and iii) factors involved in the production and degradation of FVIII, including lectin, mannose-binding 1 (*Lman1*), multiple coagulation factor deficiency 2 (*Mcfd2*), and low-density lipoprotein receptor-related protein 1 (*Lrp1*). Based on our recent experimental data in regenerating mouse livers [3], we adopted the geometric mean of the two reference genes, peptidylprolyl isomerase A (*Ppia*) and TATA box binding protein (*Tbp*), to normalize the gene expression calculated in our experiments. The gene expression profiles were correlated with the protein levels and/or activity by obtaining blood samples from the retroorbital plexus prior to the PHx and at the time of sacrifice. Samples were collected with an anticoagulant (0.1 vol 3.8% sodium citrate) and stored at -80 °C until analysis. Plasma coagulation factor activities (fibrinogen, prothrombin, factor V, VII, VIII, IX, X, XI, XII, and XIII), von Willebrand factor (VWF) antigen levels, and tissue-type plasminogen activator (t-PA) activities were measured as described below.

Experiment 2: Examination of F8 mRNA expression levels in mouse tissues during liver regeneration

C57BL/6 mice (n = 16) were either received 2/3 PHx (PHx group; n = 8) or sham-operated (non-PHx group; n = 8), and various organs (i.e., liver, lung, spleen, heart, intestine, brain, and kidney) were obtained from all of the mice 24 h after the initial procedure. This time point is known to be the peak phase in the liver regeneration process for mice [1,2]. The *F8* mRNA expression levels were analyzed in all of the extracted organs by real-time RT-PCR. To obtain a reliable gene expression profile for *F8* in the liver and other extra-hepatic organs, appropriate reference genes that are minimally affected by the PHx needed to be validated. To achieve this goal, we analyzed the expression levels of 8 commonly-used reference genes, which included: glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*); β-actin (*Actb*); hypoxanthine phosphoribosyltransferase (*Hprt1*); β-glucuronidase (*Gusb*); peptidylprolyl isomerase A (*Ppia*); TATA box binding protein (*Tbp*); transferrin receptor (*Tfrc*), and ribosomal protein L4 (*Rpl4*). The variance of the reference gene expression in all samples were assessed using two software programs, geNorm and NormFinder [18,19], which have been described elsewhere [3,20]. Using this approach, the *F8* mRNA expression were calculated following normalization with the most stable reference gene combination.

RNA isolation and quality controls

Total RNA was extracted from each tissue samples using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNase I (QIAGEN) was used to eliminate any genomic DNA contamination, and the concentration of the RNA was determined by UV spectrometry.

Reverse transcription coupled to quantitative real-time PCR (real-time RT-PCR)

Total RNA (1 μg) was reverse-transcribed using oligo $d(T)_{16}$ primers as described by the manufacturer (Omniscript RT Kit) (QIAGEN). First-strand cDNA samples were subjected to quantitative PCR amplification using a PRISM 7700 Sequence Detector (Applied Biosystems Japan, Tokyo, Japan). Coagulation factor genes, fibrinolytic factor genes, and the genes of coagulation related factors or protein as listed in Table 1 were assessed in each tissue cDNA. TaqMan probes and primers for these genes were chosen from TaqMan Gene Expression Assay (Applied Biosystems) (Table 1). The primer sets of reference genes were the same products as described in our previous report [3]. For quantification of gene expression, the cDNAs derived from total RNA extracted from pooled normal mouse livers were serially-diluted, and used to generate the reference standard curves.

Plasma analyses

Coagulation factor activities were measured by one-stage clotting assay using human plasma deficient for each coagulation factor (Sysmex, Kobe, Japan). The activities of FVIII, FIX, FXI, and FXII were measured based on aPTT using ThromboCheck APTT-SLA (Sysmex) and prothrombin, FV, FVII, and FX based on PT using ThromboCheck PT Plus (Sysmex), by a KC10A Coagulometer (Amelung, Lemgo, Germany). Plasma VWF antigen levels were assayed by sandwich ELISA using a

Table 1
Description of analyzed target gene primers used in the real-time RT-PCR assay.

Symbol	Gene Name	Assay ID	Amplicon Length (bp)
Fgb	fibrinogen, B beta polypeptide	Mm00805336_m1	154
F2	coagulation factor II (prothrombin)	Mm00438843_m1	68
F5	coagulation factor V	Mm00484202_m1	61
F7	coagulation factor VII	Mm00487329_m1	78
F8	coagulation factor VIII	Mm00433174_m1	110
F9	coagulation factor IX	Mm01308427_m1	74
F10	coagulation factor X	Mm00484177_m1	81
F11	coagulation factor XI	Mm00511167_m1	115
F12	coagulation factor XII	Mm00491349_m1	64
F13b	coagulation factor XIII, beta subunit	Mm00491938_m1	62
Serpinc1	antithrombin	Mm00446573_m1	94
Prosc	protein C	Mm00435966_m1	52
Pros1	protein S (alpha)	Mm01343426_m1	62
Plg	plasminogen	Mm00447087_m1	83
VWF	von Willebrand factor	Mm00550376_m1	63
Adamts13	a disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 13	Mm01218030_g1	77
Lman1	lectin, mannose-binding, 1	Mm00522499_m1	79
Mcfd2	multiple coagulation factor deficiency 2	Mm00454818_m1	57
Lrp1	low density lipoprotein receptor-related protein 1	Mm00464608_m1	104

capture antibody against human VWF (Dako, Glostrup, Denmark), and goat anti-human VWF-HRP antibody (Dako) as a detecting antibody. Fibrinogen levels, FXIII levels, and tPA levels were measured by the Clauss method using bovine thrombin, Berichrom FXIII chromogenic assay (Dade Behring, Marburg, Germany), and Mouse tPA Activity Assay (Oxford Biomedical Research, Oxford, MI) according to the manufacturer's instructions, respectively. For all the assessments, pooled plasma collected from 50 normal C57BL/6 mice was used as standards.

Statistical analysis

Significant differences between two groups were analyzed by 2-tailed Mann-Whitney U-test with ystat2006 software (Igakutosyosyuppan, Tokyo, Japan). $P<0.05$ was considered significant.

Results

Experiment 1: Comparison of liver mRNA expression levels and plasma levels of coagulation factors

Liver samples were analyzed for mRNA expression levels by real-time RT-PCR in both quiescent (Day 0, n = 25) and actively proliferating livers following PHx at Day 1, 2, 5 after the procedure (n = 9, 8, and 8, respectively). First, we assessed the 10 coagulation factor genes; *Fgb, F2, F5, F7, F8, F9, F10, F11, F12*, and *F13b*, which are mainly produced in the liver. Compared with values at Day 0, mRNA expression levels of all the analyzed coagulation factors were significantly reduced ($P<0.05$) (Fig. 1). All of the reduced gene expression levels gradually recovered to control (Day 0) levels by Day 5 in all except for *F7* and *F8*. The *F7* and *F8* mRNA expression levels remained significantly lower ($P<0.05$) at Day 2 and 5 compared with those at Day 0 (Fig. 1).

To correlate whether the protein activity in the plasma for the coagulation factors were concomitantly suppressed similar to the gene expression, blood was collected from each mouse and examined for their level of activity. As shown in Fig. 2, plasma activities were found to have differential activity levels during liver regeneration compared to the quiescent state (Day 0). Most of the coagulation factors (prothrombin, FV, FVII, FIX, FX, and FXII) were significantly lower at Day 1 compared to their corresponding values at Day 0. Among all of the factors examined, FVII showed the greatest decline in activity, whereby only ~30% of the activity remained at Day 1 compared to Day 0. Consistent with the recovery in liver mRNA expressions, plasma activities of many factors returned towards the Day 0 levels by the end of the experimental period (Day 5). Plasma activities of fibrinogen, FXI, and FXIII, however, showed no significant changes during the liver regeneration process. For several factors (prothrombin, FV, FIX, and VWF), there were substantial variances in Day 0 values from those of the reference pooled plasma. We assume this deviation might attribute to an individual variability in the level of plasma coagulation factor levels of mice.

In sharp contrast, plasma FVIII activity was significantly increased at Day 1 ($166.1\pm20.2\%$; $P<0.05$) compared to the quiescent state (Day 0; $110.4\pm5.1\%$), even though the *F8* mRNA expression was paradoxically suppressed at Day 1. In addition, we detected a significant increase by 2.1-fold ($P<0.05$) in the plasma antigen levels of VWF compared to the quiescent Day 0 state. Both the FVIII and VWF tended to gradually decline back towards quiescent (Day 0) values until Day 5 (Fig. 2).

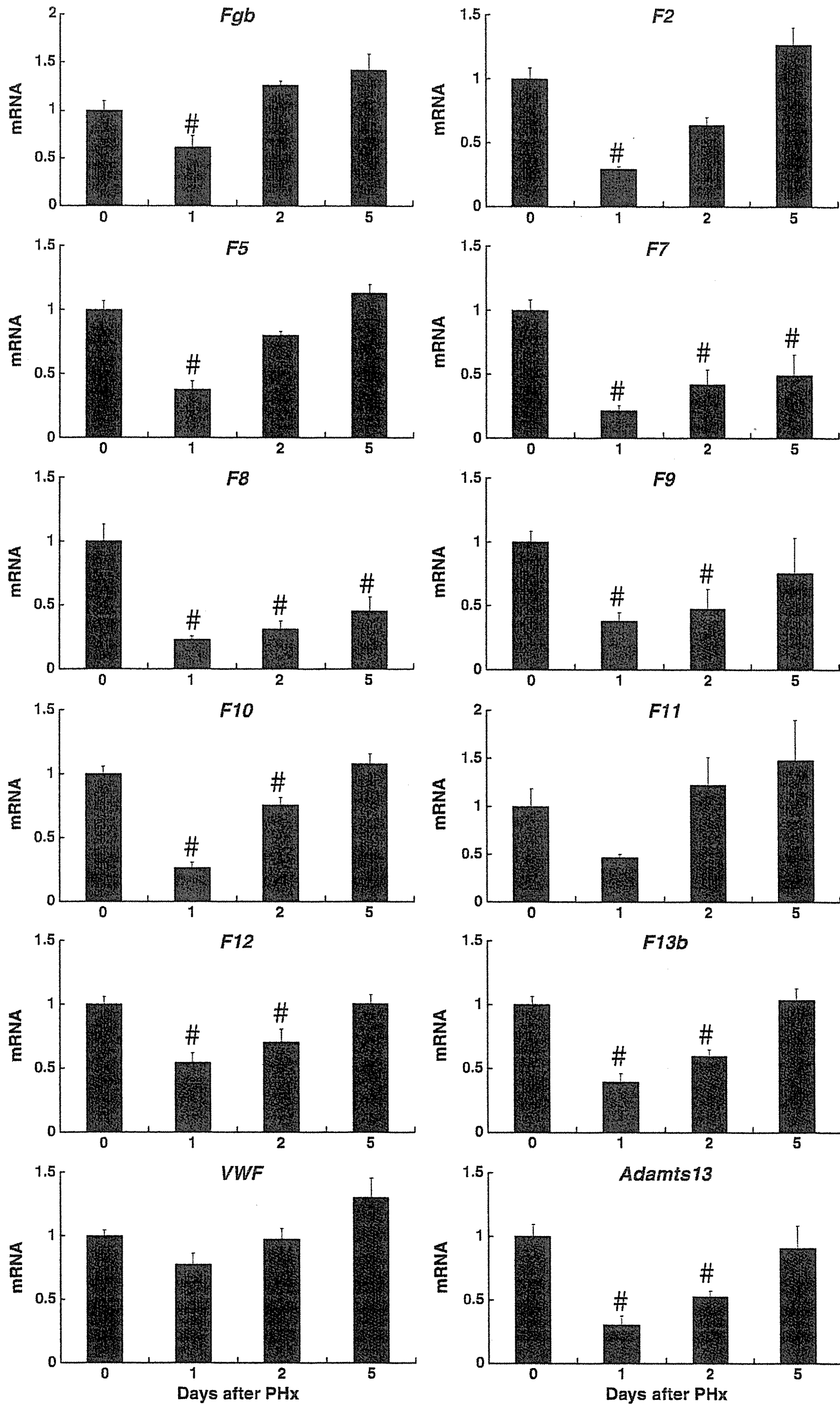
To determine whether the increased plasma FVIII activities and VWF antigen levels were associated with anesthesia and surgical stress, we performed sham-operation (i.e., abdominal incision and opening) under general anesthesia in the C57BL/6 mice (n = 10) and investigated the plasma FVIII activities and VWF antigen levels. Slight increases were observed after sham-operation in all individuals but reached no statistical significance (data not shown), which indicated that the increased FVIII and VWF in plasma were predominantly associated with liver resection and the subsequent liver regeneration process.

In addition to coagulation factors analyzed above, we also assessed the mRNA expression profiles of: i) fibrinolytic factor (*Plg*); ii) anti-coagulation factors (*Serpinc1, Prosc*, and *Pros1*); iii) other coagulation-related factors (*VWF* and *Adamts13*); and iv) factors related to FVIII production and degradation (*Lman1, Mcfd2*, and *Lrp1*). It has been documented that *Plg, Serpinc1, Prosc, Pros1*, and *Adamts13* are expressed mainly in the liver, and VWF is expressed by vascular endothelial cells throughout the body. *Lman1* and *Mcfd2* genes encode the 53-kDa ER-Golgi intermediate compartment protein, called ERGIC53, which plays important roles in the transfer of the primary translation products of *F5* and *F8* from the ER to the Golgi [21]. *Lrp1* is a hepatic cell-surface receptor protein which mediates the clearance of circulating FVIII [22]. As shown in Figs. 1 and 3, the mRNA levels of *Plg, Serpinc1, Prosc, Pros1*, and *Adamts13* were reduced at Day 1 and 2 after PHx, and the levels returned back towards the quiescent levels by Day 5. As for *Pros1*, the mRNA levels were significantly above those at Day 0 by Day 2 and 5. This reason is unclear, but it may due to the changes of some female hormones or transcription factors. The mRNA expression levels of *VWF, Mcfd2*, and *Lrp1* did not significantly change during the liver regeneration process, whereas *Lman1* mRNA levels were increased at Day 1, but returned to quiescent Day 0 levels by Day 2.

Experiment 2: Examination of F8 mRNA expression levels in mouse tissues during liver regeneration

In spite of the *F8* mRNA reduction in the liver as shown in Experiment 1, the plasma FVIII activity was paradoxically and significantly increased ($P<0.05$) during the liver regeneration phase. Because of this finding,

Fig. 1. Gene expression profiling of coagulation factors in quiescent and actively regenerating mouse livers. The gene expression profiles for 10 coagulation factors (*Fgb, F2, F5, F7, F8, F9, F10, F11, F12*, and *F13b*) and 2 hemostasis-related factors (*VWF* and *Adamts13*) in mouse livers under quiescent and regenerating status from Experiment 1 were assessed. The liver lobes removed at the time of hepatectomy were denoted as quiescent liver samples (Day 0, n = 25). The remainder of the actively proliferating liver lobes were harvested at Day 1, 2, or 5 after hepatectomy (n = 9, 8, and 8, respectively). The data were normalized to the geometric mean of *Ppia* and *Tbp* mRNA levels, and expressed as a comparative ratio to the Day 0 samples. All values were represented as mean ± SEM. $^{\#}P<0.05$ vs Day 0.


Fgb
F2
F5
F7
F8
F9
F10
F11
F12
F13b
VWF
Adamts13
mRNA
#
Days after PHx

we speculated that other extra-hepatic organs may be involved in producing FVIII to compensate for the loss of the liver production of this specific coagulation factor after the PHx. We assessed the *F8* mRNA expression levels from the following organs taken from mice with (n = 8) or without PHx (n = 8): liver, lung, spleen, heart, intestine, brain, and kidney. Prior to analyzing *F8* mRNA expression in the liver and extra-hepatic organs, we determined appropriate housekeeping reference genes that would have minimal variance between the examined organs under quiescent status or liver regeneration status using two different programs. As a result, it was elucidated that applying combination of *Hprt1*, *Rpl4*, and *Tbp*, provide the least fluctuation in the reference gene expression. Therefore, we decided to normalize the FVIII mRNA expression by calculating the geometric mean of these three genes. Using this approach, we found that *F8* mRNA levels in the liver were significantly suppressed ($P<0.05$) at Day 1 after PHx (Fig. 4), which was consistent with the results obtained in Experiment 1 (Fig. 1). However, the *F8* mRNA expression levels in all of the organs other than the liver showed no significant difference between PH group and non-PHx groups (Fig. 4). These results indicated that FVIII transcription *per se* from extra-hepatic organs were not affected by stimuli associated with liver regeneration, and that the elevated circulating plasma FVIII activity during the liver regeneration process was unlikely due to increased gene transcription of this protein.

Discussion

The present study demonstrated liver gene expression profiles of coagulation and anti-coagulation / fibrinolytic factors as well as plasma levels of coagulation factors during the active proliferation of the livers in mice. At the peak of the liver regenerative phase (i.e. Day 1 and 2), the expression levels for nearly all of the genes analyzed were temporally reduced and recovered back towards the normal levels as the regeneration event comes to the completion (i.e. Day 5). Consistent with the gene expression levels, plasma activities for all of the coagulation factors, except for FVIII, were temporally decreased during the same liver regenerative period.

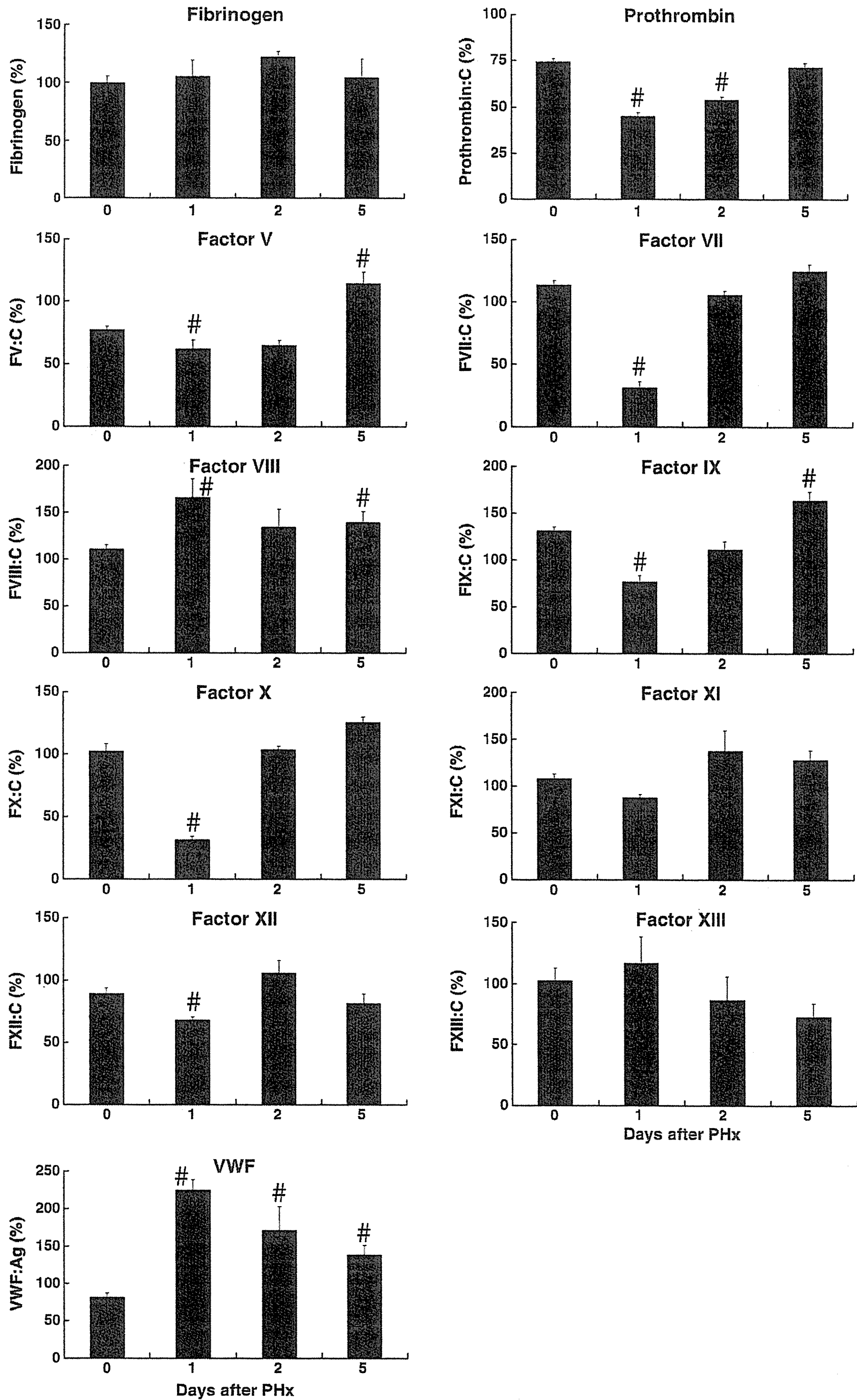
Using real-time RT-PCR, we determined that gene expression levels for the coagulation factors (*Fgb*, *F2*, *F5*, *F7*, *F8*, *F9*, *F10*, *F11*, *F12*, and *F13b*), a fibrinolytic factor (*Plg*), and anti-coagulation factors (*Serpinc1*, *Prosc*, and *Pros1*) decreased at the peak of the liver regenerative state (Day 1 and 2) following PHx, and this was followed by a rebound increase back towards to pre-hepatectomy levels at Day 5. The results are consistent with previous studies in which gene expression levels of many liver-specific proteins are temporally suppressed during the process of liver regeneration [3,23]. The time line for the changes in gene expression for the coagulation factors appears to have an inverse relationship to the DNA synthetic phase. It is known that the first 24 h period following PHx, hepatocyte DNA synthesis is initiated and begins to synchronize and peak by 48 h. Subsequently, the DNA synthesis begins to slow and returns towards the quiescent levels at Day 5 [1,2]. Our findings would suggests that the regenerating liver sacrifices the production of liver-specific proteins, which are predominantly represented as plasma proteins, to promote the cell division and growth [24]. This speculation is strengthened by our previous report in which the liver gene expressions of coagulation-related factors were significantly reduced during the progression of direct hyperplasia-mediated liver regeneration in mouse model [25].

Interestingly, the corresponding plasma activities for FVIII and other coagulation proteins, fibrinogen and FXIII, were paradoxically increased or not decreased during the same stages of liver regeneration after PHx, even though all of the mRNA expression profiles and all other coagulation factor activities were decreased. As for fibrinogen and FXIII, the half-life time of both factors are relatively long (4 to 7 days and 9 to 14 days, respectively) among all coagulation factors. This might be the reason why these two factors showed no significant decrease in plasma activities while the gene expression levels in the liver were temporally suppressed. Plasma FXIII is composed of two types of subunits, α and β, each of which is produced in different tissues (bone marrow and liver, respectively). The mRNA levels assessed in this study were FXIII β (*F13b*) and plasma activity measured mainly reflects the FXIII α function. This difference may also account for the sustained FXIII activity in plasma. In contrast, the half-life time of FVII is the shortest (1.5 - 5 h) among all coagulation factors, and this could have been associated with the drastic drop of the plasma FVII activity observed at Day 1 after PHx (27.5 % of non-PHx levels). The reason why plasma FVII levels recovered by Day 5 in spite of suppressed mRNA levels at the time point is unclear, but it is reasonable to postulate that it might represent the trauma-induced FVII activation and overestimation by the presence of FVIIa. On the other hand, the mechanism for the increased activity levels for FVIII remains to be well understood. As possible mechanisms for this paradoxical FVIII activity, we postulated the following reasons; (i) increased extrahepatic FVIII production, (ii) delayed inactivation or clearance of circulating FVIII in plasma, (iii) excessive releases of pooled FVIII from storage sites, and (iv) an alteration of intracellular trafficking pathway of FVIII.

First, the liver has been shown to be the major site of FVIII production. A previous study transplanted a normal functioning liver into a hemophilia A patient to completely correct the bleeding diathesis [26]. There is also documentation that FVIII production may be assisted by extra-hepatic tissues, such as the lung, kidney, and/or spleen [27,28]. Consistent with these previous reports, we detected *F8* mRNA in various organs, but detected no PHx-associated changes for all of the extra-hepatic organs studied. Hollestelle et al. observed the sustained plasma FVIII levels in anhepatic pig models, and they also detected no increase of *F8* mRNA in spleen, kidney, and lung [29]. Although their data as well as our data cannot totally exclude the possibility of FVIII production from organs not analyzed in the studies, it is unlikely that the induction of extrahepatic FVIII production is involved in mediating the increased FVIII activity during liver regeneration.

Second, several factors are known to be involved in delaying the loss of plasma FVIII activity. VWF acts as FVIII carrier protein and protects FVIII from proteolysis in the circulation. Increased plasma VWF may lead to enhanced binding and protection of FVIII during the liver regenerative phase and promote increased activity (Fig. 2). Mechanistically, the elevated plasma VWF levels may involve the tight regulation by ADAMTS13. In line with the recent clinical report [30], we have observed that regenerating liver temporally suppressed ADAMTS13 expression (Fig. 1), and so the reduction of ADAMTS13 may be involved in mediating the elevated plasma VWF. Alternatively, the clearance of circulating FVIII is normally mediated by low-density lipoprotein receptor-related protein 1 (*Lrp1*), a hepatic clearance receptor protein [22]. Suppression of the interaction between FVIII and Lrp1 could thus theoretically prolong the plasma FVIII half-life time. The present study showed that the *Lrp1* mRNA expression levels were constant regardless of the regeneration status, suggesting that total Lrp1 production levels from the liver had been dropped during the liver regeneration phase because of PHx-induced liver mass reduction. Taken together, the observed plasma FVIII increase could be attributed to the enhanced protection from proteolysis by higher

Fig. 2. Changes in plasma activity of circulating coagulation factors during liver regeneration. Plasma activity levels of 10 coagulation factors (fibrinogen, prothrombin, factor V, VII, VIII, IX, X, XI, XII, and XIII) and plasma VWF antigen levels were determined from mice that either had quiescent livers or were undergoing active liver proliferation from Experiment 1. Levels of prothrombin, factor V, VII, VIII, IX, X, XI, and XII were measured by one-stage clotting assay, fibrinogen levels were measured by the Clauss method, factor XIII levels were measured by chromogenic assay, and VWF antigen levels were determined by specific ELISA. The plasma samples obtained before hepatectomy were used as Day 0 samples to determine plasma activities during liver quiescence (n = 25), and the plasma samples obtained 1, 2, or 5 days after hepatectomy were used as samples to assess active proliferation at various states of liver regeneration (n = 9, 8, and 8, respectively). The data were described as percentage of pooled normal plasma, and represented as the mean ± SEM. #$P<0.05$ vs day 0.



Fibrinogen
Fibrinogen (%)
Prothrombin
Prothrombin:C (%)
Factor V
FV:C (%)
Factor VII
FVII:C (%)
Factor VIII
FVIII:C (%)
Factor IX
FIX:C (%)
Factor X
FX:C (%)
Factor XI
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Factor XII
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FXIII:C (%)
Days after PHx
VWF
VWF:Ag (%)
Days after PHx

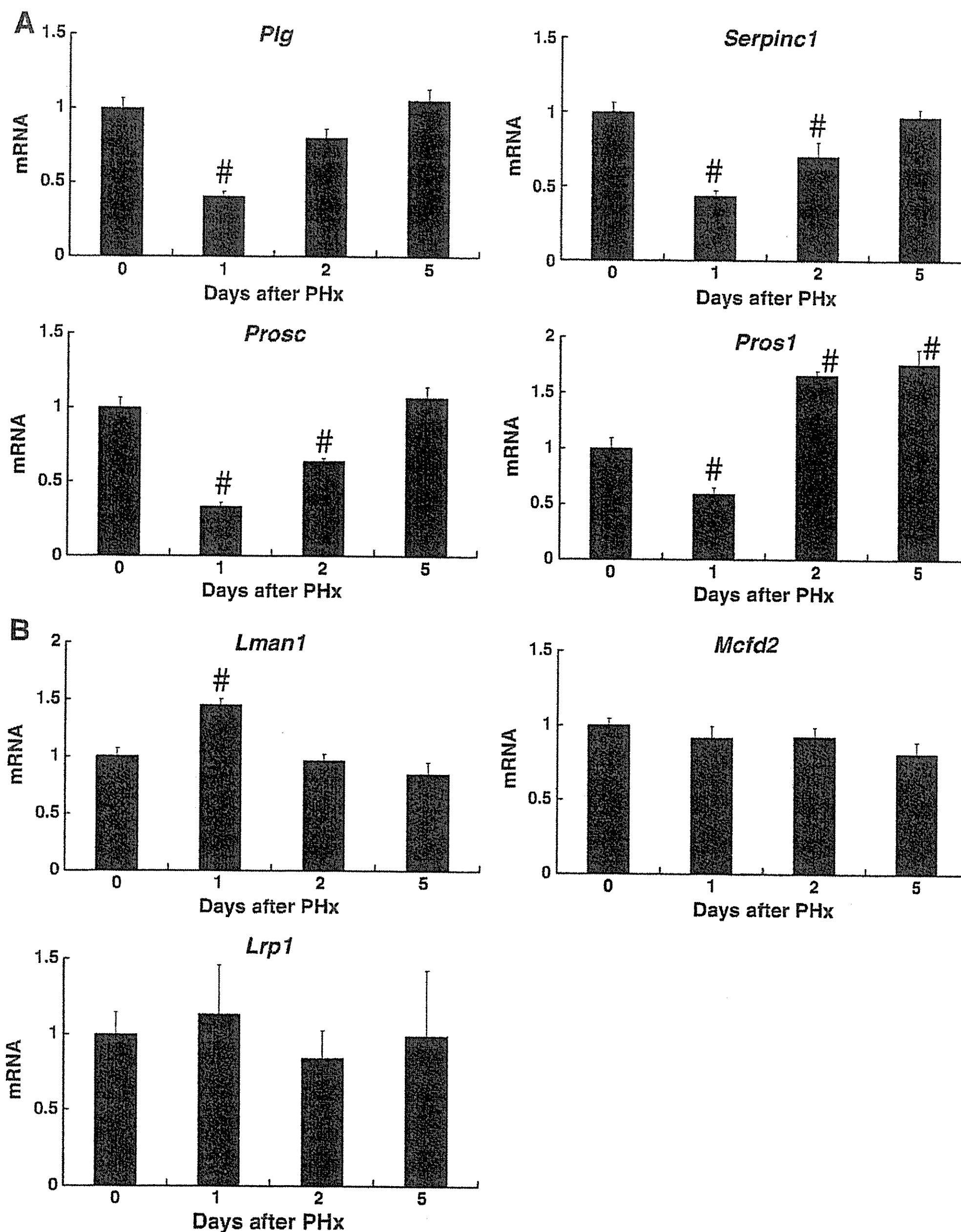

Fig. 3. The gene expression profiling of fibrinolytic and anti-coagulation factors (A), and several important factors related to factor VIII production and degradation (B) in quiescent and actively regenerating mouse livers. The gene expression levels of fibrinolytic factor (*Plg*), anti-coagulation factors (*Serpinc1*, *Prosc*, and *Pros1*), and several important factors relating to factor VIII production and degradation (*Lman1*, *Mcfd2*, and *Lrp1*) were examined from quiescent and actively regenerating mouse livers as described in Experiment 1 by quantitative RT-PCR. The same liver samples used in Fig. 1 were used for this analysis. The data were normalized to the geometric mean of *Ppia* and *Tbp* mRNA levels, and expressed as a comparative ratio to the Day 0 samples. #P<0.05 between groups.

levels of VWF and/or the reduced protein clearance by Lrp1. These speculations were supported by the previous findings by Hollestelle et al., in which the prolonged half-life time of administered FVIII was associated with the increased plasma levels of VWF in anhepatic pig models [29]. However, it is less likely that only the delayed inactivation or clearance is the major contributing factor for the observed plasma FVIII increase by more than 150% under the condition of markedly suppressed FVIII production in the liver (70% down-sizing and additional 70% suppress of gene expression in the remnant liver).

Thus, we investigated further two mechanisms responsible for the increased plasma FVIII. One possible mechanism is the excessive release of FVIII from the storage sites. Although the precise site(s) of FVIII production remains controversial [6,7,31], it has been assumed that synthesized FVIII is trafficked with VWF and partially becomes pooled into granules found within endothelial cells known as Weibel-Palade bodies [32], and the pooled contents of the granules are released by various stimuli [33]. The Weibel-Palade bodies also contain several other substances including IL-8, tPA and P-selectin, and both tPA and VWF are coordinately released in response to an agonist stimulation [33]. In this present study, the plasma levels of tPA activity were significantly increased in a similar manner of VWF and FVIII at Day 1 compared to the quiescent Day 0 period (191.2 ± 9.8 vs 100 ± 13.6 %). Another possible mechanism is the change of intracellular processing in the FVIII producing cells. The efficient FVIII secretion into the bloodstream requires the intracellular trafficking of the FVIII protein, including *Lman1* and *Mcfd2* genes, which encode proteins that forms a

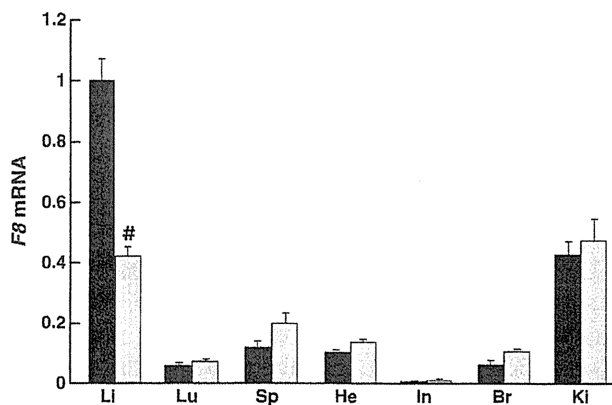

Fig. 4. Extra-hepatic gene expression of *F8* mRNA following hepatectomy in mice. Liver (Li), lung (Lu), spleen (Sp), heart (He), intestine (In), brain (Br), and kidney (Ki) were obtained from mice with hepatectomy (PHx group) or without hepatectomy (non-PHx group) at 24 h time point (n = 8 in each group) in Experiment 2. *F8* mRNA expression levels were normalized to the geometric mean of three housekeeping genes (*Hprt1*, *Rpl4*, and *Tbp*), which were validated as the most stable reference genes in this experimental condition by geNorm and NormFinder. Normalized data were expressed as a comparative ratio to the non-PHx liver samples. Black bars = non-PHx mouse group; gray bars = PHx-group mice. #*P*<0.05 vs non-PHx group.

complex that mediates FVIII transport from the endoplasmic reticulum to the Golgi apparatus [21]. During the liver regeneration phase, the present data showed that *Lman1* mRNA expression was significantly increased, and we speculate that the increase of *Lman1* expression might result in elevated levels of FVIII activity.

In conclusion, the current study showed that the process of liver regeneration involves a general reduction of gene expression for many of the coagulation cascade proteins, but there are paradoxical increases in plasma levels of FVIII and VWF. Detailed investigation revealed that this increase of plasma FVIII activity may be associated with a delay in the inactivation or clearance coupled to the release from the storage sites in endothelial cells and the alteration of *Lman1* associated intracellular trafficking pathway during liver regeneration. This finding for the increased activity of circulating FVIII may suggest that this particular coagulation factor may play an important role in maintaining the blood coagulation balance during liver regeneration, and possibly exacerbate the potential for a thrombotic episode. In addition, our observation that gene expressions of coagulation-related proteins are generally reduced in dividing cells gives an important insight to the field of hepatocyte-directed stem cell research, especially for coagulation factor production.

Conflict of interest statement

All authors have no conflict of interest to declare.

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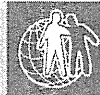
Haemophilia



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ORIGINAL ARTICLE *Inhibitors*

An analysis of factors affecting the incidence of inhibitor formation in patients with congenital haemophilia in Japan

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Summary. Studies conducted in European and North American countries have demonstrated that various factors including races affect the frequency of inhibitor formation in haemophilia patients. The present study was undertaken to analyse factors affecting the incidence of inhibitor formation in Japanese haemophilia A and B patients. Analytical data were retrospectively collected from haemophilia A and B patients born after 1988, the year when monoclonal antibody-purified factor VIII products were first marketed in Japan. Various data were collected from 184 patients (153 cases of haemophilia A; 31 cases of haemophilia B). The sample size of haemophilia B cases was too small to reveal any significant differences between the inhibitor formation group and the inhibitor-free group in any of background variables. For patients with haemophilia A, on the other hand, univariate analysis identified the severity of haemophilia and a positive family history of inhibitor development as risk factors for the formation of inhibitors. In analyses of the clotting factor products used, the incidence of inhibitor formation did not differ significantly between the group treated with plasma-derived products (29.7%) and the group treated with recombinant products (25.0%). When background variables were compared, age was higher in the group treated with plasma-derived products but none of the other background variables differed between the two groups. These results suggest that in Japanese haemophilia patients, the type of clotting factor preparations used for therapy has not influenced the incidence of inhibitor formation.

Keywords: coagulation products, haemophilia, incidence, inhibitor, risk factor

Introduction

Haemostatic treatment for patients with haemophilia has advanced considerably in the past two decades. Safe clotting factor concentrates with high haemostatic activity have become available, and increasing clinical evidence has been accumulated to confirm that regular prophylactic infusions of these products help to prevent the onset and progression of haemophilic arthropathy arising from repeated intra-articular bleeding. It is also evident, however, that the infusion of clotting factor products induce the formation of allo-antibodies (inhibitors), which inactivate factor VIII (or factor IX) in 20–30% of patients with severe haemophilia A and 3–5% of patients with severe haemophilia B. Conventional treatment protocols involving infusion of deficient clotting factors in these patients with inhibitors are poorly effective, and haemostatic control is often difficult in these circumstances, especially in patients with high-responding inhibitors. Moreover, quality of life (QOL) indices are reduced markedly in these patients [1].

Studies conducted in European and North American countries have demonstrated that various genetic factors affect the frequency of inhibitor formation. The most detailed analyses have examined the type and location of

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factor VIII gene mutations, and have revealed that the incidence of inhibitor formation was highest in haemophilia A patients with large deletions, nonsense mutations or inversions [2–9]. A higher incidence of inhibitor formation is known for Black patients [10], and a recent report suggested that differences in factor VIII haplotype contributed to this feature [11]. It is also known that within the same family some patients with haemophilia develop inhibitors whilst others do not, possibly reflecting the polymorphism of genes encoding immunomodulating cytokines [12–14] or indicating the influence of acquired factors [15–20]. With regard to the type of product used for therapy, close attention has been paid to the relationship between the incidence of inhibitor formation and the use of recombinant products or plasma-derived products [21–28].

The present study was undertaken to assess the incidence of inhibitors in patients born after 1988, the year when monoclonal antibody (mAb)-purified factor VIII products were first marketed in Japan. The analyses was restricted to patients that had been treated with clotting factor products for at least 2-years, with the goal of identifying background variables and focusing on whether or not the type of clotting factor products affected the incidence of inhibitor formation.

Methods

Selection of treatment centres included in the survey

Medical centres experienced in the treatment of haemophilia and with comprehensive records enabling a detailed investigation of the history of treatment in individual patients were invited to participate in this study.

Inclusion criteria

Patients with congenital haemophilia A or B satisfying the all following criteria were eligible to study:

1. Born between 1 January 1988 and 31 December 2006.
2. Data on the history of treatment with factor VIII (IX) products available for at least 2 years after the first infusion of clotting factor products.
3. Data on inhibitor assays available for at least 2 years after the first infusion.
4. Informed consent available in writing from the guardian (and also the patient aged 16 years and older).

Investigations

1. Background variables: name initials, gender, birth date, time (year and month) of diagnosis, factor VIII (IX) activity and blood group.
2. Complications: presence/absence of haemophilic arthropathy, hepatitis B, hepatitis C, HIV infection, haemorrhagic disease other than haemophilia and any other severe disease.
3. Neonatal history: manner of delivery and feeding method.
4. Family history: presence/absence of family history of haemophilia with or without inhibitor.
5. Treatment methods: age at the time of first infusion, and administration method of clotting factor products (on demand or regular prophylaxis).
6. Clotting factor products: the name of the clotting factor products and the number of days used during the 2-year period after the first infusion or before detection of inhibitor in patients showing inhibitor formation.
7. Severe bleeding episodes and invasive surgery: presence/absence and time of episode/surgery and the site of bleeding during the 2-year period after the first infusion.
8. Presence/absence of inhibitors: in patients showing inhibitor formation, the inhibitor level on detection, the date of measurement, peak inhibitor level and latest inhibitor level [presence/absence of immune tolerance induction (ITI) therapy if inhibitor had disappeared].

Survey period

The survey was conducted between 1 January 2008 and 31 December 2009.

Statistical analysis

The *t*-test and analysis of variance were used on numerical variables and chi-squared test was employed for nominal variables. In addition, multivariate analysis was conducted by means of logistic regression.

Ethical considerations

The study was conducted in accordance with the Japanese Ethical Guidelines on Epidemiological Studies after approval by the Nara Medical University Ethics Committee. In addition where necessary, the approval of the ethics committee of individual participating medical centres was also obtained.

Results

Analytical data were collected from 184 patients (153 cases of haemophilia A; 31 cases of haemophilia B). Of the 153 patients with haemophilia A, 41 (26.8%) developed inhibitors. In 29 of these 41 patients (70.7%), including four patients who had not received ITI, inhibitors had disappeared by the time of last evaluation. On the other hand inhibitors were persistent

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in 12 patients including 10 patients who had received ITI.

In univariate analyses, the background variables found to differ significantly between the inhibitor formation group and the inhibitor-free group were: the severity of haemophilia, family history of haemophilia patients with inhibitor (Table 1), age at the time of the first infusion of clotting factor products (Fig. 1) and factor VIII activity (Fig. 2). No other background variable differed significantly between the two groups (Table 1), including blood group and presence/absence of hepatitis A, hepatitis B and HIV infection. Logistic regression analyses of these variables demonstrated that only the family history of haemophilia patients with inhibitor had a significant influence on inhibitor formation ($P = 0.002$).

One hundred and fifty patients, excluding three haemophilia A patients who had received treatment with factor IX products, were classified into three groups according to the type of clotting factor product used during the 2-year period after the first infusion, or before the detection of inhibitor: (i) patients treated with plasma-derived products alone ($n = 37$), (ii) patients treated with recombinant products alone ($n = 104$), and (iii) patients treated with both plasma-derived products and recombinant products ($n = 9$). The incidence of inhibitor formation did not differ significantly among these three groups (29.7%, 25.0% and 22.2% respectively). Of the 46 patients who received plasma-derived products, nine received only factor VIII products containing von Willebrand factor. Among these nine patients, inhibitor formation was seen in four patients, but the incidence for this group did not differ significantly from that for the other groups. We also compared the peak inhibitor level, the status of inhibitor formation at the time of last observation and the response rate to ITI between the group treated with plasma-derived products alone and the group treated with recombinant products alone. These comparisons revealed no inter-group differences (Table 2). Furthermore, other background variables were compared in these two groups i.e. age, severity of haemophilia, family history of inhibitor, treatment method (on demand or regular prophylaxis) during the 2-year period after the first infusion, the presence/absence of intracranial haemorrhage, other severe bleeding episodes and invasive surgery. The average age of the patients at the end of the survey (31 December 2009) was significantly higher in the group treated with plasma-derived products alone than in the group treated with recombinant products alone, but no other background variable differed significantly between the two groups (Table 3).

Among the 31 patients with haemophilia B, six (19.4%) developed inhibitors. As in the haemophilia A patients, background variables in those haemophilia B patients who developed inhibitors were compared with those that remained inhibitor-free. Univariate analyses indicated that there were no significant inter-group differences in any of the background variables, possibly attributable to some extent to the small sample size.

Table 1. Background variables compared between inhibitor formation cases and inhibitor-free cases with haemophilia A.

Background variable (number of patients)	Inhibitor-free (%)	Inhibitor positive (%)	*P*-value
Severity (n = 153)			
Mild	17/112 (15.2)	0/41 (0)	0.0243
Moderate	13/112 (11.6)	4/41 (9.8)	
Severe	82/112 (73.2)	37/41 (90.2)	
Haemophilic arthropathy (n = 153)			
Present	25/112 (22.3)	14/41 (34.1)	0.2044
Family history of haemophilia (n = 148)			
Positive	46/108 (42.6)	21/40 (52.5)	0.3736
Family history of inhibitor formation (n = 122)			
Positive	3/91 (3.3)	9/31 (29.0)	0.0001
Treatment after first infusion (n = 151)*			
On demand	78/111 (70.3)	30/40 (75.0)	0.7255
Regular prophylaxis	33/111 (29.7)	10/40 (25.0)	
History of intracranial haemorrhage (n = 149)*			
Positive	11/108 (10.2)	8/41 (19.5)	0.2160
History of other severe bleeding (n = 147)*			
Positive	8/106 (7.5)	1/41 (2.4)	0.4384
History of invasive surgery (n = 148)*			
Positive	9/107 (8.4)	1/41 (2.4)	0.3577
Manner of child delivery (n = 109)			
Transvaginal	67/79 (84.8)	27/30 (90.0)	0.7198
Caesarean section	12/79 (15.2)	3/30 (10.0)	
Feeding method (n = 94)			
Breast-feeding	26/66 (39.4)	15/28 (53.6)	0.4470
Bottle-feeding	15/66 (22.7)	5/28 (17.9)	
Mixed	25/66 (37.9)	8/28 (28.6)	

*Before detection of inhibitor in inhibitor formation cases and for a 2-year period after the first infusion in inhibitor-free cases.

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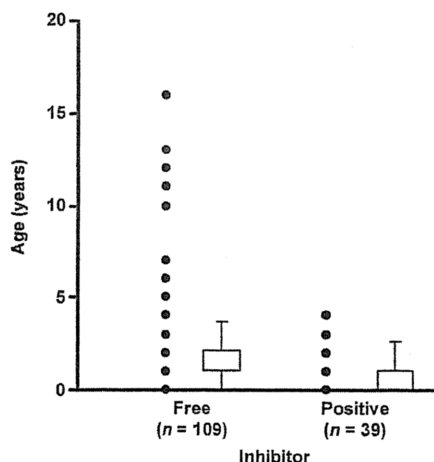

Fig. 1. Age at the time of first administration of clotting factor product (haemophilia A). Age at the time of the first infusion of clotting factor VIII products was significantly earlier in the inhibitor formation group than in the inhibitor-free group ($P < 0.001$).

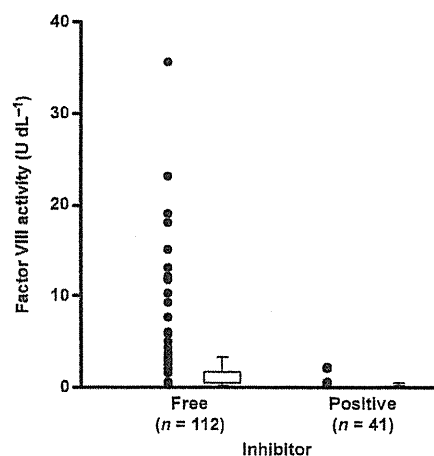

Fig. 2. Factor VIII activity. Factor VIII activity was significantly higher in the inhibitor-free group than in the inhibitor formation group ($P < 0.001$).

Table 2. Status of inhibitor formation in relation to the type of clotting factor products used during the 2-year period after the first infusion in inhibitor-free cases, or before detection of inhibitor in inhibitor formation cases.

Status of inhibitor formation	Plasma-derived products alone n = 37 (%)	Recombinant products alone n = 104 (%)	P-value
Number of inhibitor formation cases	11 (29.7)	26 (25.0)	0.7308
Peak inhibitor level			
≤5 BU mL^{-1}	2 (18.2)	7 (26.9)	0.8829
>5 BU mL^{-1}	9 (81.8)	19 (73.1)	
Status at the time of survey			
Present	4 (36.4)	7 (26.9)	0.8565
Disappeared	7 (63.6)	19 (73.1)	
Outcome of ITI			
Successful	6 (66.7)	17 (73.9)	0.9194
Ongoing	2 (22.2)	4 (23.5)	
Failure	1 (11.1)	2 (8.6)	
Cases not having received ITI among inhibitor disappearing cases	1 (14.3)	2 (10.5)	–

Table 3. Background variables in relation to the type of clotting factor products used during the 2-year period after the first infusion in inhibitor-free cases, or before detection of inhibitor in inhibitor formation cases.

Background variable	Plasma-derived products alone n = 37 (%)	Recombinant products alone n = 104 (%)	P-value
Age (years)*	14.0 ± 5.6	9.7 ± 4.3	0.0491
Severity			
Mild	4 (10.8)	12 (11.5)	0.3342
Moderate	2 (5.4)	15 (14.4)	
Severe	31 (83.8)	77 (74.0)	
Family history of haemophilia†			
Positive	20 (55.6)	42 (42.0)	0.2281
Negative	16 (44.4)	58 (58.0)	
Family history of inhibitor formation†			
Positive	1 (4.2)	8 (9.1)	0.7166
Negative	23 (95.8)	80 (90.9)	
Treatment during the 2-year period after first infusion†			
On demand	29 (78.4)	71 (68.9)	0.3795
Regular prophylaxis	8 (21.6)	32 (31.1)	
Intracranial haemorrhage†			
Present	7 (19.4)	11 (10.9)	0.3091
Absent	29 (80.6)	90 (89.1)	
Other severe bleeding†			
Present	1 (2.9)	6 (6.0)	0.7804
Absent	34 (97.1)	94 (94.0)	
Invasive surgery†			
Present	1 (2.8)	8 (8.0)	0.4903
Absent	35 (97.2)	92 (92.0)	

*Age as of 31 December 2009.
†Excluding cases where data are unavailable.

Discussion

Of the possible factors affecting the incidence of inhibitor formation in haemophilia patients, the type of factor VIII and IX products used for therapy has been attracting the closest attention. In particular, a retrospective study in France suggested that the incidence of inhibitor formation was lower in haemophilia A patients treated with single plasma-derived products than in patients treated with recombinant products [23]. Subsequently, however, the CANAL study (Concerted Action on Neutralizing Antibodies in severe haemophilia A) indicated that the incidence of inhibitor formation did not differ between patients treated with plasma-derived products (including products containing von Willebrand factor) and patients treated with recombinant products [26], and this result was consistent with a report published at about the same time in the United Kingdom [28]. All of these findings were derived from studies conducted in European and North American countries, primarily involving caucasians. It is known, however, that the formation of inhibitors following treatment with clotting factor products varies between different ethnic populations [10]. The present investigation was conducted, therefore, in Japanese haemophilia patients. The study was retrospective and

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was designed to include only those patients in whom inhibitor status could be checked for at least 2 years after the first infusion of clotting factor product. In spite of this limitation, the results on our relatively large number of patients born after 1988 (the year when mAb-purified factor VIII products were first marketed in Japan) were consistent with those of the CANAL studies. No differences in the incidence of inhibitor formation were revealed between the Japanese patients treated with plasma-derived products alone and those treated with recombinant products alone. Genetic analyses of haemophilia patients are available at some specialized facilities in Japan, but data of this nature was not comprehensively available for the present study and was not included. The incidence of inhibitor formation appeared to be very high, however, in patients from pedigrees where different family members were inhibitor positive compared to pedigrees where there was no family history of an inhibitor. This finding might have reflected an influence of gene mutation.

In the CANAL study, intensive treatment with clotting factor products at an early age appeared to be closely associated with the incidence of inhibitor formation [20], and we attempted to investigate the influence of high-dose therapy on inhibitor formation by analysing the incidence of inhibitors in patients treated for intracranial haemorrhage, other severe bleeding episodes and invasive surgery within the 2-year period prior to the detection of inhibitor. In these analyses, previous treatment for intracranial haemorrhage had been recorded approximately twice as often in the inhibitor group than in the inhibitor-free cases, although the differences were not statistically significant. In addition, the incidence of inhibitor formation did not differ between the on-demand-treated group and the group receiving regular prophylactic administration of clotting factors.

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Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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