Plasma samples (80 μl) were preincubated for 10 min with 20 μl of trigger reagent containing TF, PL, and ellagic acid (f.c. 0·5 pmol/l, 4 μmol/l, and 0·3 μmol/l, respectively). Measurements were then recorded after the addition of 20 μl reagent containing $CaCl_2$ and fluorogenic substrate (f.c. 16·7 mmol/l and 2·5 mmol/l, respectively). The development of fluorescent signals was monitored using a Fluoroskan Ascent microplate reader (Thermo Fisher Scientific, Boston, MA, USA). Data analyses were performed using the manufacturer's software to derive the standard parameters; peak thrombin, lagtime, and time to peak. It was difficult to evaluate the small changes in endogenous thrombin potential in the present study, and hence total thrombin generation at intervals from the beginning to peak level was quantified. These values (nmol/l) were divided by the sample times (min, time to peak − lagtime), and represented the mean velocity of thrombin generation until the reach to peak level, expressed as mean velocity to peak thrombin (MV-peak thrombin).

Clot waveform analysis

Activated partial thromboplastin time (PTT) measurements were performed using the MDA-II™ Haemostasis System (Trinity Biotech, Dublin, Ireland). The clot waveforms obtained were computer-processed using the commercial kinetic algorithm (Matsumoto *et al*, 2000). The minimum value of the first derivative (min1) was calculated as an indicator of the maximum velocity of coagulation. The second derivative of the transmittance data reflected the acceleration of the reaction at any given time point. The minimum value of the second derivative (min2) was calculated as an index of the maximum acceleration of the reaction. As the minimum of min2 is derived from negative changes, the data were expressed as |min2|. Given that |min2| values were well-correlated with the levels of FVIII:C (Matsumoto *et al*, 2000), |min2| was analysed in the present study.

Data analyses

All experiments were performed at least four separate times, and the average and standard deviation values are shown. Nonlinear least squares regression analyses were performed using KALEIDAGRAPH (Synergy Software, Reading, PA, USA).

Results

Neutralizing effects of FVIII in in vitro *models of HA with inhibitors*

To examine whether the different epitopes of anti-FVIII inhibitor alloAbs affected the neutralizing ability of FVIII, *in vitro* models reflecting potential therapeutic protocols were prepared using mixtures of FVIII and anti-FVIII alloAbs, as described in Materials and methods. We considered that anti-FVIII inhibitors at 2·5 BU/ml were neutralized by FVIII at 1 U/ml in circulating blood. Coagulation function at various reaction times was evaluated by thrombin generation assays. Fig 1 shows representative curves for the different classes of inhibitor. Case 2 illustrates an anti-A2 epitope with type 1 pattern, Case 3 an anti-C2 type 1, and Case 6 an anti-C2 type 2 inhibitor. With both anti-A2 type 1 (Case 2) and anti-C2 type 1 (Case 3) a time-dependent reduction in thrombin generation (a decrease of peak thrombin and a delay of time to peak) was evident. In particular, thrombin generation in the presence of anti-C2 type 1 was significantly lower than that in anti-A2 type 1. However, the anti-C2 type 2 inhibitor (Case 6) showed a small decrease in thrombin generation over a 2-h incubation period. Results with other

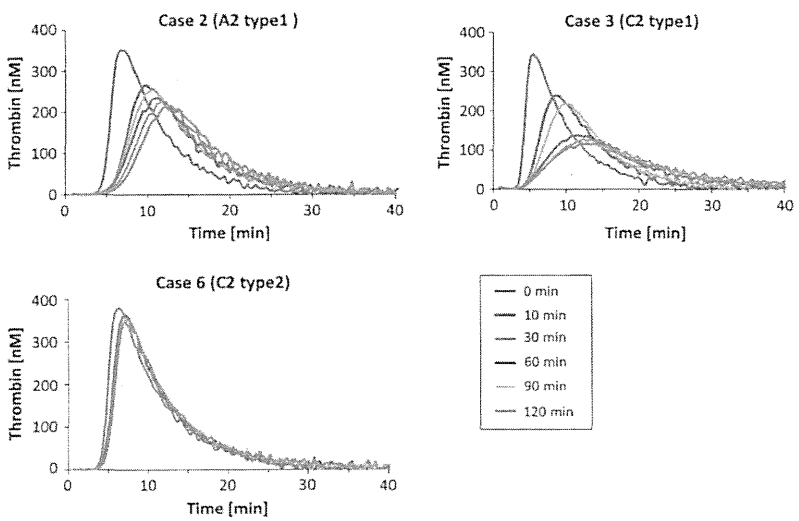

Fig 1. Thrombin generation in time-related reactions of FVIII with anti-FVIII alloantibodies – Inhibitor IgG samples [final concentration (f.c.) 2·5 BU/ml] from Cases 2, 3, and 6 were added to FVIII-deficient plasma, and incubated with FVIII (f.c. 1 U/ml). Samples were obtained at the indicated reaction times (0, 10, 30, 60, 90, and 120 min) and were mixed with tissue factor (0·5 pmol/l), ellagic acid (0·3 μmol/l), and phospholipid (4 μmol/l), prior to the addition of fluorogenic substrate and $CaCl_2$ at the start of the assay as described in Materials and methods. Representative curves from each case are illustrated.

inhibitor samples (Cases 1, 4, and 5) showed similar patterns based on epitope classification (data not shown).

Kinetics of thrombin generation in the presence of anti-FVIII alloAbs with different epitopes

To further examine the relationship between inhibitor epitopes and the neutralizing effects of FVIII in thrombin generation assays, peak thrombin and MV-peak thrombin obtained from thrombin generation curves were analysed using the formula of single exponential decay (Fig 2A-a,b, respectively). The results are summarized in Table II. In both instances, mixtures without or with the addition of FVIII (1 U/ml) prior to incubation with inhibitor IgGs (2·5 BU/ml) were regarded as 0% or 100%, respectively. Levels of peak thrombin with anti-C2 type 1 inhibitors (Cases 4 and 5) were reduced to *c.*10% and 20%, respectively within 30-min incubation. Using another antibody with similar properties (Case 3), peak thrombin was reduced to *c.*50% of the initial level at 30-min incubation, and was decreased to *c.*30% at 2-h incubation. With both of the anti-A2 type 1 inhibitors (Cases 1 and 2), peak thrombin was >50% of the initial level after 2 h. In contrast, the anti-C2 type 2 inhibitor (case 6) produced a small decrease in peak thrombin generation over the 2-h incubation period. Compared to normal IgG (control), therefore, anti-C2 type 1 and anti-A2 type 1 inhibitors depressed the peak thrombin by 10- to 30-fold and *c.* 5-fold, respectively, whilst anti-C2 type 2 moderated activity by only *c.* 1·5-fold (Table II). Analyses of the alternative parameter, MV-peak thrombin, showed similar results, and illustrated a good correlation between peak thrombin and MV-peak thrombin measurements. It appeared, therefore, that the inhibition of thrombin generation by anti-C2 type 1 and anti-A2 type 1 inhibitors were, respectively, 10- to 20-fold and *c.* threefold more potent than that in anti-C2 type 2 in these assays. The findings suggested that the different epitopes of FVIII inhibitors could significantly affect the neutralizing ability of therapeutic FVIII infusions.

Physiologically, FVIII circulates as a complex with VWF, a macromolecule that protects and stabilizes the cofactor. Experiments as described above were repeated, therefore, using FVIII-VWF complex (1 U/ml) in place of FVIII alone. There appeared to be less decrease in peak thrombin formation (Fig 2B-a) and MV-peak thrombin measurements (Fig 2B-b) in the presence of VWF, in the order of anti-C2 type 1 (Cases 3, 4 and 5), anti-A2 type 1 (Cases 1–2), and anti-C2 type 2 (Case 6). In particular, with all three anti-C2 type 1 inhibitors, the thrombin generation measurements were less affected in the presence of FVIII-VWF (Fig 2B) compared with FVIII alone (Fig 2A). The ratios of these parameters in the presence of VWF relative to its absence (B)/(A) ranged between 0·59 and 0·89 (Table II), supporting the protective

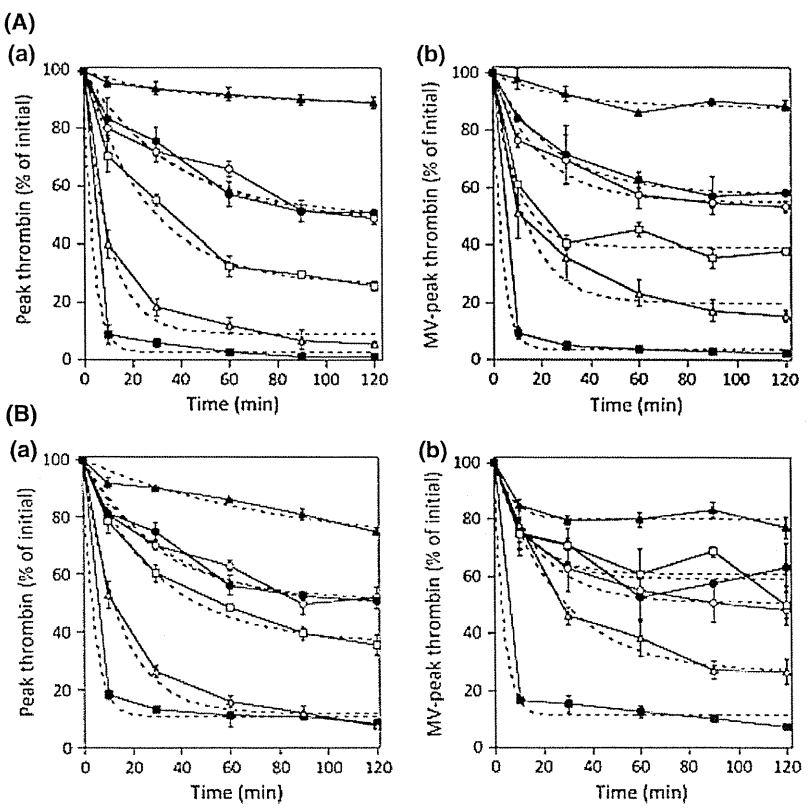

Fig 2. Time-dependent decreases in peak thrombin or mean velocity (MV)-peak thrombin in mixtures of FVIII or FVIII-VWF with anti-FVIII alloantobodies (alloAbs)- Mixtures with alloAb IgG [final concentration (f.c.) 2·5 BU/ml] and FVIII-deficient plasma were incubated with FVIII (*panel* A) or FVIII-VWF (*panel* B; f.c. 1 U/ml). Thrombin generation was measured at the indicate times as described in Materials and methods. The symbols used are, ○; Case 1, ●; Case 2, □; Case 3, ■; Case 4, △; Case 5, and ▲; Case 6, respectively. The values of peak thrombin (*panel* a) or MV-peak thrombin (*panel* b) in the absence or presence of FVIII (or FVIII-VWF) prior to incubation with inhibitor IgGs were regarded as 0% or 100%, respectively. Peak thrombin and MV-peak thrombin measurements at time zero were 300–400 nmol/l and 500–700 nmol/l/min, respectively. The data obtained in (A and B) were fitted using the formula of single exponential decay (*dotted lines*).

function of VWF. In contrast, the results with the anti-C2 type 2 inhibitor (Case 6) suggested higher decrease rate of thrombin generation in the presence of FVIII-VWF compared with FVIII alone ((B)/(A); *c.* 2·0), whereas with both of the anti-A2 type 1 inhibitors (Cases 1 and 2), the presence of VWF had little effect on the kinetics ((B)/(A); *c.* 1·0). The VWF-interactive sites on the FVIII molecule are located in the acidic regions of the A3 and C2 domains (Foster *et al*, 1988; Saenko *et al*, 1994), and our results supported the concept that the characteristics of the anti-FVIII alloAbs with different epitopes governed interactions with FVIII and FVIII-VWF.

Effects of anti-FVIII mAbs in the in vitro *neutralization models*

Anti-FVIII inhibitor alloAbs are polyclonal, and to confirm specificity, therefore, similar experiments were repeated using anti-FVIII mAbs with well-defined epitopes. The measurements of peak thrombin (Fig 3A) and MV-peak thrombin (Fig 3B) observed with anti-C2 mAbESH4 (anti-C2 type 1) were rapidly depressed to *c.*30% of the initial level after a 30-min incubation. In contrast, thrombin generation was maintained at *c.*75% after 30 min, and decreased to *c.*65% after 2 h with anti-A2 mAbJR8 (anti-A2 type 1), and was reduced by only *c.*20% after 2 h with mAbESH8 (anti-C2 type 2). These results were similar to those obtained with polyclonal FVIII antibodies, and demonstrated that compared to normal IgG, these sensitive parameters of thrombin generation were significantly inhibited by mAbESH4, (*c.* 20-fold), but were less affected by mAbJR8 (*c.* three to sixfold) and mAbESH8 (*c.* twofold; Table III).

Inhibition of thrombin generation with mAbESH4 was slightly weaker in assays using FVIII-VWF than with FVIII alone, and VWF had very little effect on the results with mAbJR8 (Fig 3). The ratios of these parameters in the

Table II. Decrease rates of peak thrombin and MV-peak thrombin with anti-FVIII alloAbs.

	Peak thrombin			MV-peak thrombin		
Case	(A) FVIII (-fold) ×10/min	(B) FVIII-VWF(-fold) ×10/min	(B)/(A) ratio	(A) FVIII (-fold) ×10/min	(B) FVIII-VWF(-fold) ×10/min	(B)/(A) ratio
1	0·30 ± 0·08 (5·0)	0·31 ± 0·08 (5·1)	1·03	0·35 ± 0·10 (5·0)	0·36 ± 0·12 (5·1)	1·02
2	0·29 ± 0·04 (4·8)	0·30 ± 0·07 (5·0)	1·03	0·36 ± 0·06 (5·1)	0·39 ± 0·10 (5·6)	1·07
3	0·63 ± 0·06 (10)	0·50 ± 0·06 (8·3)	0·79	0·94 ± 0·14 (13)	0·55 ± 0·09 (7·9)	0·59
4	2·03 ± 0·31 (33)	1·65 ± 0·30 (27)	0·81	2·30 ± 0·19 (33)	2·05 ± 0·46 (29)	0·89
5	1·19 ± 0·19 (19)	0·89 ± 0·07 (14)	0·74	1·09 ± 0·22 (16)	0·69 ± 0·07 (9·9)	0·63
6	0·09 ± 0·02 (1·5)	0·18 ± 0·04 (3·0)	2·00	0·12 ± 0·05 (1·7)	0·29 ± 0·04 (4·1)	2·40
Control	0·06 ± 0·01 (1·0)	0·06 ± 0·01 (1·0)	—	0·07 ± 0·01 (1·0)	0·07 ± 0·01 (1·0)	—

Values were calculated by nonlinear least squares regression from the data shown in Fig 2 using single exponential decay.

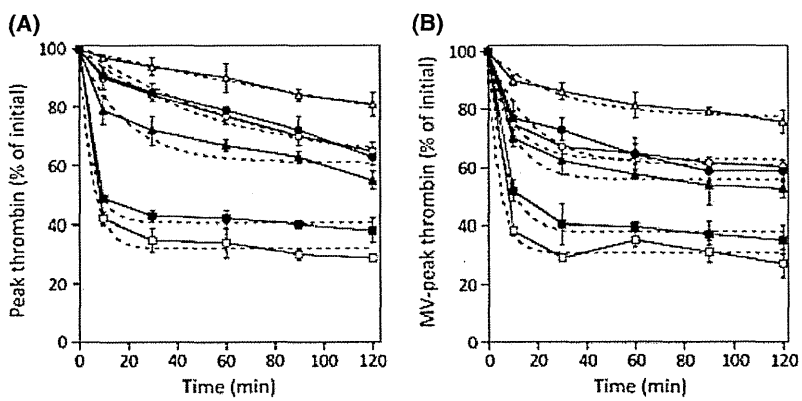

Fig 3. Time-dependent decreases in thrombin generation parameters in mixtures of FVIII or FVIII-VWF with anti-FVIII monoclonal antibodies (mAbs) – Mixtures with mAb IgG [final concentration (f.c.) 2·5 BU/ml] and FVIII-deficient plasma were incubated with FVIII or FVIII-VWF (f.c. 1 U/ml) and thrombin generation parameters determined at the indicated times. The symbols used are, ○; JR8/VWF(−), ●; JR8/VWF(+), □; ESH4/VWF(−), ■; ESH4/VWF(+), △; ESH8/VWF(−), and ▲; ESH8/VWF(+). Peak thrombin (*panel* A) or MV-peak thrombin (*panel* B) in the absence or presence of FVIII (or FVIII-VWF) prior to incubation with inhibitor IgGs were regarded as 0% or 100%, respectively. Peak thrombin and MV-peak thrombin measurements at time zero were 300–400 nmol/l and 500–700 nmol/l/min, respectively. The data in (A and B) were fitted using the formula of single exponential decay (*dotted lines*).

Table III. Decrease rates of peak thrombin and MV-peak thrombin with anti-FVIII mAbs.

	Peak thrombin			MV-peak thrombin		
Case	(A) FVIII (-fold) ×10/min	(B) FVIII-VWF(-fold) ×10/min	(B)/(A) ratio	(A) FVIII (-fold) ×10/min	(B) FVIII-VWF(-fold) ×10/min	(B)/(A) ratio
JR8	0·18 ± 0·04 (3·0)	0·17 ± 0·05 (2·9)	0·97	0·42 ± 0·12 (6)	0·46 ± 0·80 (6·5)	1·09
ESH4	1·03 ± 0·29 (17)	0·86 ± 0·12 (14)	0·82	1·56 ± 0·35 (22)	1·10 ± 0·11 (15)	0·70
ESH8	0·09 ± 0·03 (1·5)	0·25 ± 0·06 (4·1)	2·78	0·18 ± 0·07 (2·6)	0·50 ± 0·14 (7·0)	2·77
Control	0·06 ± 0·01 (1·0)	0·06 ± 0·01 (1·0)	—	0·07 ± 0·01 (1·0)	0·07 ± 0·01 (1·0)	—

Values were calculated by nonlinear least squares regression from the data shown in Fig 3 using single exponential decay.

presence of VWF relative to its absence (B)/(A) were 0·7–0·8 and *c.*1·0, respectively (Table III). In contrast, the thrombin assays using mAbESH8 appeared to be significantly enhanced in the presence of FVIII-VWF compared FVIII alone ((B)/(A); *c.* 2·8), probably showing a lower neutralizing effect of FVIII-VWF. These data demonstrated a similar tendency to those obtained with polyclonal alloAbs, and again indicated that the different epitopes of anti-FVIII inhibitors could affect the outcome of neutralization therapy using either FVIII or FVIII-VWF complex concentrates.

Clot waveform analysis in mixtures of FVIII or FVIII-VWF with anti-FVIII inhibitors

The neutralizing effects of FVIII and FVIII-VWF on the FVIII inhibitors with different epitopes were further compared using another global coagulation assay, clot waveform analysis. The anti-FVIII alloAbs or mAbs were incubated with FVIII or FVIII-VWF for 2 h, and measurements of coagulation acceleration (|min2|) were derived from clot waveforms obtained in samples as described in Materials and methods (Fig 4). The |min2| values observed with Cases 1 and 2 and mAbJR8 (anti-A2 type 1) were approximately half (0·27–0·29) of those with normal IgG (*c.* 0·55), and were little affected by the presence of VWF. In contrast, the |min2| observed with Cases 3–5 and mAbESH4 (anti-C2 type 1) were much lower (0·06–0·15) than those with anti-A2 type 1, and were significantly elevated in the presence of VWF (1·4–2·0-fold higher) compared with FVIII alone. The |min2| data obtained for Case 6 and mAbESH8 (anti-C2 type 2) were modestly lower, relative to control (0·5 and 0·35, respectively), but were further reduced in the presence of VWF, reflecting a potentially lower neutralizing ability of FVIII-VWF with this type of antibody. These results were consistent with those obtained in thrombin generation assays.

The levels of FVIII:C in samples obtained after a 2 h incubation, measured by one-stage clotting tests, did not appear to be significantly related to the different epitopes of anti-FVIII alloAbs and mAbs, although FVII:C levels obtained in Case 6 were relatively high. The findings suggested, therefore, that conventional assays of this nature would be unlikely to provide suitable laboratory data for evaluating the clinical effects of neutralization therapy.

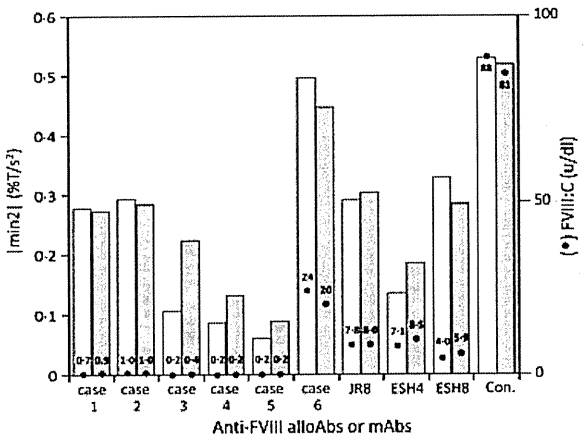

Fig 4. Clot waveform analysis after a 2-h incubation of FVIII or FVIII-VWF with anti-FVIII antibodies – Mixtures of anti-FVIII alloantibody (alloAb) or monoclonal antibody (mAb) IgG [final concentration (f.c.) 2·5 BU/ml] and FVIII-deficient plasma were incubated with FVIII or FVIII-VWF (f.c. 1 U/ml) for 2 h, prior to clot waveform analysis as described in Materials and methods. The values of |min2| obtained from mixtures containing FVIII or FVIII-VWF are represented by *white bars* or *grey bars* respectively. The figures above the symbols (•) within the bars indicate the levels of FVIII:C coagulant activity (FVIII:C) measured in one-stage clotting assays. Con., control.

Discussion

Anti-FVIII inhibitors are classified into two groups, based on the immunological response in patients with inhibitors treated with FVIII concentrates. So called 'low responders' are those in which inhibitor titres remain at <5 BU/ml even after considerable FVIII therapy. Conversely, 'high responders' are those in which inhibitor levels increase significantly above 5 BU/ml after administration of FVIII. In general, haemostatic treatment in HA patients with inhibitors depends on neutralization therapy or bypassing therapy, for low responders or high responders, respectively. The clinical effectiveness of neutralization therapy, however, could depend on differences between the reactivity of exogenous FVIII with specific anti-FVIII inhibitors, and changes in established strategies may be beneficial in some circumstances. This concept remains to be fully explored, however,

and for this purpose in the present study, we have devised *in vitro* models of neutralization therapy using mixtures of FVIII (1 U/ml) and anti-FVIII inhibitor alloAbs or mAbs (2·5 BU/ml) in FVIII-deficient plasma.

Differences in the *in vitro* neutralizing effects of FVIII observed in this study were dependent on the particular epitope of anti-FVIII inhibitors. The addition of FVIII alone appeared to decrease the potency of the inhibitor in the order of anti-C2 type 2 ≫ anti-A2 type 1 > anti-C2 type 1. Anti-C2 and anti-A2 type 1 inhibitors showed similar rapid decreases of FVIII:C (<1 U/dl) in one-stage clotting assays (data not shown). In both the thrombin generation assay and one-stage clotting assay, anti-C2 inhibitors showed the rapid decrease even at lower concentrations, but anti-A2 inhibitors showed moderate decrease (data not shown), suggestive of the presence of different inhibitory mechanisms (probably due to FVIIIa-PL interaction and FVIIIa-FIXa interaction, respectively). This potent effect could contribute to the much greater inhibition seen with anti-C2 type 1. On the other hand, equivalent effects of FVIII-VWF were observed in the order of anti-C2 type 2 ≫ anti-A2 type 1 ≥ anti-C2 type 1. Furthermore, with the anti-C2 type 2 inhibitors, FVIII-VWF appeared to have diminished haemostatic properties compared with those of FVIII alone. These different effects identified using the *in vitro* coagulation model inferred that clinical responses in HA patients with low responding inhibitors could be optimized by making a choice between FVIII and FVIII-VWF. The data suggest that the durability of therapeutic FVIII and FVIII-VWF might be predicted by determining the precise epitopes of the individual inhibitors.

The current evidence suggests that the different neutralizing effects of FVIII and FVIII-VWF were centred on precise inhibitory mechanisms related to the functional properties of FVIII. The anti-C2 type 1 antibodies prevent FVIIIa binding to PL surfaces (Shima *et al*, 1993), and, hence, intrinsic FXase complexes that are responsible for the propagation phase of blood coagulation could not be formed. The anti-A2 type 1 and anti-C2 type 2 antibodies prevent FVIIIa-FIXa association (Fay & Scandella, 1999) and thrombin (and FXa)-catalysed activation of FVIII (Meeks *et al*, 2007; Matsumoto *et al*, 2012), respectively. It seems likely, therefore, that although both coagulation mechanisms are essential for the expression of intrinsic FXase activity, the overall effects of these specific inhibitors were less prominent than those of the anti-C2 type 1 antibodies, and it may be that FVIIIa-PL interactions play the dominant role in the expression of FXase activity.

VWF binds non-covalently to the N-terminus of A3 and C2 domains in FVIII (Foster *et al*, 1998, Saenko *et al*, 1994). The neutralizing effects of FVIII-VWF complex in our experiments, therefore, could be especially relevant. The epitopes of anti-C2 type 1 inhibitors overlap with the VWF-binding site(s) on FVIII, and as could be expected, the time-dependent neutralization of anti-C2 type 1 antibodies by FVIII-VWF was enhanced compared with FVIII alone, reflecting the protective role of VWF. Moreover, the results with anti-A2 type 1 inhibitors were little affected by the presence of VWF, whilst the neutralization with anti-C2 type 2 inhibitors was more attenuated with FVIII-VWF than with FVIII alone. This apparent paradox might be explained by the effects of the inhibitors on thrombin-catalysed proteolysis of FVIII. Thrombin-catalysed cleavage of FVIII at Arg^{1689} liberates FVIII from VWF, resulting in the generation of FVIIIa cofactor activity (Lollar *et al*, 1988). The anti-C2 type 2 antibodies (case 6 and mAbESH8) could, therefore, have inhibited the release of FVIII from VWF (Saenko *et al*, 1996) and disturbed thrombin-catalysed FVIII activation. Our findings suggest that this type of antibody might retain full inhibitory activity in patients treated with FVIII alone, but could be expected to have reduced potency in therapeutic protocols using FVIII-VWF.

Accurate measurements of blood coagulation *in vitro* are essential for complete clinical assessment of clotting function. Measurements of FVIII:C using conventional activated partial thromboplastin time-based one-stage clotting assays demonstrated little difference between the various types of inhibitor, but the global coagulation assays, thrombin generation and clot waveform analysis, facilitated identification of epitope-dependent reactions with FVIII and FVIII-VWF. The one-stage assays have been utilized for many years for routine laboratory assessment of clotting factor activity, but only partially reflect coagulation in a non-physiological environment. In the present study, the inhibitor titre used (2·5 BU/ml), was determined by the sample after a 2-h incubation in one-stage clotting assay. However, in global coagulation assays (thrombin generation assay and clot waveform analysis), coagulation function after a 2-h incubation revealed mild reduction, probably representing the quite different implication of inhibitor titre, especially anti-C2 type 2 inhibitors, between one-stage clotting assay and comprehensive coagulation assay. The global assays have recently been utilized for monitoring haemostatic treatment using bypassing agents in HA patients with inhibitors (Turecek *et al*, 2003; Barrowcliffe, 2008), and it seems evident that these techniques could also provide useful data for the clinical management of neutralization therapy using FVIII and FVIII-VWF concentrates.

Our previous results have shown that both rFVIIa and APCC, used as bypassing agents, could activate FVIII directly even in the presence of anti-FVIII inhibitor alloAbs (Yada *et al*, 2011, 2013). In patients with anti-C2 type 1 inhibitors, in particular, inactivation of FVIIIa by APCC was moderated by a delay in cleavage at Arg^{336}, and resulted in relatively persistent levels of activated FVIII. Our present findings add further details regarding epitope-dependent reactions with FVIII and FVIII-VWF, and indicate that further studies of this nature could lead to the development tailor-made treatment protocols. In addition, the data emphasize that detailed analysis of inhibitor epitopes could help to establish important guidelines in general for the haemostatic treatment of HA patients with inhibitors.

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Conflict of interest

The authors have no direct or indirect conflicts of interest.

Authorship contributions

KY performed the research, KY and KN analysed the data and wrote the paper, KN designed the research study, KN and MS interpreted the data.

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Repression of factor VIII inhibitor development with apoptotic factor VIII-expressing embryonic stem cells

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Abstract

Development of factor VIII (fVIII)-neutralizing antibodies, called inhibitors, is a challenging problem in the management of hemophilia A patients. We explored the possibility of pretreatment with apoptotic fVIII-expressing embryonic stem (ES) cells to prevent the development of fVIII inhibitors. Murine ES cells integrated with the human *F8* gene were differentiated into embryoid bodies, dissociated to a single cell suspension, subjected to hypo-osmotic shock to induce apoptosis, and intraperitoneally injected into hemophilia A mice. Inhibitors were induced by periodic intraperitoneal injections of recombinant human fVIII (rhfVIII). In the groups in which intraperitoneal injections of rhfVIII began at 1-3 weeks after pretreatment, the titers of inhibitors were significantly lower after the third administration of rhfVIII compared with that in the control group in which apoptotic Ainv18 ES cells (without the human *F8* gene) were used for pretreatment, and continued to show lower levels until the sixth administration of rhfVIII. These results suggest that pretreatment with apoptotic hfVIII-expressing ES cells might be promising for the prevention of fVIII inhibitor development in hemophilia A patients.

Introduction

Hemophilia A is an X-linked bleeding disorder resulting from an abnormality in the coagulation factor VIII (fVIII) gene. Hemophilia A patients require life-long supplementation with intravenous fVIII. The risk of infectious diseases has dramatically decreased as a result of the development of highly pure plasma-derived and recombinant fVIII. Nonetheless, development of fVIII-neutralizing antibodies, called fVIII inhibitors, remains a challenging problem for the treatment of hemophilia A. Approximately 25-35% of severe type hemophilia A patients develop inhibitors that reduce or completely negate the benefits of replacement therapy.[1,2] Furthermore, a major issue is that all gene therapy trials for hemophilia are subject to the risk of developing inhibitory antibodies.[3] Although immune tolerance induction therapy (ITI) using high amounts of fVIII is effective for eradicating inhibitors, the extremely high cost required for this type of therapy hampers its worldwide application. Moreover, ITI is ineffective in about 30% of hemophilia A patients with inhibitors.[4] Thus, it is desirable to develop a novel method to prevent the development of fVIII inhibitors. To this end, several attempts have been made as follows. Madoiwa *et al.* successfully induced immune tolerance for human fVIII (hfVIII) by intrathymic injection of hfVIII into neonatal hemophilia A mice.[5] Furthermore, induction of long-term fVIII tolerance can be achieved by administration of lentiviral vectors carrying a canine fVIII transgene to neonatal hemophilia A mice.[6] Although these approaches were effective, they have several disadvantages such as difficulties in the procedures or the risk of viral vectors causing adverse genetic modification of patients.

The use of apoptotic cells for immune tolerance induction is in line with the current perception that dendritic cells induce peripheral tolerance by capturing cells that normally die during cell turnover. Because apoptotic cells are immunologically *silent*,[7] they exert a tolerogenic influence in adaptive immune responses.[4,8-10] Therefore, a method to prevent antibody production with apoptotic cells has an immunological rationale and is sufficiently promising based on recent studies.[11,12] However, such an approach has several disadvantages that should be overcome for practical use in the clinical setting of hemophilia A treatment. One of the most concerning issues is the source of fVIII-expressing cells. While the most appropriate cells are autologous cells from an immunological viewpoint, patient-derived cells have a genetically abnormal *F8* gene. Thus, *ex vivo* transfection of the *F8* gene into patient-derived fibroblasts has provided a breakthrough for this approach. Su *et al.* demonstrated that injection of fVIII expression vector-modified apoptotic syngeneic fibroblasts achieves suppression of fVIII inhibitor development in hemophilia A mice.[12] However, the significant issues in this approach are the complex procedures such as collection of fibroblasts from each patient and transfection of the *F8* gene into these cells. Therefore, fVIII-expressing cells derived from histocompatible stem cells [such as embryonic stem (ES) cells or induced pluripotent stem (iPS) cells] will be the most suitable. Although we have observed that iPS cells can be differentiated into liver-like populations that show a strong expression level of fVIII mRNA,[13] fVIII protein expression levels have not yet been examined. Therefore, we attempted to use genetically engineered (hfVIII-transgenic) ES cells as fVIII-expressing stem cells. We explored the possibility of fVIII inhibitor prevention by pretreatment with apoptotic fVIII-secreting ES cells in anticipation of the common and broad usage of iPS cells.

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Key words: hemophilia, fVIII inhibitors, prevention, embryonic stem cells, apoptosis.

Contributions: YS, conception and design of the study, interpretation of data, and revisions of the manuscript; SK and KT, animal experiments, interpretation of data, literature searches, and preparation of manuscript; TK and JK, cell culture, animal experiments, and collection of data; AK, preparation of ES cells and supervising genetic manipulation; MS supervising study and critical revisions of the manuscript.

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Materials and Methods

Mice

fVIII-deficient mice carrying a stop mutation in exon 16 of the *F8* gene were generously given to us by Prof. Yoichi Sakata (Research Division of Cell and Molecular Medicine, Jichi Medical University, Shimotsuke, Japan) and used as a model of hemophilia A.[14] These hemophilic mice were mated with C57BL/6 mice for at least eight generations to create C57BL/6 fVIII knockout mice. The genotypes of hemophilic mice were

confirmed by polymerase chain reaction analysis of genomic DNA extracted from peripheral leukocytes.[14] The mice were maintained as an inbred colony and were between 8 and 12 weeks of age at the beginning of the study. All mice were housed under specific pathogen-free conditions in the animal facility of Nara Medical University Research Center. Blood samples were obtained by orbital plexus bleeding and anticoagulated (9:1) with 0.105 M sodium citrate. Plasma samples were obtained by centrifugation of the blood at 3600 g for 10 min at room temperature, divided into aliquots, and frozen at –80°C until analysis. All experiments using mice were approved by the Nara Medical University Animal Use Committee and performed in accordance with the applicable guidelines and regulations.

Induction of factor VIII inhibitors

Development of fVIII inhibitors in C57BL/6 fVIII knockout mice was induced by weekly intraperitoneal administrations of 4 IU recombinant hfVIII (rhfVIII; Kogenate FS, Bayer Yakuhin, Osaka, Japan) (approximately 100 IU/kg body weight).

Assay for factor VIII inhibitors

The titers of fVIII inhibitors were assessed by the Bethesda assay using the obtained plasma samples according to a previous report with minor modification.[15,16] In brief, 50 μL of doubling diluted mouse plasma with Owren's Veronal Buffer (more diluted if needed) was incubated with 50 μL of normal pooled human plasma at 37°C for 2 h. Residual human fVIII activity was measured in a one-stage assay using 50 μL of FVIII-deficient human plasma (Sysmex, Kobe, Japan) and a 50 μL sample from the previous incubation. Samples were mixed with 100 μL of phospholipid activator (APTT-SLA, Sysmex), incubated at 37°C for 3 min, and then mixed with 100 μL of 20 mM/L $CaCl_2$. Clotting times were measured with a coagulometer (KC 10, Amelung, Lemgo, Germany). Coagtrol N (Sysmex) was diluted with Owren's Veronal Buffer to produce a standard curve of fVIII activity. The measurements were made in the linear portion of the response range.

Cell preparations

Murine Tet-WT-F8 ES cells, in which human *F8* gene expression was induced by the tetracycline analog doxycycline, were prepared as described previously.[17] Briefly, Ainv18 ES cells were transfected with the WT-F8-plox targeting plasmid (wild-type human fVIII gene-loading, lox-targeting plasmid) by electroporation to yield tet-WT-F8,[18] after which the transfectants were selected with G418 as described previously.[19] Tet-WT-F8 ES cells were maintained on mouse embryonic fibroblasts as a feeder layer, passaged twice on gelatin-coated dishes, and then induced to form embryoid bodies (EBs) for 6 days. The EBs were stimulated with doxycycline for the last 2 days. Then, EBs were dissociated to a cell suspension by digestion with 0.25% trypsin/EDTA. As a control, Ainv18 ES cells without the human *F8* gene were treated in the same manner.

Apoptosis of the dissociated ES cells was induced according to the osmotic shock method by Liu *et al.*[20] Briefly, 1×10^6 cells were washed in RPMI 1640 twice, and resuspended in 1 mL hypertonic medium (0.5 M sucrose, 10% wt/vol polyethylene glycol 1000, and 10 mM Hepes in RPMI 1640, pH 7.2) for 10 min at 37°C. Then, 10 mL prewarmed hypotonic medium (40% H_2O, and 60% RPMI 1640) was added, and the cells were incubated for an additional 2 min at 37°C. Immediately after the incubation, the cells were centrifuged, washed twice with ice-cold phosphate-buffered saline (PBS), and used as apoptotic cells. Induction of apoptosis of the dissociated ES cells was confirmed by flow cytometry analysis with annexin V/7-amino-actinomycin D staining (*data not shown*).

Administration of apoptotic embryonic stem cells

Dissociated and apoptosis-induced Tet-WT-F8 ES cells or control Ainv18 ES cells in 200 μL PBS (2×10^5 cells) were intraperitoneally injected into hemophilia A mice. Intraperitoneal injections of rhfVIII for the induction of fVIII inhibitor development were started at 3 days, 1, 2, and 3 weeks after administration of the apoptotic Tet-WT-F8 ES cells (group D3, W1, W2, and W3, respectively) and after administration of Ainv18 ES cells (control). After the first intraperitoneal injection of rhfVIII, blood sampling was performed just prior to the next intraperitoneal injection of rhfVIII (Figure 1).

Statistical analysis

Data were presented as the mean ± standard error of the mean (SEM). Differences between groups were assessed by the Kruskal-Wallis test, followed by Dunn's multiple-comparison post hoc analysis. A P value of less than 0.05 was defined as statistically significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

Development of factor VIII inhibitors in hemophilia A mice

Figure 2 shows the progressive increase of the fVIII inhibitor titer with weekly intraperi-

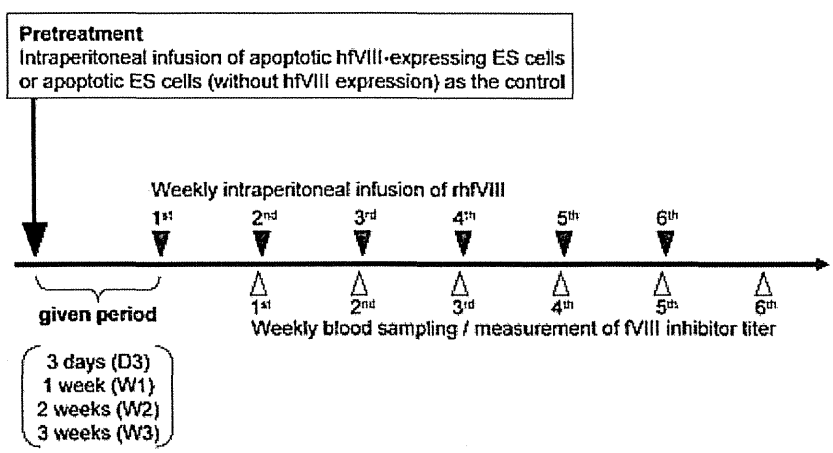

Figure 1. Timeline of the experimental procedure.

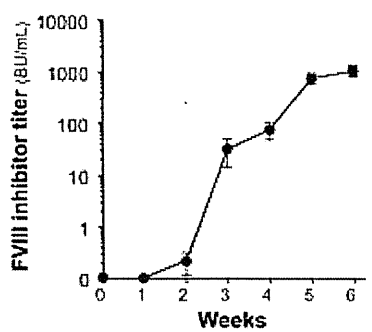

Figure 2. Progressive increase of factor VIII (fVIII) inhibitor titers in hemophilia A mice by weekly intraperitoneal administrations of recombinant human fVIII (rhfVIII) (n=6-8). Inhibitor titers reached a plateau after the fifth administration of rhfVIII.

toneal administration of rhfVIII to non-pretreated hemophilia A mice (n=8). The fVIII inhibitor titers appeared after the second administration of rhfVIII (0.22±0.38 BU/mL). Then, inhibitor titers increased with every following rhfVIII administration and reached 1687±309 BU/mL after the six administrations.

Intraperitoneal injection of apoptotic factor VIII-expressing cells attenuates the development of factor VIII inhibitors

The fVIII inhibitor titers were undetected in both treated and untreated groups before intravenous rhfVIII administration. Those of pretreatment groups at each blood collection point are shown in Figure 3. Pretreatment with apoptotic Ainv18 ES cells (without the human *F8* gene), regardless of the period between pretreatment and the first administration of rhfVIII, resulted in no difference in fVIII inhibitor development compared with that in non-pretreatment groups (data not shown). The fVIII inhibitor titers of a group in which intraperitoneal injections of rhfVIII were started at 1 week after administration of Ainv18 ES cells were shown in Figure 3 as a representative control group (Cont).

The fVIII inhibitor titers observed in pretreatment group D3 appeared higher than that in the control group after the second blood sampling but without significance. However, in W1, W2, and W3 groups, the fVIII inhibitor titers were significantly lower than that in the control group at the third blood sampling ($P<0.05$, $P<0.01$, and $P<0.05$, respectively). The results obtained during the rest of the observation period also showed a similar tendency of lower fVIII inhibitor titers in W1, W2, and W3 groups than those in the control group.

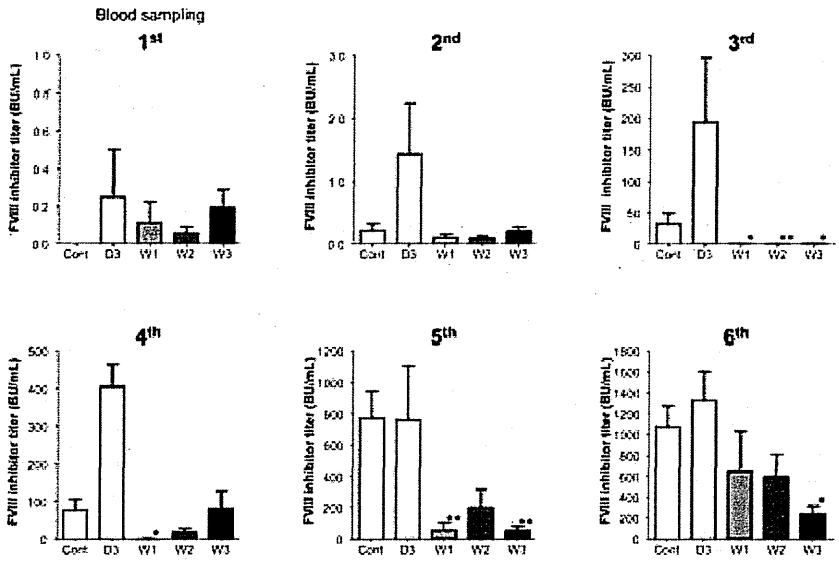

Figure 3. Effects of intraperitoneal injection of apoptotic factor VIII (fVIII)-expressing embryonic stem cells on fVIII inhibitor development. Intraperitoneal administrations of recombinant human fVIII (rhfVIII) for the induction of fVIII inhibitor development were started at 3 days, 1, 2, and 3 weeks after administration of the apoptotic cells (D3, W1, W2, and W3 groups, respectively) (n=4-8) and 1 week after administration of Ainv18 cells (Cont) (n=6). Data are presented as the mean ± standard error of the mean. *P<0.05, **P<0.01, compared with the control group.

Discussion

In the current study, we examined the possibility of pretreatment with hfVIII-expressing ES cells for the prevention of fVIII inhibitor development. The efficacy for the reduction of fVIII inhibitor titers depended on the period between pretreatment with apoptotic hfVIII-expressing ES cells and the first administration of rhfVIII. In the D3 group, pretreatment with apoptotic hfVIII-expressing ES cells resulted in rising inhibitor titers rather than a reduction. However, in groups with a greater than 1 week interval, significant suppressive effects on fVIII inhibitor development were achieved by the pretreatment. These observations suggest that a specified period is required to suppress immune response to fVIII by prior injection of apoptotic hfVIII-expressing ES cells.

The molecular weight of the hfVIII protein is very large, as much as 280 kDa, and multiple epitopes in this protein are recognized by fVIII inhibitors.[21] Thus, it is a critical issue to clarify whether the fVIII inhibitors that bind multiple epitopes of the fVIII protein can be suppressed by apoptotic hfVIII-expressing cells. Lei *et al.* found that full-length antigen expressed by B cells can induce immune tolerance for multiple epitopes in a target protein,[22] because proteolytic cleavage of the protein, selection of the resulting peptides, and peptide presentation are executed by the host's own APCs. Our successful results in the present study support this previous notion. We have no need for identification of the precise peptide epitopes of the fVIII inhibitors or the establishment of ES cells that express each epitope corresponding to a fVIII inhibitor.

The mechanism by which pretreatment with apoptotic hfVIII-expressing ES cells suppresses fVIII inhibitor development remains to be elucidated. Although further investigations are required to address this issue, it might be attributed to the inhibitory effects of the apoptotic ES cells on the proliferation of fVIII-responsive effector T cells by antigen-specific $CD4^+CD25^+$ regulatory T cells as described by Su *et al.*[12]

In this study, rhfVIII was injected not intravenously but intraperitoneally. A previous report describes that inhibitor titer elevated to 122.5 BU/mL after five biweekly intravenous injections of 50 U/kg rhfVIII (total amount of 250 U/kg) in hemophilia mouse.[5] In our study, inhibitor titer did not exceeded 122.5 BU/mL until total amount of administered rhfVIII reached 500 U/kg, suggesting that our protocol may be inefficient compared to that of the previous report. The difference of administration route as well as applied dose and administration interval may exert influence. Large size of molecules are absorbed slowly through the peritoneal lymphoid system through the stomata of the milky spot.[23] As intraperitoneally injected fVIII with a molecular size of more than 280 kDa would be therefore absorbed more slowly than intravenously injected fVIII, immune response against rhfVIII might wane. Furthermore, the peritoneal immune system, which contains distinct immune cells such as B1 cells,[24] may respond to foreign rhfVIII differently from the circulating immune system. Apoptotic ES cells were also administered intraperitoneally. Intraperitoneal administration of apoptotic cells could be applied as intraperitoneal catheters have been used for intraperitoneal chemotherapy. A comparative controlled study of administration routes, intravenous and intraperitoneal should be required to clarify which route is more effective. Furthermore, modification of the protocol for administration of apoptotic fVIII-expressing ES cells, including changes of the administrating cell dose or administration frequency, would be required for complete suppression of fVIII inhibitor development.

Thus, our methodology is not necessarily directly applicable to hemophilia patients with

inhibitors. Nevertheless, our study opens up a whole new avenue for the prevention of fVIII inhibitor development. Although the ES cells used in this study were genetically engineered (hfVIII transgenic), iPS cell-derived fVIII-secreting cells will be established in the near future.[25] In addition, establishment of an iPS cell bank may allow the prevalence of this approach for fVIII inhibitor prevention.[26]

Conclusions

Our results in this study suggest the effectiveness of pretreatment of apoptotic fVIII-expressing ES cells on repression of factor VIII inhibitor development. In addition, a specific period might be required to induce immune tolerance by pretreatment with such apoptotic cells. We believe that this approach has potential as a future preventive therapy against fVIII inhibitor development in hemophilia A patients.

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Two haemophilia patients with inhibitors who became ambulatory after physiotherapy under haemostatic cover with bypassing agents

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For haemophilia patients who develop inhibitory antibodies to factor VIII (FVIII) or FIX, haemostatic therapy becomes more complicated and difficult than for patients without inhibitors. Treatment of haemarthroses should be aggressive to prevent recurrent bleeding and chronic and debilitating joint damage [1]; otherwise severe mobility impairment may be exhibited from a young age. Bypassing agents are the recommended haemostatic treatment for inhibitor patients [2], and currently recombinant activated factor VII (rFVIIa; NovoSeven®, Novo Nordisk, Chiyoda-ku, Tokyo, Japan) and plasma-derived activated prothrombin complex concentrate (pd-aPCC; FEIBA®, Baxter, Chuo-ku, Tokyo, Japan) are available for use in Japan.

In patients with severe haemophilia without inhibitors, prophylactic therapy can reduce bleeding and resultant joint damage [3]. Use of bypassing agents to prevent bleeding in inhibitor patients prior to surgical procedures is well described [4]; however, prophylaxis with bypassing agents in non-surgical scenarios remains under investigation for therapeutic benefit and safety [5]. Here, we report two previously non-ambulatory haemophilia A patients with inhibitors, who became ambulatory following physiotherapy accompanied by haemostatic therapy with bypassing agents.

Patient 1 was a 19-year-old male who had no family history of bleeding disorders, but a haemorrhagic stool 2 days after birth led to a diagnosis of severe haemophilia A, and on-demand replacement therapy was started. An inhibitor [67 Bethesda Units (BU) mL−1] was detected when this patient was 7 years of age, and haemostatic therapy was switched to bypassing agents. Frequent joint haemorrhage and damage resulted in the patient becoming non-ambulatory at approximately 12 years of age. Immune tolerance induction (ITI) was conducted when the patient was 14-years old but was unsuccessful (inhibitor titre rose to 2700 BU mL−1). Subsequently, repeated haemarthroses in the ankle, knee and hip, accompanied by progressive muscular atrophy in both legs, resulted in difficulties standing, and the patient was admitted to hospital for physiotherapy.

The patient's laboratory findings and the Arnold–Hilgartner classification of joint arthropathy at hospital admission are given in Table 1 and Fig. 1 respectively.

Table 1. Laboratory data for patient 1 and patient 2 at hospital admission.

	Patient 1	Patient 2
Haematology		
White blood cell count (cells per μL)	3800	6300
Red blood cell count (cells per μL)	4.68 × 106	5.02 × 106
Haemoglobin (g dL−1)	14.2	14.7
Haematocrit (%)	42.9	43.0
Platelet count (cells per μL)	155 × 103	166 × 103
Coagulation and fibrinolysis		
Prothrombin time (s)	13.5	11.7
Activated partial thromboplastin time (s)	111.0	121.8
FVIII:C (%)	<1	<1
FVII:C (%)	77.0	108.6
FVIII inhibitors (BU mL−1)	84.6	17.9
Thromboelastography		
Reaction time (min)	>150	189.0
Angle (min)	Undetectable	55.6
Maximum amplitude (mm)	Undetectable	Undetectable
Blood chemistry		
γ-glutamyl transpeptidase (IU L−1)	18	26
Aspartate aminotransferase (IU L−1)	19	26
Alanine aminotransferase (IU L−1)	22	22
Lactate dehydrogenase (IU L−1)	126	301
Total bilirubin (mg dL−1)	0.57	1.55
Creatine kinase (IU L−1)	81	81
Sodium (mEq L−1)	143	140
Potassium (mEq L−1)	4.2	4.5
Chlorine (mEq L−1)	102	106
Blood urea nitrogen (mg dL−1)	12.7	11.4
Creatinine (mg dL−1)	0.55	0.67
Total protein (mg dL−1)	7.7	7.0
Total cholesterol (mg dL−1)	170	105
C reactive protein (mg dL−1)	0.05	0.96

FVIII:C, FVIII clotting activity; FVII:C, FVII clotting activity.

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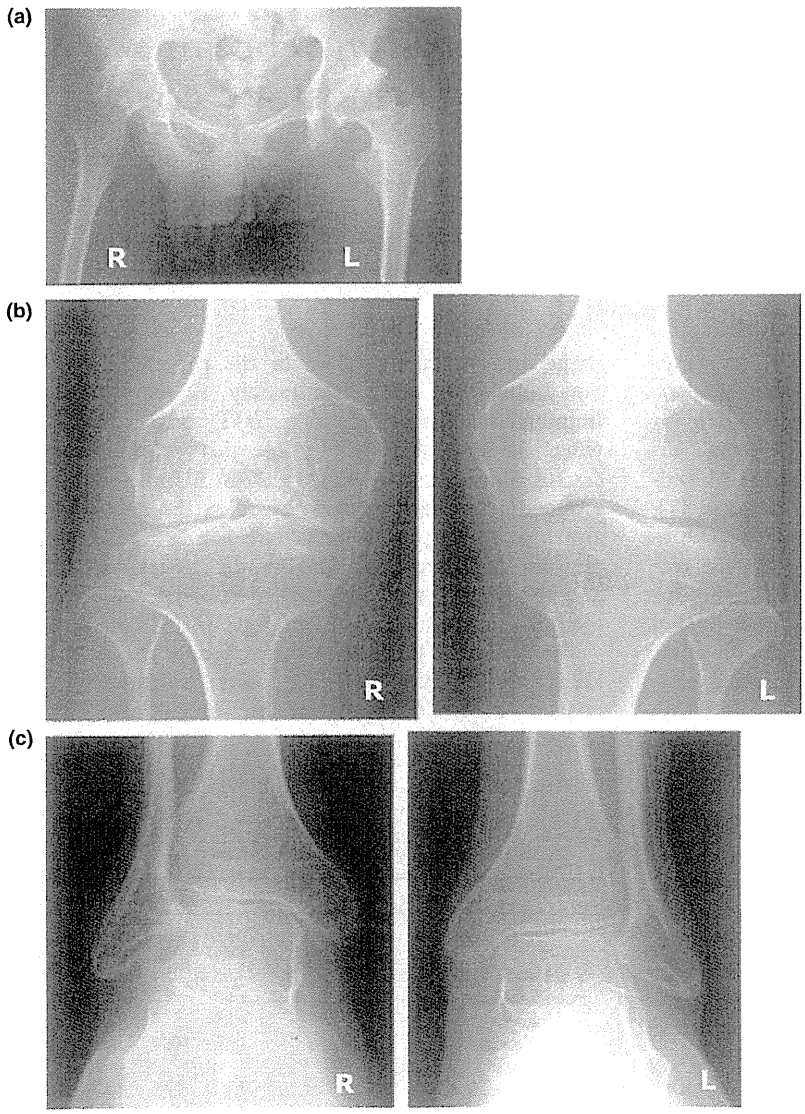

Fig. 1. Patient 1: Degree of progression of arthropathy (Arnold–Hilgartner classification) at hospitalization. (a) Hip joints: right, stage V; left stage III. (b) Knee joints: right, stage III; left, stage II. (c) Ankle joints: right, stage III; left, stage II. R, right; L, left.

Prior to hospital admission, each bleeding episode was treated on-demand with pd-aPCC (59 U kg−1) given 4–9 times (every 6–8 h over 3–4 days), and if haemostasis was not achieved, rFVIIa (94 μg kg−1, 3–4 doses, 3-h intervals) was given. Following haemostasis, no physiotherapy was attempted for 1–2 weeks.

During the patient's hospital stay, physiotherapy (five times per week) focused on strength-training in the lower legs, in combination with pd-aPCC (78 U kg−1 day−1, three times per week). After 21 days in hospital, the patient began to stand up, after 53 days he began walking with a frame, and on day 74 he started to walk with a single crutch. During this time, no intra-articular haemorrhages were observed; however, a right elbow bleed occurred on day 102. As pd-aPCC dosing was time-consuming and the patient's lifestyle improved, on day 116, regular haemostatic coverage was switched to rFVIIa (94 μg kg−1, three times weekly). The patient continued to do well, with no further haemorrhages, and was discharged after 4.5 months in hospital.

After the patient was discharged, prophylaxis with rFVIIa (94 μg kg−1, three times weekly) was continued. The patient used a cane and an assistive device to correct the difference in the length of his legs. He took various independent trips, walking and using public transport. A haemorrhage in his right elbow occurred 1 month after discharge. This and subsequent bleeds were managed with rFVIIa (118 μg kg−1) at 2-h intervals up to three doses. Following haemostasis, the patient rested for 1–2 weeks before continuing rehabilitation. Generally, treatment

of bleeding episodes with three rFVIIa 118 μg kg−1 doses achieved haemostasis relatively quickly in comparison with the patient's previous history of poorly controlled bleeds.

Patient 2 was a 19-year-old male with a family history of haemophilia; a maternal uncle died at 12 years of age, and the patient had a sibling who was diagnosed with haemophilia in utero. When the patient was 5 months of age, clotting difficulty was observed when bloods were drawn; severe haemophilia A was diagnosed and on-demand replacement therapy was started. An inhibitor (10 BU mL−1) was detected when he was 3 years of age, and treatment was switched to bypassing agents. Repeated haemorrhages in the left knee resulted in joint deterioration.

When the patient was 17 years of age, on-demand treatment with rFVIIa at his local clinic was increased to 228 μg kg−1, with additional 114 μg kg−1 doses two to six times (every 3 h), if required.

At 18 years of age, the patient started having difficulty walking and tried to prevent bleeding by not bearing weight on his left leg. He was subsequently admitted to our hospital for physiotherapy. Laboratory findings and Arnold–Hilgartner classification of arthropathy at hospitalization are shown in Table 1 and Fig. 2 respectively.

Physiotherapy focused on strength-training in the lower legs, combined with rFVIIa prophylaxis (114 μg kg−1 day−1, three times weekly). However, on days when rFVIIa was not administered, haemorrhage occurred after physiotherapy, so dosing was increased to five times per week. Subsequently, the number of bleeding episodes decreased. After 27 days in hospital, the patient began walking with a frame, and after 43 days he could walk with two crutches. After 50 days in hospital, self rehabilitation training was undertaken to enable the patient to take a holiday, and the dosing of rFVIIa was increased to 114 μg kg−1 every day.

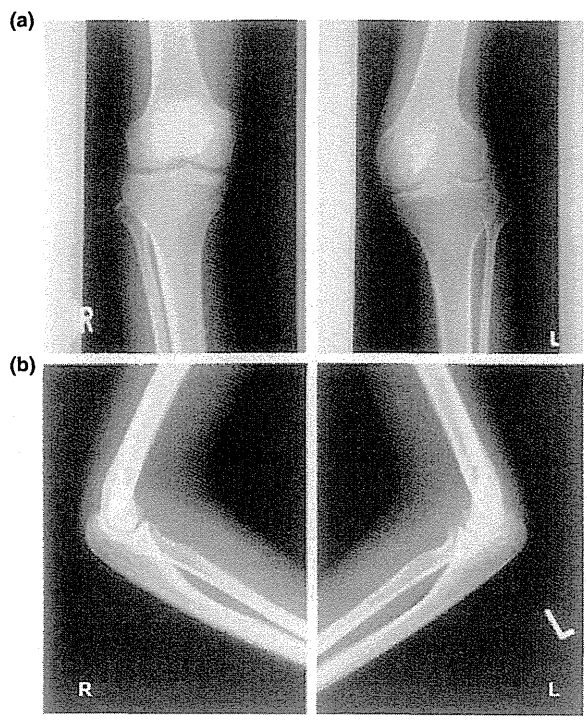

Fig. 2. Patient 2: Degree of progression of arthropathy (Arnold–Hilgartner classification) at hospitalization. (a) Knee joints: left, stage III. (b) Elbow joints: left, stage IV. R, right; L, left.

As self-injection was difficult, and taking into consideration the patient's desired lifestyle, an intravenous Hickman catheter was placed in his right subclavian vein to allow easier administration of rFVIIa, and dosing was gradually reduced. No further joint bleeds occurred and the patient was discharged on day 250.

After discharge, the patient wore a long-leg brace and continued regular treatment with rFVIIa 114 μg kg−1 day−1 (three times weekly). If bleeding occurred, rFVIIa (153 μg kg−1) was administered at 2-h intervals, which achieved haemostasis more quickly than the patient's historical responses.

The experiences described above support those of other groups reporting reduced bleeding frequency using regular haemostatic coverage with bypassing agents [6]. Due to the short half-life of rFVIIa (2.5 h), regular doses of rFVIIa were considered unlikely to prevent bleeding. However, such treatment has been reported to successfully suppress bleeding episodes in inhibitor patients undergoing rehabilitation and ITI [7]. In addition, a randomized, prospective clinical trial showed that 3 months of secondary prophylaxis with rFVIIa (270 or 90 μg kg−1 day−1) reduced bleeding frequency (including joint bleeds) vs. on-demand treatment [8]. Furthermore, such secondary rFVIIa prophylaxis tended to improve the health-related quality of life in the patients in this trial [9]. In patients with more advanced haemarthropathy, suppression of joint deterioration by treatment with bypassing agents may be more difficult [10].

To summarize, our observations demonstrate that after the combined use of physiotherapy and bypassing agents, haemophilia patients with inhibitors who were non-ambulatory due to progressive haemarthropathy, were able to take more control of their daily activities. We believe that this management programme facilitated effective prevention of bleeding as well as improvement in ambulation, and may have helped the patients to differentiate sensations of haemorrhage from those of muscle fatigue (allowing the early administration of bypassing agents if they did experience a haemorrhage). To conclude, management programs combining physiotherapy and haemostatic therapy with bypassing agents can help in the

prevention and progression of otherwise life-changing haemarthropathy.

Author contributions

M. Kajiwara treated the patients and wrote the article. M. Shima and A. Yoshioka treated the patients and reviewed the manuscript. All authors have approved the final submitted version.

Disclosures

M. Kajiwara received a fee for preparing materials for a Novo Nordisk Symposium. M. Shima and A. Yoshioka have received consultant and speaker's fees from Novo Nordisk and Baxter. Editorial assistance to the authors during the preparation of this manuscript was provided by Sharon Eastwood (medical writer, PAREXEL) and financially supported by Novo Nordisk in compliance with international guidelines for good publication practice.

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OFFICIAL COMMUNICATION OF THE SSC

Towards standardization of clot waveform analysis and recommendations for its clinical applications

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Introduction

Automated coagulation analyzers can provide a wealth of information in addition to that provided by conventional clotting tests such as the prothrombin time (PT) and the activated partial thromboplastin time (APTT), which are often considered of limited use for clinical purposes [1]. One particular type of analysis, the clot waveform, which was originally described using the Multichannel Discrete Analyzer (MDA series; Organon, Technika, Durham, NC, USA), defines changes in light transmittance that occur during the process of clot formation. A number of recent reports have described the use of this type of automated clotting instrument for clot waveform analysis (CWA), and there appears to be significant advantages in using this assay for the assessment of global coagulation function. In this communication, we propose standardization of methods for the CWA using currently available clotting analyzers and overview the potential clinical applications.

Principles of clot waveform analysis

Visualization of clot waveforms

Changes in light transmittance or absorbance are determined by continuous measurements during the APTT and are designated the clot waveform (CW) (Fig. 1). This clotting process is categorized into three parts: the pre-coagulation, coagulation and post-coagulation phases. Pre-coagulation is described as the first segment of the trace, from the beginning of the signal to the onset of coagulation. After the onset of coagulation, light transmittance is decreased or absorbance is increased by the formation of fibrin, and this is defined by a slope in the waveform. At the end of coagulation, light transmittance or absorbance tends to stabilize and is characterized again by a linear segment. If fibrinolysis is enhanced due to acquired or congenital abnormalities of hemostasis, light transmittance may increase or absorbance may decrease again in the post-coagulation phase.

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Coagulation analyzer

There are two types of clotting machines for CWA. One utilizes a system to detect transmittance during the APTT clotting reaction, and is represented by the MDA-II or CS series. In this type, transmittance is decreased after initiation of clotting (Fig. 1A). The other type monitors the absorbance, and is represented by the ACL series. In this type, 0% absorbance defines the pre-coagulation phase, and the absorbance increases after the initiation of clotting (Fig. 1B). Other analyzers having similar features should also be able to provide CWA data easily, particularly if manufacturers incorporate the relevant software. Even if an automated CWA is not available, there are several analyzers that are able to provide adequate raw data (Table S1). CWA is possible using such analyzers by statistical evaluation of this raw data of transmittance or absorbance (Fig. S1).

Recommended method for standardization of CWA

APTT assay

Plasma should be prepared from fresh citrated whole blood as for the standard APTT assay. Pooled plasma from nor-

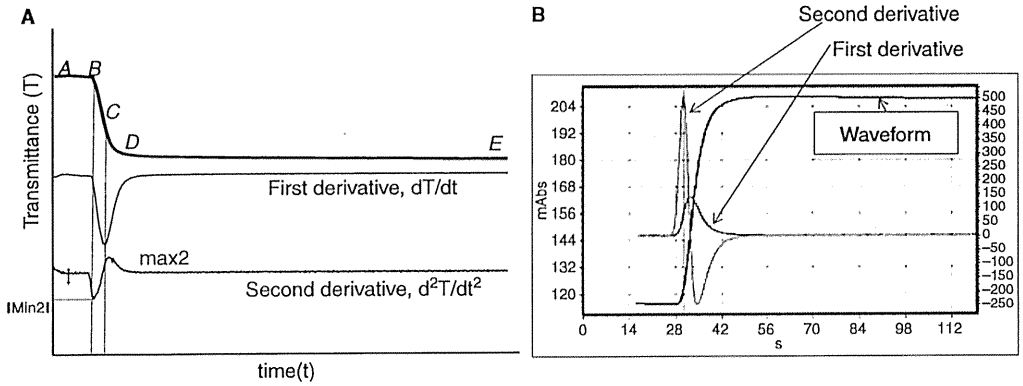

Fig. 1. APTT clot waveforms (A) Clot waveform of normal plasma by monitoring of transmittance (CS2000i). The upper trace shows the changes in light transmittance observed during the performance of APTT with normal reference plasma by CS200i (Sysmex Kobe, Japan). Point 'A' marks the beginning of the recording by the instrument, which occurs 8 s after the addition of CaCl2. Point 'B' indicates the initiation of coagulation, namely fibrin formation. The clot waveform is separated into the pre-coagulation phase (A–B), the coagulation phase (B–D) and the post-coagulation phase (D–E). (B) Clot waveform of normal plasma by monitoring of absorbance (ACL-Top). The upper blue colored trace shows the changes in absorbance observed during the performance of APTT with normal reference plasma by ACL-Top (Instrumentation Laboratory). The red colored curve is the first derivative of the absorbance corresponding to the coagulation velocity. The light blue colored biphasic curve is the second derivative of the absorbance corresponding to the coagulation acceleration.

mal individuals or commercial normal plasma is available as reference plasma. Colorless APTT reagents, without opacity, are recommended to detect sensitive changes in transmittance or absorbance, and for subsequent precise measurements of the various parameters. Although any APTT reagent that fulfills this criteria should be usable for CWA, among the reagents tested so far (Table S1), Thrombocheck APTT-SLA/0.02M $CaCl_2$ is suitable for MDA-II and CS series and HemosIL SynthASil for ACL-Top series. Other instrument and reagent combinations should also be possible to use but these need to be tested. APTT reagents used for the detection of anti-phospholipid antibodies are not recommended for the CWA because their sensitivity for assessing low clotting function is not sufficiently high. The APTT for CWA is performed in a similar manner to that for the standard APTT assay.

CWA parameters

The first derivative of the transmittance reflects coagulation velocity, and the second derivative reflects coagulation acceleration. Clotting time (CT), maximum coagulation velocity (Min1), maximum coagulation acceleration (Min2) and maximum coagulation deceleration (Max2) are common basic parameters. Among these measurements, Min2 has been reported to be correlated with clotting function in hemophilia [2].

Clinical applications of CWA

Clotting function of various bleeding disorders

Initial evaluation of clotting function by CWA is undertaken by qualitative assessment of the CW pattern. In particular, two characteristic CW patterns are observed in various coagulation abnormalities compared with normal reference plasma (Fig. S2). In normal plasma, the pre-coagulation phase is short and the slope, reflecting the coagulation phase, is steep. In factor (F) XII, X, IX, VIII, V and II deficiencies, the pre-coagulation phase is prolonged but the changes in slope are different [1]. Changes in slope are more evident in FVIII and FIX deficiencies than in other deficiencies. Thus, qualitative analysis of CW may have diagnostic value in various clinical settings of impaired clotting function.

Evaluation of clotting function in hemophilia A and B

While assays of FVIII:C and FIX:C are most important for the clinical management of hemostasis in patients with hemophilia, CWA provides a potentially widely available platform for assessment of global hemostasis in these patients [3] (Figs S3 and S4). This assay could then also provide a novel method not only for diagnosis and correlations with the bleeding phenotype but also for monitoring of hemostasis in cases of replacement therapy for serious hemorrhage or surgery.

Furthermore, the aPTT CWA is also useful for assessing very low levels of FVIII or FIX, for example less than 1 IU dL^{-1}. Studies in a number of patients with severe HA diagnosed by conventional clotting assays, demonstrated that CWA patterns differed from patient to patient. The APTT clotting time was prolonged in all patients with severe HA, but there was variation in the slope [2]. Using mixtures of severe HA plasma and exogenous FVIII ranging from zero to 1.0 IU dL^{-1}, the slope and the APTT clot time and the min2 appeared to change in a dose-dependent manner. Similarly, in further studies

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

Correlation between clinical severity and CWA parameters

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

Monitoring hemostatic therapy in the patients with inhibitors

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

Clotting function of acquired hemophilia

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

Advantages and limitations

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

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

Addendum

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

Disclosure of Conflict of Interests

M. Shima is supported for APTT reagents from Sysmex.

Supporting Information

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

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

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

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Coagulation potential of immobilised factor VIII in flow-dependent fibrin generation on platelet surfaces

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Summary

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

Keywords

Factor VIII, von Willebrand factor, flow, fibrin generation, haemophilia

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Introduction

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

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

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

Materials and methods

Blood collection

This work was approved by the institutional review board of Nara Medical University. Blood was collected from five non-smoking healthy volunteers, who had not taken any medications in the previous two weeks. Two different ways of blood collection (anticoagulation by 1/10th volume of 3.8% sodium citrate or 20 μM of argatroban; Tanabe-Mitsubishi Co., Tokyo, Japan) were employed for evaluation of intra-thrombus fibrin generation under flow. For the citrated blood, 50 μg/ml of corn trypsin inhibitor (CTI; Haematologic Technologies Inc, Essex Junction, VT, USA) was added

into the blood sample to minimise the contact activation of blood, then 8 mM of $CaCl_2$ was added to initiate blood coagulation just prior to perfusion. With regards to the argatroban-treated blood, this relatively low argatroban concentration was determined to allow gradual thrombin generation without the flow path occlusion by gross clot formation during blood perfusion, which makes it suitable for the evaluation of fibrin generation, as well as platelet adhesion and aggregation, when the time lag from blood drawing to the perfusion start was strictly adjusted among experiments, as described (7, 8).

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

Human native VWF (FVIII-VWF complex) was purified from cryoprecipitate as previously described (9). FVIII-free VWF was obtained by the rechromatography of purified FVIII-VWF complex with Separose-CL6B in the presence of 0.35 M $CaCl_2$ as described (10), and the complete depletion of FVIII was confirmed by ELISA assay for FVIII as previously described (11). Glass plates which had been coated with purified FVIII-free VWF as described (8, 12) were reacted with recombinant FVIII (Kogenate FS provided by Bayer Pharmaceutical Co., Osaka, Japan) at varying concentrations (0 as a negative control, 0.1, 0.5, 1, 2.5, 5, and 10 units (IU)/mL) for 2 hours (h) at room temperature. After non-adherent proteins were washed out, the amount of FVIII immobilised to the glass-bound VWF was quantified by ELISA-based assay. Briefly, a rubber ring (diameter: 8 mm) was placed on a VWF-coated glass on which various amounts of FVIII was immobilised. A peroxidase-conjugated anti-FVIII human polyclonal antibody previously described (11) was then reacted to a glass surface inside the rubber ring, followed by the routine ELISA assay procedures. The final reactant with enzyme activity inside the ring was collected, transferred to an ELISA-plate, and the enzyme intensity was determined at the wave length of 492 nm, reflecting the amount of surface-immobilised FVIII.

In vitro perfusion studies

In perfusion studies for the evaluation of platelet adhesion and aggregation, whole blood anticoagulated with argatroban was immediately incubated with the fluorescent dye DiOC6 (1 μM; Molecular Probes Inc., Eugene, OR, USA) for 10 minutes (min) at 37°C to label platelets, allowing visualisation of platelet-surface interactions by confocal laser scanning microscopy (CLSM, FV300; Olympus Co., Tokyo, Japan), as described (7, 13-15). DiOC6-labelled platelets were aspirated through the chamber by a syringe pump (Model CFV-3200, Nihon Kohden Co., Ltd., Tokyo, Japan), producing a 250 (typical low) or 1500 (typical high) shear rate at the 37°C situation, as described (7, 13-16). Fluorescent images were viewed by CLSM at 1-μm intervals from the VWF surface to a height of 60 μm from the surface, and used to calculate the percentage of the area covered by adhering platelets (surface coverage) and each thrombus volume in a defined area at the indicated time points as described (7, 15). Briefly, surface coverage of platelet thrombi was evaluated based on sliced images at 2-μm height from the VWF surface, and total volume of platelet thrombi in a defined area was calculated by summing all sliced images of identical portions using the image-analysing computer software (Image Pro Plus version 4.5; Planetron, Tokyo, Japan).

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

Preparation of synthetic "acquired" haemophilic blood

After informed consent, plasma samples were obtained from a Japanese patient with severe haemophilia A and a high-titre inhibitor, and the anti-FVIII inhibitor IgG (human alloantibody) was puri-

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

Synthetic haemophilic blood	Inhibitor titre in whole blood (BU/ml)	Plasma FVIII:C (%)	Remaining inhibitor titre in plasma (BU/ml)	aPTT (sec)	aPTT with CTI (sec)
#1	5	1.1	12.2	108.2 ± 3.2	166.2 ± 5.2
#2	10	<1.0	22.0	111.7 ± 4.3	178.4 ± 7.1
#3	20	<1.0	44.0	112.5 ± 4.5	172.4 ± 4.8
Control	0	100	0	38.5±2.3	84.2±2.5

aPTT; activated partial thromboplastin time, BU; Bethesda units, CTI; corn trypsin inhibitor (50 □g/ml).