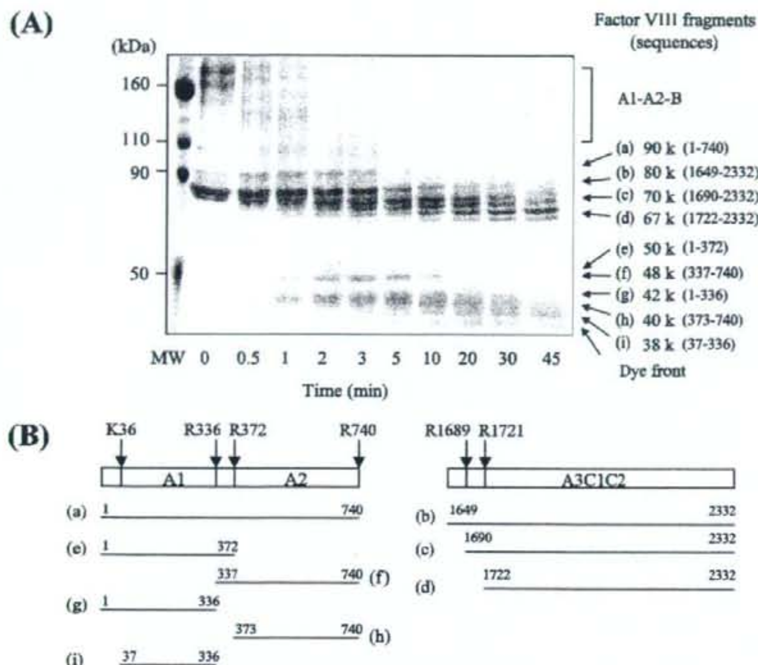

FIGURE 3. **Time course of plasmin-catalyzed proteolysis of factor VIII.** *A*, SDS-PAGE of factor VIII (1.5 μM) was incubated with plasmin (20 nM) in the presence of phospholipid vesicles (10 μM) as described under "Materials and Methods." At the indicated times, the reaction was terminated, and samples were run on an 8% gel and stained with GelCode Blue. The molecular weight and amino acid sequence of each fragment were obtained from given molecular weight marker (*MW*, *lane 1*) and N-terminal sequence analysis. *B*, schematic representation of the cleavage sites in factor VIII by plasmin and the generated cleaved factor VIII fragments. The characters from *a*–*i* correspond to *A* and *B*.

indicated that plasmin cleaved the cofactor at four sites in the heavy chain, Lys^{36}, Arg^{336}, Arg^{372}, and Arg^{740}, and at two sites in the light chain, Arg^{1689} and Arg^{1721}. These sites appeared to be identical to those cleaved by factor Xa (5, 13). The sequences of several factor VIII products generated from cleavage by plasmin were estimated from the N-terminal sequence, the molecular weights, specificity for cleavage of plasmin, and Western blotting (as described in next paragraph) using anti-factor VIII monoclonal antibodies analyzed recognizing epitope. The amino acid sequences of the 90-, 50-, 48-, 42-, 40-, and 38-kDa fragments derived from the heavy chain were shown to be residues 1–740, 1–372, 337–740, 1–336, 373–740, and 37–336, respectively, and were designated as A1-A2, $A1^{1-372}$, $A1^{337-372}$-A2, $A1^{1-336}$, A2, and $A1^{37-336}$, respectively. The amino acid sequences of the 70- and 67-kDa fragments derived from the light chain were residues 1690–2332 and 1722–2332, respectively, indicating that the light chain was cleaved by plasmin in the order Arg^{1689} and Arg^{1721}. The relationship between these fragments is shown schematically in Fig. 3*B*.

Cleavage of the Factor VIII Heavy Chain by Plasmin—Procoagulant factor VIII activity correlates well with the pattern of limited proteolysis of the heavy chain, as represented by cleavage at Arg^{372} by thrombin and factor Xa for activation, and cleavage at Arg^{336} by APC and factor Xa for inactivation. We therefore focused on plasmin cleavage of the factor VIII heavy chain using Western blotting (Fig. 4). Products of proteolysis were visualized using two anti-A1 monoclonal antibodies recognizing the N terminus (58.12, Fig. 4*A*) or C terminus (C5, Fig. 4*B*) of A1 and one anti-A2 monoclonal antibody (JR8, Fig. 4*C*). The cleavage sites of the heavy chain by plasmin and epitope regions of factor VIII monoclonal antibodies are schematically illustrated in Fig. 4*D*. In the presence of factor VIII (500 nM), plasmin (10 nM), and phospholipid, the heavy chain (A1-A2-whole B) was initially converted into A1-A2 subunits by cleavage at Arg^{740} (Fig. 4, *A*–*C*). Subsequently, the $A1^{337-372}$-A2 fragment appeared to be generated more rapidly than the A2 product (Fig. 4*C*, *inset*), suggesting that cleavage at Arg^{336} occurred more quickly than that at Arg^{372}. Consequently the $A1^{1-336}$ and $A1^{337-372}$-A2 fragments were generated as intermediate products. These intermediate products were not seen, however, at the 10- and 20-min time points using the anti-A1 antibodies (Fig. 4, *A* and *B*, *arrows*). Because the 58.12 and C5 antibodies recognize the N- and C-terminal regions in A1, respectively, the failure to detect $A1^{1-336}$ and $A1^{337-372}$-A2 fragments suggests that complete cleavage at Lys^{36} and Arg^{372} by plasmin generated terminal products $A1^{37-336}$ and A2. An alternative product, $A1^{1-372}$, generated initially by cleavage at Arg^{372} was further proteolyzed at Arg^{336} and Lys^{36}, also resulting in the terminal product $A1^{37-336}$. These results demonstrated that plasmin proteolyzed the heavy chain into $A1^{37-336}$ and A2 subunits by two cleavage pathways, a more predominant cleavage at Arg^{336} and a minor cleavage at Arg^{372}.

Cleavage of the A1 Subunit of Factor VIII(a) by Plasmin, APC, and Factor Xa—The cleavage sites within the A1 subunit are Arg^{336} for APC and Arg^{372}, Arg^{336}, and Lys^{36} for factor Xa and plasmin. The changes associated with the activity of factor VIII incubated with proteases in these studies were different, however, suggesting alternative mechanisms. Therefore, to investigate this further, the A1 cleavage pattern mediated by plasmin, APC, and factor Xa was analyzed by Western blotting using anti-A1 antibody 58.12 (Fig. 5*A*). The addition of APC mixed with cofactor protein S cleaved factor VIII at Arg^{336} and resulted in the appearance of $A1^{1-336}$ fragments in a time-dependent manner (Fig. 5*A*, *panel b*). Factor Xa cleaved initially at Arg^{740} followed by cleavages at Arg^{372} and Arg^{336} (Fig. 5*A*, *panel c*), consistent with the earlier reports (5). However, plasmin cleaved the A1 subunit at Arg^{336} much more rapidly than at

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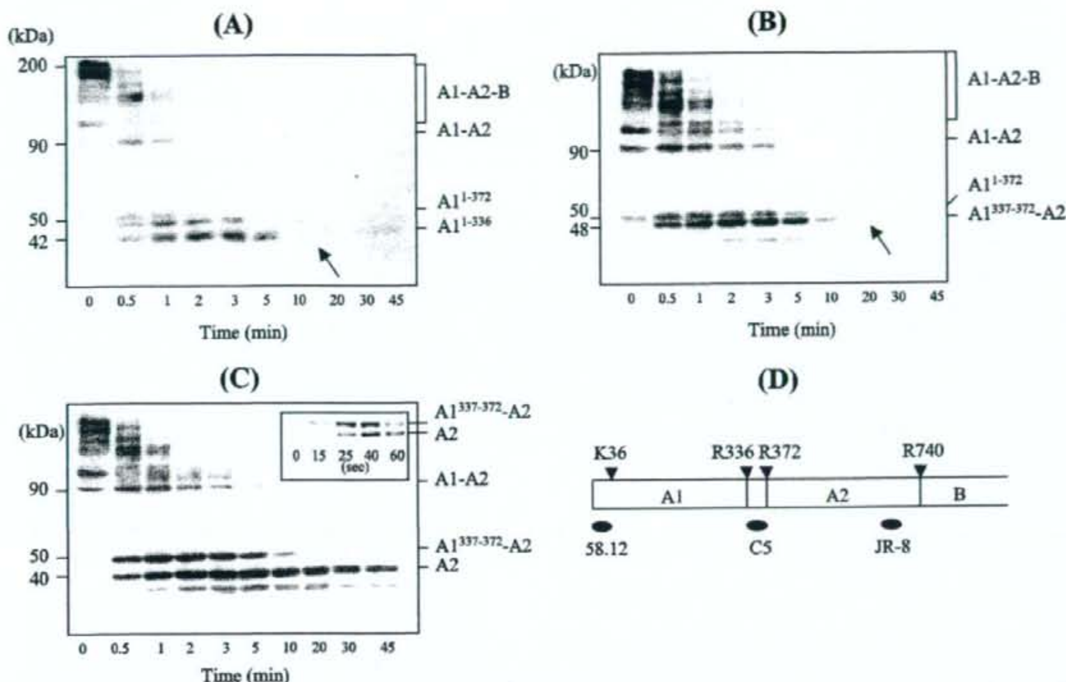

FIGURE 4. **Time course of plasmin-catalyzed cleavage of the heavy chain of factor VIII.** Factor VIII (500 nM) was incubated with plasmin (10 nM) in the presence of phospholipid vesicles (10 μM) for the indicated times as described under "Materials and Methods." Samples were run on an 8% gel followed by Western blotting using anti-A1 (58.12, *A*), anti-A1 (C5, *B*), or anti-A2 (JR8, *C*) monoclonal antibodies. *D* shows a schematic presentation of the domain organization of the heavy chain, location of plasmin-catalyzed cleavage sites, and epitope regions of monoclonal antibodies. The *inset* in *C* represents the appearance of $A1^{337-372}$-A2 and A2 bands at an early time phase (within 60 s). The *arrow* in *A* or *B* shows the disappearance of $A1^{1-336}$ or $A1^{337-372}$-A2 band by proteolytic cleavage at Lys^{36} or Arg^{372}, respectively.

Arg^{372}, followed by cleavage at Lys^{36} at an early time point (Fig. 5*A*, *panel a*). These data were consistent with distinct rate-dependent cleavage patterns of the A1 subunit by plasmin, APC, and factor Xa, although the cleavage sites appeared to be similar or identical.

Additional experiments, using active factor VIIIa, in place of inactive factor VIII, were devised to quantitate the kinetics of A1 cleavage. Western blotting using 58.12 antibody (Fig. 5*B*) was utilized to assess both the proteolysis of the A1 subunit in factor VIIIa (*left panels*) and the rate of loss of intact $A1^{1-372}$ subunit (*right panel*). The Western blots were analyzed by scanning densitometry, and the data points were extrapolated using a single exponential decay equation. The rate of cleavage of A1 in factor VIIIa (100 nM) by plasmin (4 nM) was 1.53 ± 0.16 min^{-1} and was ~3.2 to more than 20-fold greater than that mediated by APC (0.48 ± 0.06 min^{-1}) and factor Xa (0.06 ± 0.01 min^{-1}). These findings were in keeping with earlier reports (15, 35) that inactivation of factor VIIIa can be attributed to A1 proteolysis at Arg^{336} (and Lys^{36}) and that similar cleavage governs plasmin-catalyzed inactivation of factor VIII.

Role of Individual Factor VIIIa Subunits in A1 Subunit Cleavage by Plasmin—To further study the roles and mechanisms of each subunit in factor VIII(a) for plasmin-mediated proteolysis, various factor VIII(a) fragments (100 nM) were used as substrates for plasmin (4 nM), followed by the Western blotting using the anti-A1 58.12 antibody for detection (Fig. 6). Because this antibody, recognizing the N terminus of the A1 subunit, detects $A1^{1-336}$ fragments but not $A1^{37-336}$ fragments, the appearance or disappearance of $A1^{1-336}$ can be attributed to cleavage at Arg^{336} or Lys^{36}, respectively. The $A1^{1-336}$ fragments derived from factor VIII (Fig. 6*A*), factor VIIIa (Fig. 6*B*), and intact heavy chain (Fig. 6*C*) were identified at an early time point after the addition of plasmin. With A1/A3-C1-C2 dimers (Fig. 6*D*), little $A1^{1-336}$ was detected, and with isolated A1 subunits (Fig. 6*E*), the $A1^{1-336}$ fragment was generated very slowly after the addition of plasmin, respectively. Factor VIII(a) and the intact heavy chain contain the A2 domain, but this is not present in A1/A3-C1-C2 dimers or isolated A1subunits. Our results supported the view that cleavage at Arg^{336} may be modulated by the A2 domain (Fig. 6, *panel a*). Interestingly, the $A1^{1-336}$ fragments derived from factor VIII (Fig. 6*A*) and factor VIIIa (Fig. 6*B*) disappeared within ~5 min after the addition of plasmin. In contrast, with the intact heavy chain (Fig. 6*C*) and isolated A1 subunit (Fig. 6*E*), the $A1^{1-336}$ fragments persisted strongly even at the 30 min-time point. With the A1/A3-C1-C2 dimer (Fig. 6*D*), the $A1^{1-372}$ fragment disappeared after the addition of plasmin, without the appearance of $A1^{1-336}$. It therefore seemed likely that cleavage at Lys^{36} in the dimer form

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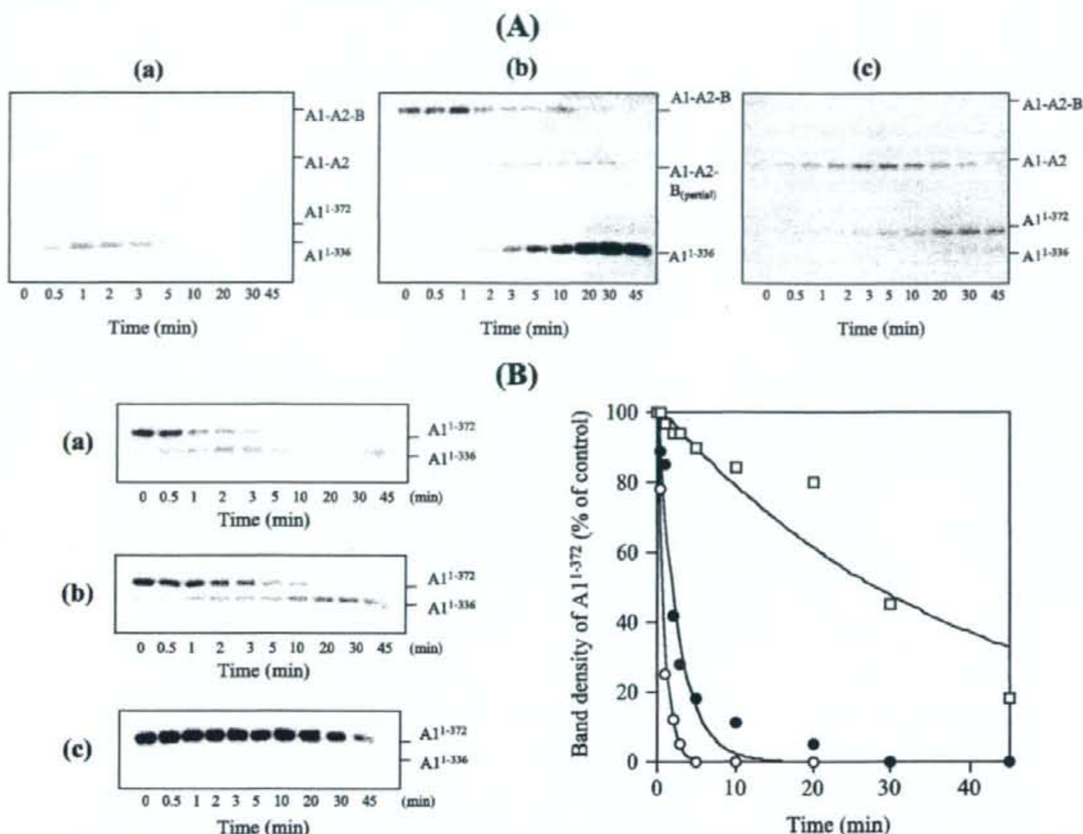

FIGURE 5. **Comparison with A1 cleavage in factor VIII(a) by plasmin, APC, and factor Xa.** Factor VIII (*A*, 100 nM) or factor VIIIa (*B*, 100 nM) was incubated with 4 nM plasmin (*panel a*), APC (*panel b*) together with protein S (40 nM), and factor Xa (*panel c*) in the presence of phospholipid vesicles (10 μM) for the indicated times as described under "Materials and Methods." Samples were run on 8% gel followed by Western blotting using an anti-A1 antibody (58.12). *Right panel* in *B* shows the data obtained by quantitative densitometry of the intact $A1^{1-372}$. The symbols used are as follows: *open circles*, plasmin; *closed circles*, APC; and *open squares*, factor Xa. The band density of $A1^{1-372}$ at the time 0 point was designated as 100%. Data were extrapolated using a single exponential decay curve.

was predominant, probably with little cleavage at Arg^{336}. The factor VIII(a) molecule and the dimer contain A3-C1-C2 subunits, but these are not constituents of the heavy chain or isolated A1. Our findings therefore suggest that cleavage at Lys^{36} was likely regulated by the presence of the A3-C1-C2 subunit (Fig. 6, *panel b*). Taken together, these data demonstrated that specific cleavages at Arg^{336} and Lys^{36} by plasmin appeared to be selectively modulated following interaction of plasmin with the A2 and A3-C1-C2 subunit, respectively.

DISCUSSION

In normal hemostasis there is a controlled balance between coagulation and anti-coagulation or fibrinolysis. An imbalanced state between these systems leads to pathologic thrombosis or hemorrhage consequent to the excessive activation and inactivation of coagulation and fibrinolytic factors (36, 37). In clinical disseminated intravascular coagulation, the course of the disease is complicated by uncontrolled proteolysis of important clotting factors (38). During the development of disseminated intravascular coagulation, numerous proteases are likely to be active simultaneously, and in particular, plasmin is likely to be a major instigator of the observed proteolysis (39, 40). In this context, the proteolysis of fibrinogen and/or fibrin by plasmin has been extensively studied and documented in the literature (41). It is less widely appreciated, however, that other mechanisms involving thrombolytic proteases may promote a hemorrhagic response, and that plasmin may have an anticoagulant effect by moderating the activity of coagulation cofactors factor V or factor VIII and limiting excessive activation of the coagulation system. An earlier study (42) reported that an increase in the plasma concentration of plasmin correlated with decreased factor VIIIa activity. This study was therefore undertaken to examine the relationship between factor VIII and plasmin.

We observed that plasmin inactivated factor VIII activity rapidly after an initial weak increase (~2-fold) in activity in a

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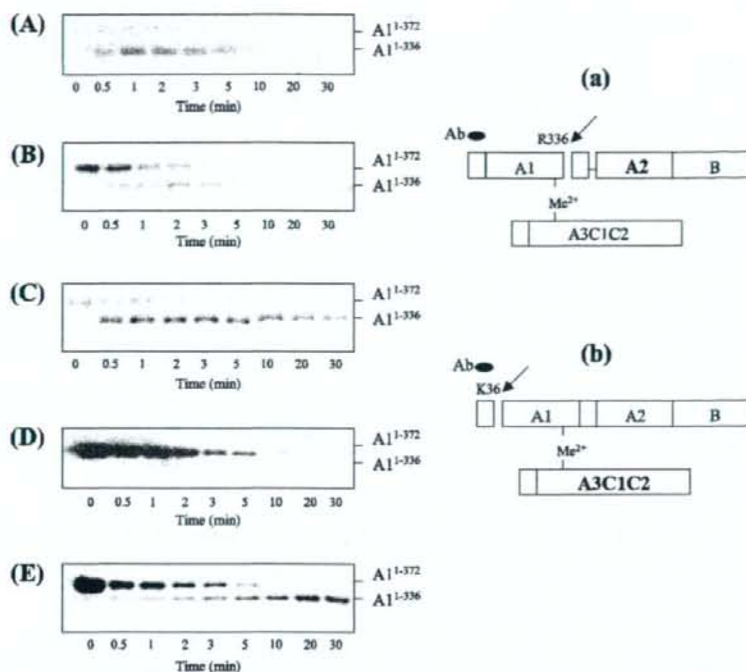

FIGURE 6. **Effects of factor VIII(a) subunit on Lys[36] and Arg[336] cleavages in A1 subunit by plasmin.** Equivalent concentrations (100 nM) of factor VIII (*A*), factor VIIIa (*B*), intact heavy chain (*C*), A1/A3-C1-C2 dimer (*D*), or A1 subunit (*E*) were incubated with plasmin (4 nM) in the presence of phospholipid vesicles (10 μM) for the indicated times as described under "Materials and Methods." Samples were run on 8% gel followed by Western blotting using an anti-A1 antibody (58.12). *Right panels* schematically illustrate the relationship between the cleavage at Arg[336] (*panel a*) or Lys[36] (*panel b*) and the A2 or A3-C1-C2 subunit (*bold*), respectively.

clotting-based assay. This phospholipid-independent response was similar for both native factor VIII and active factor VIIIa (data not shown). However, the phospholipid-independent action of plasmin on factor VIII was different from that reported for other clotting factors. For example, the inactivation of factor Va by plasmin appeared to be phospholipid/Ca^{2+}-dependent (17), and the mechanism has been described to be associated with critical residues 307–348 of the factor V molecule (18). Similarly, plasmin-induced cleavage and inactivation of factor X appeared to be lipid-dependent (20). Factor VIII and factor V are structurally homologous (43), and our data imply distinct similarities in the action of plasmin on factor VIII and factor V.

Factor VIII circulates in a noncovalent complex with VWF. Critical sites for factor VIII interaction with VWF have been localized to the N-terminal acidic region of the A3 domain (8), to the C terminus of the C2 domain (44), and within the A3-C1 domain (32, 45), supporting the view that the A3-C1-C2 environment forms an extended surface for interaction with VWF. In our experiments neither VWF nor phospholipid affected plasmin-catalyzed factor VIII inactivation. It therefore appeared that plasmin interaction with factor VIII did not involve VWF and phospholipid-interactive sites and was not dependent on activated and/or unactivated forms of the cofactor. These findings indicated that plasmin may be a unique anticoagulant protease for factor VIII. Activation and inactivation of factor VIII by other proteases, APC (32, 33) and factor Xa (46, 47), are markedly affected by the presence of VWF. Interestingly, an earlier report by Rick and Krizek (22) demonstrated that factor VIII activation and inactivation by plasmin was affected by the presence of platelets but not by phospholipid vesicles. The reason for this difference was not clear, but it may be that the conformation of factor VIII bound to platelets may be more susceptible to plasmin compared with that bound to isolated phospholipid moieties. Our studies were performed using phospholipid vesicles, and further experiments are in progress to investigate possible differences in reaction between phospholipid and intact platelets.

A combination of SDS-PAGE and N-terminal sequence analysis confirmed that limited proteolysis of factor VIII occurred at four positions in the heavy chain, Lys^{36}, Arg^{336}, Arg^{372}, and Arg^{740}, and at two sites in the light chain, Arg^{1689} and Arg^{1721}, resulting in the generation of three terminal products, $A1^{37-336}$, A2, and A3-C1-$C2^{1722-2332}$ within 1 h of adding plasmin. The amino acid specificity of these cleavage sites is in agreement with the known preference of the protease for hydrolyzing either arginine or lysine residues (48). The six plasmin cleavage sites included those lysed by thrombin and factor Xa that result in activation of factor VIII cofactor, and those lysed by APC and factor Xa that result in inactivation. Surprisingly, the plasmin cleavage sites were identical to those observed after interaction of factor VIII with factor Xa (5, 13), but one of the APC cleavage sites, Arg^{562}, was not affected by plasmin. We also observed the A2 doublet with an unidentified band (Fig. 4*C*). The N-terminal sequences of the double bands were identical (data not shown). Therefore, this is unclear at the present time. Fay *et al.* (7) reported that although the doublet bands derived by the heavy chain cleavage by thrombin and factor Xa could be observed, the origin of the bands was unclear.

Proteolysis at Arg^{372} and Arg^{1689} is essential for generating factor VIIIa cofactor activity (6). Recently, Nogami *et al.* (49) reported that failure of proteolysis at Arg^{740} resulted in markedly low cofactor activity, indicating that cleavage at the A2-B junction may be an essential step in the process of pro-cofactor activation. In our studies, the initial activation of factor VIII by plasmin appeared to be associated with three cleavage sites.

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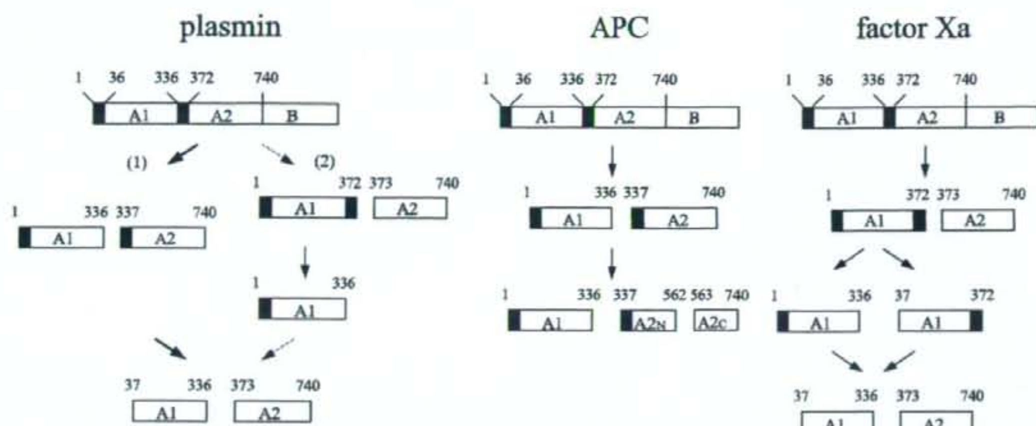

FIGURE 7. **A schematic comparison of the proposed pathways for the cleavage of factor VIII heavy chain by plasmin, APC, and factor Xa.**

Therefore, we focused attention on inactivation of factor VIII, and it was notable that cleavage by plasmin at Arg^{336} within A1 subunit was rapid compared with that at Arg^{372} between the A1 and A2 junction. This predominant cleavage at Arg^{336} rather than at Arg^{372} was in contrast to the cleavage process reported for factor Xa. Furthermore, the cleavage at Arg^{336} was very rapid compared with similar cleavage induced by APC. It is well known that serine proteases, including APC and factor Xa, inactivate factor VIII(a) following cleavage at Arg^{336} (15, 35). Inactivation occurs because of an altered interaction between the A2 subunit and the truncated A1 and results in the loss of a factor X-interactive site within residues 337–372 (14) and an increase in the K_m value for substrate factor X (12, 13). Our data using a clotting-based assay and SDS-PAGE supported the concept that the degree of factor VIII inactivation is likely to be dependent on the proportion of unactivated molecules, activated molecules, and decay following subunit dissociation, the more rapid the cleavage at Arg^{336} in the A1 subunit, the more rapid the inactivation of factor VIII(a).

A relatively recent report described that additional cleavage at Lys^{36} altered the conformation of the A1 subunit and limited productive interaction with the A2 subunit (13). Consequently, there appeared to be approximately half the level of activity of factor VIIIa compared with the $A1^{1-336}$ subunit (13). In this study, proteolysis by plasmin at Lys^{36} occurred more rapidly than that observed by factor Xa. These results suggested that the proteolysis at Lys^{36} might contribute to some extent to the rapid inactivation of factor VIII(a) by plasmin. No influence on inactivation of factor VIII by cleavage at Arg^{1721} in the light chain has been reported (10). In this context our data again demonstrated that cleavage at Arg^{336} was a major mechanism responsible for plasmin-catalyzed inactivation of factor VIII.

A comparison of the proteolysis of the factor VIII heavy chain by plasmin, APC, and factor Xa is schematically illustrated in Fig. 7. These mechanisms have been well described. APC cleaves factor VIII at Arg^{336} within the A1 domain, followed by Arg^{562} within A2 (50). Factor Xa cleaves initially at Arg^{372} and then at Lys^{36} and Arg^{336} (35). Interestingly, the terminal products derived from plasmin cleavage are identical to those produced by factor Xa. Although plasmin-catalyzed cleavage of the heavy chain may involve the two sites, Arg^{336} and Arg^{372}, within the A1 domain, cleavage at Arg^{336} would appear to be the predominant pathway.

The factor VIII-APC interactive sites have also been localized to the A3 domain (32, 51). In addition, Nogami *et al.* (35, 47) demonstrated that factor Xa-catalyzed reactions at Arg^{336} and Lys^{36} are likely selectively regulated by interactions with the A3-C1-C2 and A1 subunits, respectively. The data from this study using gel analysis, indicated that cleavage at Arg^{336} was selectively enhanced by plasmin associated with the A2 subunit, and cleavage at Lys^{36} was regulated by the A3-C1-C2 subunit, although conformational changes of the A1 subunit associated with factor VIII(a) could not be completely excluded. On this basis, we suggest that this mechanism of plasmin activity is distinct from that of factor Xa, although the cleavage sites are identical for both proteases. Plasmin is composed of a heavy chain containing five kringle domains and a light chain containing the catalytic domain. It is reactive with numerous proteins represented typically by lysine-binding site interaction with fibrin (41). It is therefore atractive to speculate that clustered basic residues of lysine (and arginine) found within both A2 and A3-C1-C2 sequences but not the A1 domain (2, 3) provide the natural target for plasmin in the factor VIII molecule.

The physiological significance of plasmin-catalyzed cleavage of factor VIII, resulting in activation and inactivation of cofactor function, remains to be fully determined. However, even very low concentrations (4 nM) of protease, generated from high plasma concentrations of proenzyme, plasminogen (~2.4 μM), would be sufficient to promote a catalytic rate ~3.7- and 12-fold greater than APC and factor Xa, respectively. Our data imply that small amounts of plasmin generated in the fibrinolytic response might contribute to the up- and down-regulation of blood coagulation. Furthermore, the plasmin-catalyzed inactivation mechanism of other clotting factors such as factor Va (18) and factor IX (19) has been also reported. In particular, factor V has similar conformation to factor VIII and similar

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activation/inactivation mechanisms. Therefore, we also suggest the presence of a regulatory role of plasmin through direct proteolytic reaction in the coagulation reaction as well as fibrinolytic activity.

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ORIGINAL ARTICLE

The measurement of low levels of factor VIII or factor IX in hemophilia A and hemophilia B plasma by clot waveform analysis and thrombin generation assay

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Summary. *Background:* Precise assessment of clotting function is essential for monitoring of hemostatic treatment for hemophilias A and B. *Materials and methods:* Clot waveform analysis and thrombin generation assays were performed on factor (F) VIII- and FIX-deficient plasmas, which had been reconstituted with known amounts of recombinant FVIII (rFVIII) and affinity-purified FIX respectively. Clot waveforms were assessed qualitatively and quantitatively by measuring the parameters clotting time, maximum coagulation velocity (Min1), and maximum coagulation acceleration (Min2). The thrombin generation assay was also assessed qualitatively and measurements made of time to peak and peak height. *Results:* Overall results obtained with both assays showed good correlation for both clotting factors confirming that the changes in clotting waveform reflected changes in thrombin generation. Both assays demonstrated a predictable dose response to the addition of FVIII or IX. However, clot waveform analysis was more sensitive than the thrombin generation assay, particularly in detecting very low levels (0–0.1 IU dL^{-1}) of both factors. *Conclusions:* These data suggest that the application of clot waveform analysis to the routine management of the hemophiliacs could increase our understanding of the clinical significance of low levels of FVIII and FIX that cannot be measured by assays in current use. This may be particularly useful in the management of hemophiliacs with inhibitors or undergoing gene therapy.

Keywords: clot waveform, factor IX, factor VIII, hemophilia, thrombin generation.

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Introduction

Hemophilias A (factor [F] VIII deficiency) and B (FIX deficiency) are the most commonly encountered congenital bleeding disorders. In each case the clinical severity of the disorder generally correlates very well with the level of its clotting activity measured by factor assay. By convention, according to the level of factor activity measured, patients are classified into three clinical categories: severe (< 1.0 IU dL^{-1}); moderate (1.0–5.0 IU dL^{-1}); and mild (> 5.0 IU dL^{-1}). Most patients in the severe category, i.e. with undetectable factor levels by conventional assay, have frequent spontaneous bleeds unless they receive regular prophylactic factor replacement therapy. In contrast, individuals in the moderate category bleed infrequently and usually only after trauma. However, clinical heterogeneity is observed with some patients in the severe category on the basis of factor assay exhibiting a moderate severity clinical phenotype. Similarly, occasionally patients in the moderate severity category exhibit a severe clinical phenotype with frequent episodes of spontaneous bleeding.

The basic approach to the management of the patients with hemophilias A and B is replacement therapy with FVIII or FIX concentrates respectively and major improvements in the frequency and severity of bleeding episodes are achieved by relatively minor elevations (≥1.0 IU dL^{-1}) of factor level. As a result of this and the introduction of highly purified viral-inactivated plasma-derived and recombinant factor concentrates of both FVIII and FIX, severely affected individuals may now be offered safe and effective regular prophylactic therapy. As a result, it is possible to reduce substantially the frequency and severity of bleeding episodes and thus significantly improve the quality of life of severely affected patients. Accumulated clinical experience suggests that the key objective of prophylactic factor replacement therapy is to maintain the circulating factor level at ≥1.0 IU dL^{-1}. Consequently, regular laboratory monitoring is essential to achieve this goal. As routine assays, such the activated partial thromboplastin time (aPTT), do not have sufficient sensitivity or specificity for factor levels in this range, monitoring is usually performed by specific factor assay

of FVIII or FIX. However, the measurement of FVIII or FIX in the very low range is difficult and the assays are frequently adversely affected by assay variables, such as the FVIII- or FIX-deficient plasmas used as substrates. As a consequence, it is possible that the factor levels recorded may have been underestimated and thus explain the occasional lack of correlation between the clinical and laboratory phenotypes. For example, significant clinical improvement following FIX gene therapy with only a minimal increase in circulating FIX levels has been reported [1]. These observations together with the recognition that only small quantities of theses critical clotting factors are required to achieve relatively normal hemostasis strongly suggest that a more accurate and sensitive method for the day-to-day monitoring of low levels of these clotting factors is required.

Clot waveform analysis, using the MDA-II system (bio-Mérieux, Durham, NC, USA), is a recently developed technique for monitoring hemostasis. The MDA-II is a fully automated coagulometer, which determines clotting end points photo-optically. During the performance of routine clotting assays such as aPTT or PT, it is possible to obtain a continuous measurement of the changes in light transmittance that occur as the test citrated plasma sample clots [2]. Thus, clot waveform analysis can be performed at the same time as the regular clotting end point is determined for the aPTT and PT assays. The waveform produced may be mathematically processed and several parameters derived which characterize the form of the wave observed (Fig. 1). Determination of the first derivative of the transmittance data provides a measurement of the velocity of coagulation at any given time point on the curve, whereas the second derivative is a measurement of acceleration or deceleration in the reaction. Preliminary investigations suggested that this technique provided useful information for determining the likely clinical phenotype of patients with very low factor levels [3]. Moreover, because the information could be derived during the performance of routine assays such as the aPTT, these additional data are easily obtained and thus facilitate prompt decision-making in patients who frequently present as medical emergencies.

As thrombin is the terminal enzyme in the coagulation cascade, it is logical that the measurement of thrombin generation should be proposed as the most appropriate way to determine an individual patient's overall hemostatic potential [4]. A number of different methods of achieving this have been proposed and applied to the evaluation of both hypercoagulable and hypocoagulable states and their management [5,6]. Thus, in this report we have compared the sensitivity and accuracy of waveform analysis with a test of thrombin generation in determining low levels of FVIII and FIX.

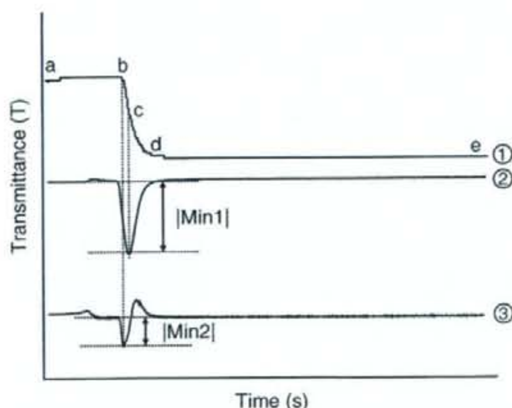

Fig. 1. Activated partial thromboplastin time (aPTT) clot waveform of normal plasma. The upper trace shows the recording of the changes in light transmittance (T) observed over time (t) during the performance of a normal diluted aPTT. The middle trace shows the first derivative (dT/dt: |Min1|) derived from these transmittance data. The lower trace shows the second derivative (d^2T/dt^2: |Min2|). Point 'a' marks the beginning of the recording by the instrument which occurs 8 s after the addition of $CaCl_2$; point 'b' the initiation of coagulation, i.e. the index of the second derivative (clot time); point 'c' the midpoint of coagulation, i.e. the index of the first derivative.

Materials and methods

Plasma samples

Normal pooled plasma was prepared from 10 normal healthy individuals. Blood was drawn into evacuated anticoagulant tubes [nine volumes of blood to one volume of 3.8% (w/v) trisodium citrate solution]. After centrifugation for 15 min at 1500 g the platelet poor plasma was stored at −80 °C and thawed at 37 °C immediately prior to performing the assays described. Test samples of known FVIII concentration were prepared by the addition of a known concentration of recombinant FVIII (rFVIII; Kogenate™ FS; Bayer, Osaka, Japan) to congenital FVIII-deficient plasma (George King, Overland Park, KS, USA) and a series of known concentration, ranging from 0 to 100.0 IU dL^{-1}, prepared by serial dilution. Test samples of known FIX concentration were prepared by an identical procedure. However, as rFIX is not available in Japan, affinity-purified plasma-derived FIX (Christmassin-M; Mitsubishi Pharma, Osaka, Japan) was used to make the serial dilutions.

Modified activated partial thromboplastin time test

The aPTT tests were performed on the MDA-II® Hemostasis System (bioMérieux) using a commercially available aPTT reagent consisting of synthetic phospholipids (phosphatidylethanolamine 40 μM, phosphatidylcholine 76 μM, phosphatidylserine 12 μM), and ellagic acid (APTT-SLA; Sysmex, Kobe, Japan; [7]). In order to obtain equivalent assay conditions to those employed in the thrombin generation assay (see below), the reagent was diluted to obtain the same final concentration of phospholipids (PE:PC:PS = 10 μM:19 μM:3 μM) as in the thrombin generation assay.

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Factor VIII:C and factor IX:C assays

FVIII:C was measured by a one-stage aPTT-based clotting assay on the MDA-II® Hemostasis System (bioMérieux). Chemically depleted FVIII-deficient plasma was used as the substrate (bioMérieux). A standard curve was prepared using Verify Reference Plasma (bioMérieux) in serial doubling dilutions (1:10 to 1:5120) in 0.05 M imidazole saline buffer containing 0.05% sodium azide (pH 7.3). The Verify Reference Plasma is calibrated by the manufacturer against an International FVIII Standard (IRP-SSC1 or 2). Each test sample was diluted to 1:10 in imidazole saline buffer. FIX:C was also measured by an identical procedure but using congenital FIX-deficient plasma as the substrate (George King).

Modified aPTT waveform analysis

Waveform analysis of the optical data obtained from the modified aPTT assay was performed on the MDA-II® Hemostasis System as described previously and the data automatically processed by algorithms built into the software. The data were processed subsequently by export research tools (WIT/WET) provided by bioMérieux.

The first derivative of the transmittance (dT/dt) reflects the coagulation velocity at each time point along the waveform plot of changes in light transmission, which in turn reflects the conversion of fibrinogen to a fibrin clot. In the studies described the minimum value of the first derivative (Min1), defining the maximum velocity of change in light transmission achieved (point 'c' – Fig. 1), was calculated as an indicator of the maximum velocity of coagulation achieved at any given concentration of FVIII or IX. The second derivative of the transmittance data (d^2T/dt^2) reflects the acceleration of the reaction at any given time point. The minimum value of the second derivative (Min2), measured at point 'b', was also calculated as an index of the maximum acceleration of the reaction achieved. This occurs at the time that clotting is initiated and is the trigger for determining the clot time in the MDA-II® Hemostasis System [2].

Thrombin generation assay

The measurement of thrombin generation was performed essentially according to the method of Hemker *et al.* with minor modification [8,9]. Defibrinated test and control plasma samples were prepared by treatment with reptilase (Zeria Pharmaceutical Co., Tokyo, Japan). Each was diluted with synthetic phospholipids and 20 pM recombinant tissue factor (TF; American Diagnostica, Stanford, CT, USA) and incubated for 5 min at 37 °C. Thrombin generation was initiated by the addition of 50 mM $CaCl_2$ together with the chromogenic substrate for thrombin (S-2238; Chromogenix, Milan, Italy). Absorbance at 405 nm was measured every 30 s on a DU-640 spectrophotometer (Beckman, Fullerton, CA, USA). Purified α-thrombin (Sigma, St Louis, MO, USA) was serially diluted and used to prepare a standard curve as previously described by Hemker *et al.* [8,9]. The peak thrombin generation rate (peak height, PH) and the time to peak (TTP) were obtained from this curve (Fig. 2).

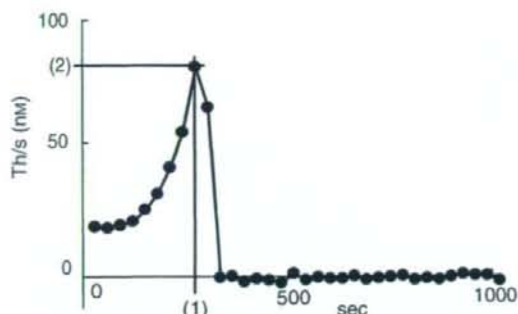

Fig. 2. Thrombin generation parameters of normal plasma. (1) Time to peak (s; TTP); (2) peak thrombin generation rate [nM; peak height (PH)].

Statistical analysis

The significance of the differences between the clot waveform and thrombin generation parameters were determined by paired Student's t-test analysis of the results obtained from multiple measurements ($n = 10$) on samples with varying levels of FVIII:C or FIX:C prepared by the addition of FVIII- or FIX-deficient plasma. The correlation between parameters was evaluated by Pearson's correlation coefficient.

Results

Correlation between quantitative clot waveform analysis and thrombin generation

In order to evaluate whether the clot waveform parameters measured reflect the total thrombin generated at any given level of FVIII or IX, the clot waveform parameters, clotting time, Min1 and Min2 and the thrombin generation parameters, TTP and PH were measured in plasma samples with known concentrations of FVIII or IX and the correlation coefficients between the results obtained for each data set determined (Table 1). In FVIII-deficient plasma, good correlation between clot waveform parameters and thrombin generation parameters was obtained in both range of dilution of FVIII. However, in the case of FIX the correlation was also good, in the case of the high range of FIX dilution.

The effects of varying concentrations of FVIII on clot waveform analysis and thrombin generation in hemophilia A plasma

Recombinant FVIII was added to congenital FVIII-deficient plasma to achieve test samples with known levels of FVIII. Initially, samples with final concentrations from 1.0 to 100.0 IU dL^{-1} were prepared and waveform analysis carried out and transmittance data obtained during the performance of

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Table 1 Correlation between clot waveform parameters and thrombin generation parameters

	Thrombin generation	
	TTP	PH
FVIII (0–100 IU dL^{-1})		
Clot time	0.8078	−0.8153
Min1	−0.9084	0.9190
Min2	−0.8551	0.8642
FVIII (0–1 IU dL^{-1})		
Clot time	0.9860	−0.8950
Min1	−0.9966	0.8636
Min2	−0.9983	0.8721
FIX (0–100 IU dL^{-1})		
Clot time	0.8975	−0.9064
Min1	−0.9292	0.9237
Min2	−0.8608	0.8372
FIX (0–1 IU dL^{-1})		
Clot time	0.6523	−0.8285
Min1	−0.7008	0.8115
Min2	−0.6746	0.8073

TTP, time to peak; PH, peak height.

a modified aPTT assay on the MDA-II® Hemostasis System. Changes in the clot waveform followed the calculated FVIII concentration in a dose-dependent manner (Fig. 3A). The clotting times shortened and the slope of the waveform became steeper as the concentration of FVIII was increased. Notably, the waveform was substantially modified toward normality by the addition of only 1.0 IU dL^{-1} FVIII. Similarly, thrombin generation, measured on the same samples, also showed a dose-dependent response to the concentration of FVIII with the PH increasing and the TTP shortening as the concentration was increased (Fig. 3B). However, qualitatively the thrombin generation curve appeared to be less sensitive to the effect of low concentrations of FVIII, i.e. 1.0 IU dL^{-1}, than the clot waveform patterns. In order to explore this apparent difference in sensitivity in more detail, further dilutions of rFVIII in deficient plasma with a lower range of values (0–1.0 IU dL^{-1}) were prepared and tested in both assay systems. In the case of the waveform assay, multiple measurements ($n = 10$) of clot time, Min1 and Min2 were made at each FVIII dilution and the difference between the mean values obtained for each parameter tested for statistical significance. An identical approach was taken with the thrombin generation assay using the measurements of TTP and PH in the analysis. Again the clot waveform profile was improved in dose-dependent manner even at very low concentrations of FVIII (Fig. 4A). Moreover, The differences between the FVIII concentration intervals 0–0.2, 0.2–0.5, and 0.5–1.0 IU dL^{-1} were all statistically significant ($P < 0.01$ – Student's t-test) for all three parameters measured (Table 2). Although thrombin generation was also improved, with the TTP shortening in a dose-dependent manner, the peak values did not appear to change substantially over this range of FVIII concentrations (Fig. 4B). In addition, although the differences in TTP and PH were significant for the concentration intervals 0–1.0 IU dL^{-1} ($P < 0.01$), these were not significant for the intervals 0–0.2, 0.2–0.5, and 0.5–1.0 IU dL^{-1} ($P > 0.05$; Table 3). These results suggest that the clot waveform profile mirrors the rate of thrombin generation measured but is more sensitive than this assay in measuring very low levels of FVIII. Of particular significance is that waveform analysis appears to be capable of discriminating differing levels of FVIII that cannot be detected by current one-stage clotting or chromogenic assays, i.e. <1.0 IU dL^{-1}.

The effects of varying concentrations of FIX on clot waveform analysis and thrombin generation in hemophilia B plasma

Similar experiments to those performed with FVIII in hemophilia A plasma were performed with the addition of varying concentrations of highly purified FIX to hemophilia B

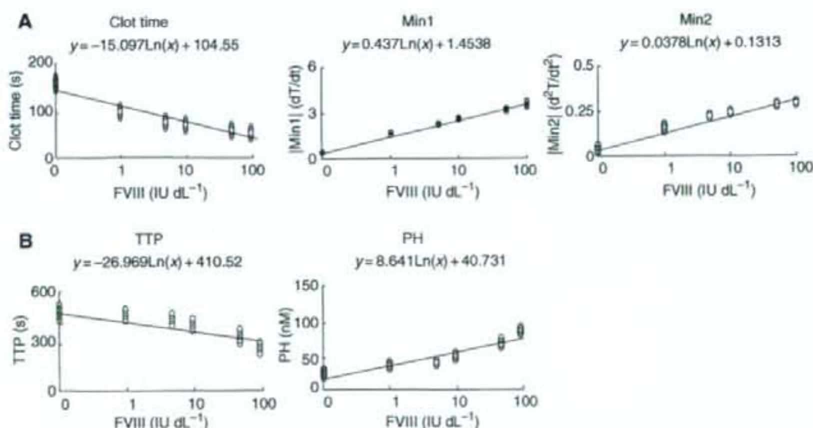

Fig. 3. Effects of addition of increasing concentrations of recombinant FVIII- to FVIII-deficient plasma on clot waveform and thrombin generation (FVIII 0–100 IU dL^{-1}). (A) Clot waveform parameters; (B) thrombin generation parameters.

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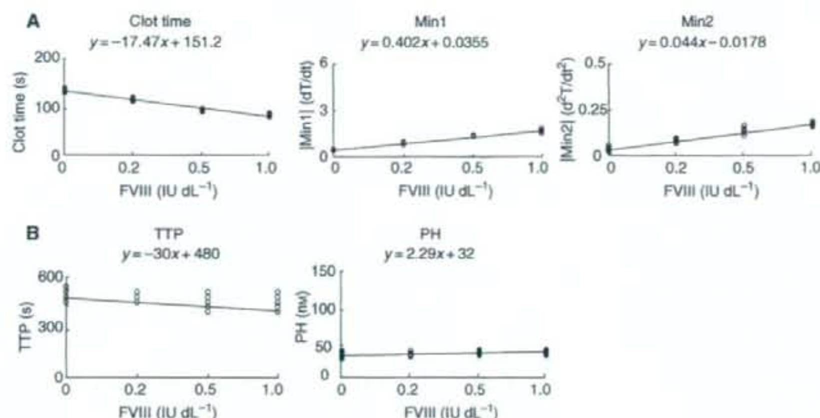

Fig. 4. Effects of addition of increasing concentrations of recombinant FVIII- to FVIII-deficient plasma on clot waveform and thrombin generation (FVIII 0–1.0 IU dL^{-1}). (A) Clot waveform parameters; (B) thrombin generation parameters.

Table 2 Changes in waveform parameters at varying concentrations of FVIII and FIX

	0.0	0.2	0.5	1.0
Factor VIII (IU dL^{-1})				
Clot time (s)*	136.5	114.3	94.4	84.9
Min1 (%T s^{-1})*	0.4012	0.8723	1.2803	1.6037
Min2 (%T s^{-2})*	0.0264	0.0686	0.1200	0.1570
P-value†	← < 0.01 →	← < 0.01 →	← < 0.01 →	
Factor IX (IU dL^{-1})				
Clot time (s)*	114.8	95.5	84.7	76.9
Min1 (%T s^{-1})*	1.4645	2.2724	2.7634	3.2189
Min2 (%T s^{-2})*	0.1367	0.2210	0.2623	0.3022
P-value†	← < 0.01 →	← < 0.01 →	← < 0.01 →	

*n = 10.
†Student's *t*-test.
T, transmittance.

Table 3 Changes in thrombin generation parameters at varying concentrations of FVIII and FIX

	0.0	0.2	0.5	1.0
Factor VIII (IU dL^{-1})				
TTP (s)*	450	420	390	360
P-value†	←——— < 0.01 ———→			
	← > 0.05 →	← > 0.05 →	← > 0.05 →	
PH (nM)*	34.1	35.5	41.6	39.7
P-value†	← > 0.05 →	← > 0.05 →	← > 0.05 →	
Factor IX (IU dL^{-1})				
TTP (s)*	390	390	390	360
P-value†	←——— < 0.01 ———→			
	← > 0.05 →	← > 0.05 →	← < 0.01 →	
PH (nM)*	36.0	38.8	40.5	44.4
P-value†	←——— < 0.01 ———→			
	← > 0.05 →	← > 0.05 →	← < 0.05 →	

*n = 10.
†Student's *t*-test.
TTP, time to peak; PH, peak height.

plasma. Both clot waveform and thrombin generation rate curves showed a dose-dependent relationship to FIX concentrations in the range of 1.0–100.0 IU dL^{-1} (Fig. 5A,B). In comparison with the waveform pattern of response to FVIII, the changes in the slopes were progressively steeper as the concentration of FIX was increased. Actual measurement of the waveform parameters Min1 and Min2 confirmed this impression with higher values for each being observed for FIX at equivalent concentrations of FVIII. In each case the differences were statistically significant ($P < 0.01$). In this range of FIX concentrations (1.0–100.0 IU dL^{-1}) the thrombin generation curve showed the same dose dependent response to that observed with FVIII. However, when samples with FIX concentrations in the range of 0–1.0 IU dL^{-1} were tested, no dose–response was noted in the thrombin generation assay (Fig. 6B) and the differences between the mean values of multiple determinations ($n = 10$) of both TTP and PH at each concentration were only significant for the intervals 0.5–1.0 and 0–1.0 IU dL^{-1} for TTP and 0.5–1.0 and 0–1.0 IU dL^{-1} for PH (Table 3). In contrast, a clear dose–response waveform profile could be seen for each concentration of FIX (Fig. 6A) and the differences observed between the mean values of multiple determination ($n = 10$) of clot time, Min1 and Min2 at each concentration were all statistically significant ($P < 0.01$) for each intervals (Table 2).

Discussion

The laboratory assessment of FVIII and FIX activity is a basic requirement for the diagnosis and management of hemophilias A and B. Indeed, by convention, stratification of the expected clinical severity with regard to bleeding is based on the determination of the baseline clotting factor activity. Although there is generally a good correlation between the levels recorded and clinical severity, occasionally discrepancies are reported. Unfortunately, because of the difficulty in quantifying clinical

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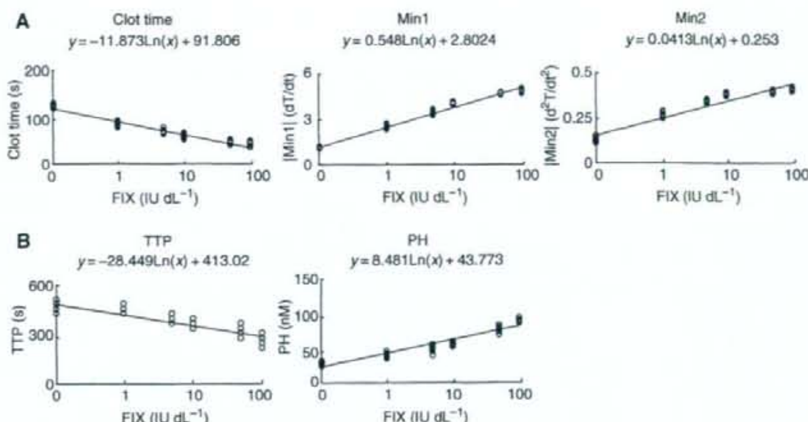

Fig. 5. Effects of addition of increasing concentrations of affinity purified FIX- to FIX-deficient plasma on clot waveform and thrombin generation (FIX 0–100 IU dL^{-1}). (A) Clot waveform parameters; (B) thrombin generation parameters.

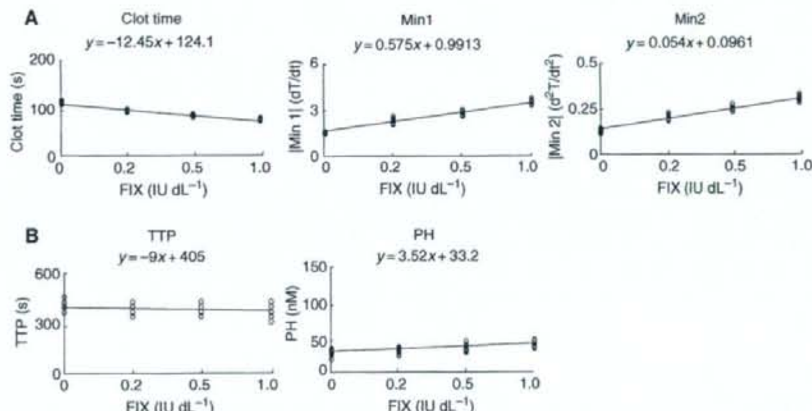

Fig. 6. Effects of addition of increasing concentrations of affinity-purified FIX- to FIX-deficient plasma on clot waveform and thrombin generation (FIX 0–1.0 IU dL^{-1}). (A) Clot waveform parameters; (B) thrombin generation parameters.

parameters of severity, such observations remain anecdotal. In addition, the methodology for the quantification of clotting factor activity is far from ideal. Apart from the usual issues of pre-analytical and analytical variables, it has been questioned whether the methods in general use reflect the full basal hemostatic potential and its modification by therapy. A particular disadvantage of current assay systems is their inability to measure very low levels of clotting factor activity. Although anecdotal, there is clear evidence that the concentration of clotting factors required to maintain relatively normal hemostasis is in the very low range. This either cannot be measured or measured only with difficulty. Consequently, priority should be given to developing and evaluating laboratory methods that increase both the specificity and sensitivity of quantifying clotting factor activity in the hemophilias. Two such approaches are the determination of thrombin-generating potential and clot waveform analysis. As thrombin is the terminal enzyme of the coagulation cascade in which FVIII and FIX are essential participants, it would seem logical to measure total thrombin generation as the ultimate expression of the hemostatic deficit that would result from their deficiency. Persuasive theoretical and experimental data has been presented in support of this approach [8–10]. Application to routine clinical practice presents more difficulty but suitable methods have been developed and show promise, particularly in the management of FVIII bypass therapy [11]. In clot waveform analysis, the process of fibrin formation during the performance of routine coagulation assays such as the PT and aPTT is monitored photo-optically. Information on the velocity and acceleration of the reaction, i.e. fibrinogen to fibrin conversion, may then be derived from the data. As fibrinogen is the substrate for thrombin, it is likely that these data also reflect

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thrombin generation. Preliminary investigations have confirmed that it appears to be an extremely sensitive method of performing qualitative and quantitative observations in patients with severe hemophilia A. In this report we have extended these observations to include hemophilia B. Parallel studies have also been performed comparing the sensitivities of the two method in measuring FVIII and IX and determining whether or not the changes in waveform pattern and parameters correlate with changes in thrombin generation at low concentrations of FVIII and IX.

The thrombin generation assay used was based on the original method of Hemker and Beguin with minor modification [4]. In order to compare the results of thrombin generation with clot waveform analysis under the same assay conditions, we used recombinant TF and $CaCl_2$ as a trigger for the reaction. At the present time there is no agreement as to the optimal approach to expressing the results obtained [12]. A number of different approaches have been suggested. Chantarangkul *et al.* have proposed that the potential should be expressed as a percentage of the reaction rate (fluoro-units min^{-1}) for the test sample vs. the value obtained with a sample of pooled normal plasma [13]. However, as the rate of thrombin generated is not constant, it is questionable whether this approach would reflect total thrombin generation. Turecek *et al.* have used peak thrombin, lag phase, peak time, and thrombin potential at 60 min [11]. Peak thrombin generation rate and the TTP were derived from the thrombin generation rate curve and the total thrombin generated and the average thrombin generation rate from the cumulative thrombin-generated curve. Clot waveform analysis was performed as described previously [3, 14] but again, in order to standardize the assay conditions used in the two systems, the aPTT reagent was diluted to provide equivalent concentrations of phospholipid to that used in the thrombin generation assay.

Excellent correlation was obtained between all the clot waveform and thrombin generation parameters in the measurement of both FVIII and FIX in the concentration range of 1.0–100.0 IU dL^{-1}. This was also observed for FVIII in the very low range (0–1.0 IU dL^{-1}). There was less correlation between the results with FIX in the very low range (0–1.0 IU dL^{-1}) but the insensitivity of the thrombin generation assay to these very low levels of FIX clearly contributed to this. The statistically significant differences observed in the waveform parameters Min1 and Min2 at any given concentration of FVIII vs. FIX are interesting and warrant further investigation. Given affinity-purified plasma derived rather than rFIX was used and compared in identical experiments using rFVIII, we considered the possibility that the differences may reflect this choice. However, identical results were obtained when affinity-purified FVIII was substituted for rFVIII in the studies described (data not shown). Thus, it appears more likely that the differences reflect the differing roles that the two clotting factors, one a serine protease and the other a co-factor, in the development of tenase activity [10].

Thus, these data confirm that clot waveform analysis does accurately reflect thrombin generation in a dynamic way. Moreover, it should be emphasized that these data are derived from a routine assay, the aPTT, performed on citrated rather than defibrinated plasma. This has major implications in substantially simplifying and facilitating the collection of such information in routine clinical practice. Furthermore, clot waveform analysis appeared to be more sensitive than the thrombin generation assay for the detection of the hemostatic effect for levels of FVIII or FIX at or < 1.0 IU dL^{-1} either by qualitative examination of the curves or quantification of the parameters used.

Given that these studies were performed on plasma specimens, which were contrived to have varying levels of either FVIII or FIX, caution should exercised in making direct extrapolations to monitoring these clotting factors in clinical practice. Nonetheless, these observations do suggest that clot waveform analysis may be very useful in improving the precision by which a hemophiliac is assigned to any particular category of severity. It may also be particularly useful in monitoring the response to therapy where relatively small increments in factor level, immeasurable by current routine assays, may exert a clinically significant effect. Kasuda *et al.* recently reported the utility of clot waveform analysis in monitoring the hemostatic benefit of continuing with the use of FVIII concentrates in patients with FVIII inhibitors who would otherwise been treated with FVIII bypassing agents [15].

In conclusion, good correlation of the changes in clot waveform with thrombin generation, particularly in the case of FVIII, has been demonstrated. The method is sensitive to very low levels of both factors and useful qualitative and quantitative information can be obtained without excessive pre-analytical manipulation of the sample and during the performance of a commonly used routine coagulation test, the aPTT. It is suggested that the application of this methodology should increase our understanding of the clinical significance of factors levels that could not be measured previously and thus improve the management of hemophiliacs, particularly in the areas of inhibitor and gene therapy.

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LETTER TO THE EDITOR

Higher recovery of factor VIII (FVIII) with intermediate FVIII/von Willebrand factor concentrate than with recombinant FVIII in a haemophilia A patient with an inhibitor

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The first line of therapy for acute bleeding in patients with low-responding factor VIII (FVIII) inhibitors is FVIII concentrates (1). Most inhibitors, recognizing the FVIII light chain, inhibit von Willebrand factor (VWF) and phospholipid binding to FVIII (2,3), and appears to be less active *in vitro* against plasma-derived FVIII concentrates containing VWF (pdFVIII/VWF) than VWF-free FVIII concentrates (4–7). These findings suggest that pdFVIII/VWF might be therapeutically more effective than recombinant FVIII (rFVIII) in patients with FVIII inhibitors. However, no clinical studies supporting this concept have been reported. In the present study, we have compared the recovery of FVIII activity (FVIII:C) after treatment with pdFVIII/VWF and rFVIII for massive intramuscular bleeding that occurred during regular infusion of FVIII for immune tolerance induction (ITI) therapy in a young male haemophilia A patient with an inhibitor.

Immune tolerance induction therapy was commenced in our patient at the age of 9 years (18 October 1999) with the administration of 100 U kg^{-1} of rFVIII (Recombinate™; Baxter Healthcare Corp., Westlake Village, CA, USA) daily for 3 weeks, followed by infusions three to four times a week. Inhibitor levels fluctuated for 3 years after ITI therapy were initiated (maximum inhibitor level, 152.0 BU mL^{-1}) and regular infusions of FVIII were continued. The number of bleeding episodes appeared to decline, and since January 2003, the inhibitor level has been kept constant within a low range from 0.9 to 2.1 BU mL^{-1}. He was admitted into our hospital with severe pain in his right buttock and walking difficulties on 6 January 2004. He had suffered from painful swelling in his right buttock 2 weeks before admission without improvement in spite of daily infusions of FVIII (100 U kg^{-1}). A subcutaneous haematoma (7 × 8 cm) was evident on the right buttock, with heat sensation, and impaired flexion and extension of the right hip joint. Computer tomography scanning demonstrated a massive intramuscular haematoma, measuring 10 cm in diameter, in the right gluteus maximus and gluteus medius muscles. On admission, 12 h after infusion of 4000 U (87 IU kg^{-1}) of rFVIII, the FVIII inhibitor titre was 1.7 BU mL^{-1}. As the bleeding manifestations had not responded to the infusion of rFVIII, 4000 U of activated prothrombin complex concentrate (Feiba Immuno™; Baxter Healthcare Corp.) were administered. Nevertheless, the swelling and pain in the right buttock increased. Subsequently, replacement therapy with the same dose of rFVIII (4000 IU) was administered and continued (Fig. 1). Clinical symptoms gradually improved and the patient was discharged on 30 January. Regular prophylaxis in this patient is now maintained using FVIII/VWF concentrate.

The inhibitor titre in this patient remained constant, within the range of 1.5–2.0 BU mL^{-1}, throughout the present series of investigations. Therefore, it was possible to compare the recovery

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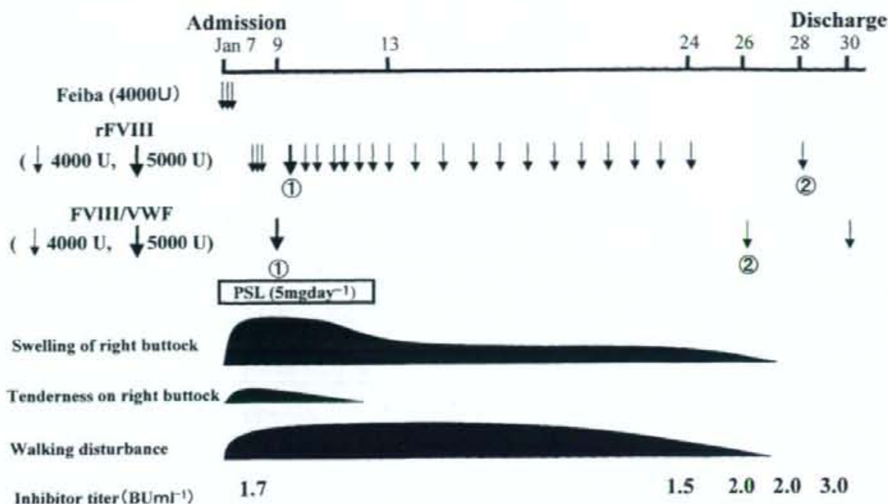

Fig. 1. Clinical course after admission and haemostatic treatment including comparative recovery tests. No. 1, the first recovery test; No. 2, the second recovery test.

of rFVIII with that of FVIII/VWF under similar conditions. For this purpose, FVIII:C recovery was assessed twice with each product during the course of replacement therapy for the intramuscular haematoma. The first comparison was performed on 9 and 11 January when the inhibitor titre was 1.7 BU mL^{-1}. The second comparison was performed on 26 and 28 January when the inhibitor titre was 2.0 BU mL^{-1}. In the first instance, 5000 U (109 IU kg^{-1}) of FVIII/VWF (Confact F^{TM}; The Chemo-Sero-Therapeutic Research Institute, Kaketsuken, Kumamoto, Japan) were compared with an identical dose of rFVIII (Recombinate^{TM}; Baxter Healthcare Corp.). On the second occasion, 4000 U (87 IU kg^{-1}) of the same products were administered. FVIII:C was measured before infusion, and at 30 min, 1 and 2 h after infusion. Levels of FVIII:C were much higher following treatment with FVIII/VWF than with rFVIII, and higher levels were maintained for at least 2 h after infusion (Fig. 2 and 3). In the second recovery test, when the inhibitor titre was slightly higher (2.0 BU mL^{-1}), initial differences were more pronounced following infusion of FVIII/VWF, although the survival time of circulating FVIII:C appeared to be diminished (Fig. 3).

These *in vivo* findings were in keeping with the suggestion that the presence of VWF or the complex formation between FVIII and VWF minimized the neutralizing activity of the inhibitor antibody. To further confirm this *in vitro*, residual FVIII:C was measured after incubation of the inhibitor with different doses of rFVIII in the absence or presence of purified VWF. The residual FVIII:C was much higher in the presence of VWF at each dose of rFVIII (Fig. 4).

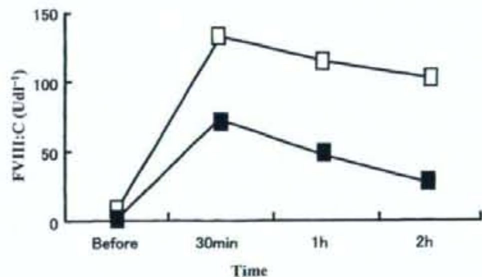

Fig. 2. The recovery of factor VIII (FVIII):C in the first test. □-□, FVIII concentrates containing von Willebrand factor complex concentrates; ■-■, recombinant FVIII concentrates.

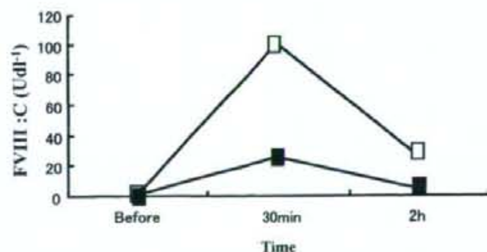

Fig. 3. The recovery of factor VIII (FVIII):C in the second test. □-□, FVIII concentrates containing von Willebrand factor complex concentrates; ■-■, recombinant FVIII concentrates.

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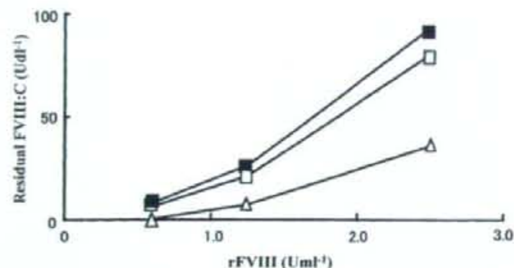

Fig. 4. The inhibitory effect of von Willebrand factor (VWF) on the anti-factor VIII:C activity of the patient's alloantibody. △-△, buffer control; □-□, in the presence of VWF at a final concentration of 5 mg mL^{-1}; ■-■, in the presence of VWF at a final concentration of 10 mg mL^{-1}.

From these data, we predicted that VWF and/or the complex formation between VWF and FVIII blocked antibody binding to FVIII. To confirm this, we developed an enzyme-linked immunosorbent assay to assess the competitive inhibitory effect of fluid phase FVIII/VWF and rFVIII on the binding of inhibitor antibody (IgG_4) to immobilized rFVIII. The addition of rFVIII in the fluid phase competitively inhibited the IgG_4 antibody binding to the immobilized rFVIII in a dose-dependent manner. Similarly, FVIII/VWF also inhibited antibody binding in a dose-dependent manner, but the effect was less marked than that of rFVIII alone (Fig. 5). We further examined if the inhibitor antibody blocked FVIII binding to VWF using the BIAcore system (Biacore AB, Uppsala, Sweden). Recombinant FVIII bound to VWF immobilized on a BIAcore CM5 tip in a dose-dependent manner. The Kd was 0.26 nM (Fig. 6a). Preincubation of 4 nM rFVIII with antibody IgG for 1 h, inhibited binding to VWF in a dose-dependent

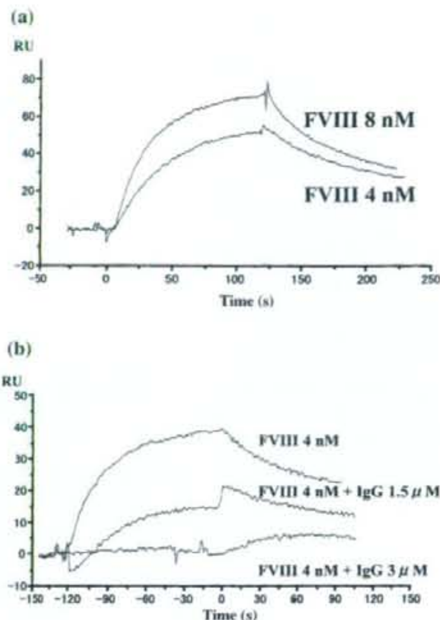

Fig. 6. The effect of the inhibitor IgG on the binding of factor VIII (FVIII) to von Willebrand factor (VWF). (a) Interaction of FVIII with VWF immobilized to BIAcore CM5 tip on surface plasmon resonance (SPR) assay. (b) The effect of the inhibitor IgG on the binding of FVIII to VWF.

manner. At a final concentration of 3 μM antibody IgG, no significant binding was observed (Fig. 6b).

The inhibitor in our present case was a typical anti-FVIII alloantibody. The major IgG subclass was IgG_4, and immunoblotting identified binding to the 80-kDa light chain of purified FVIII and the 72-kDa light chain fragment of thrombin-treated FVIII

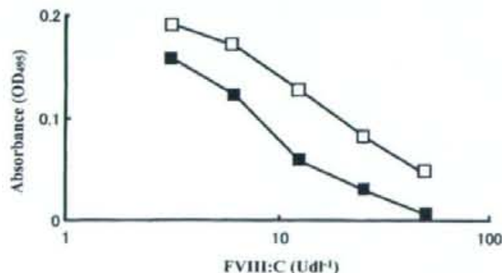

Fig. 5. The blocking effect of factor VIII concentrates containing von Willebrand factor (FVIII/VWF) or recombinant FVIII (rFVIII) on binding of the anti-FVIII IgG_4 antibody to immobilized rFVIII. □-□, FVIII/VWF complex concentrates; ■-■, rFVIII concentrates.

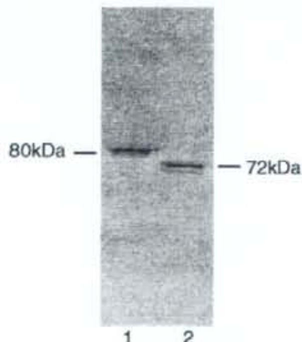

Fig. 7. Immunoblotting analysis of the inhibitor alloantibody. Lane 1, factor VIII (FVIII); lane 2, thrombin-treated FVIII.

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(Fig. 7). The VWF-binding site has been found within the FVIII light chain, and most inhibitors recognizing the light chain have been shown to inhibit FVIII binding to VWF (3,8). Several studies *in vitro* have demonstrated that the reactivity of these light-chain inhibitors is weaker against FVIII/VWF than free FVIII (4–7), and Kallas and Talpsep (7) emphasized that clinical studies were warranted to determine whether haemophilia A patients, with relatively high amounts of FVIII light-chain antibodies in their plasma, might benefit from infusions of FVIII concentrates containing VWF.

We have confirmed the *in vitro* findings and shown for the first time *in vivo* that FVIII/VWF concentrates might be superior to rFVIII for the haemostatic management of patients with the low-responding inhibitor antibodies. As these studies, our patient has received regular prophylactic treatment with intermediate FVIII/VWF concentrate. He has had no bleeding episodes other than minor subcutaneous bleeds for more than 1 year and his inhibitor titre has decreased to <1.0 BU mL^{-1}. Although FVIII/VWF concentrates are generally favoured for VWD, the present findings suggest that this type of therapeutic product should also be considered for both acute haemostatic and prophylactic treatment in the haemophilia A patients with inhibitor.

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ORIGINAL ARTICLE

Naked plasmid DNA transfer to the porcine liver using rapid injection with large volume

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The naked plasmid DNA transfer method of rapid injection with large volume has been useful for gene therapy in experimental study. However, only small animals like rodents have usually been reported on. In this study, the authors attempted to transfect naked plasmid DNA to the porcine liver by modified hydrodynamic method. We decided to transfer plasmid DNA to a part of the liver using the angiocatheter to reduce the liver damage. To discern the condition of injection, naked plasmid DNA-encoding green fluorescent protein (GFP) was transferred for use as a marker gene. The GFP gene expression was markedly observed in gene-transferred pig livers. In large animals, not only the naked gene quantity, the solution volume containing the plasmid DNA and the injection speed, but also the additional treatments of the portal vein and the hepatic artery preparation were crucial. We found that the following injection condition were needed: plasmid DNA, 3 mg; the solution volume, 150 ml and the injection speed, 5 ml/s. The portal vein and the hepatic artery were clamped during gene delivery and the blood flow of the portal vein was flushed out using normal saline. Cytotoxic T-lymphocyte antigen 4-immunoglobulin (CTLA4-Ig) gene was used to test for secretory protein. CTLA4-Ig gene was injected with a large volume of solution via the hepatic vein to the left outer lobe of the liver selectively. CTLA4-Ig was detected in the pig blood at a maximum serum level of 161.7 ng/ml 1 day after gene transfer, and the CTLA4-Ig was detected for several weeks. Our new technique of inserting a catheter into only a selected portion of the liver reduced liver toxicity and increased gene transfer efficiency. This is the first report of successful gene transfer, using a hydrodynamic method, to the segmental liver in pigs, and achieved more than enough secretory protein for the clinically therapeutic level in pigs.
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Keywords: *gene transfer; plasmid DNA; hydrodynamic; porcine; liver; CTLA4-Ig*

Introduction

Non-viral gene transfer is a useful technique to reduce the side effects of virus vector.[1] The hydrodynamic method has shown that plasmid DNA can be injected rapidly into blood vessels with a large volume of solution, with high-level gene expression to the liver[2–4] and skeletal muscle[5–7] reported. The hydrodynamic method is an easy and useful technique for gene experiments. Also, the mechanism of hydrodynamic gene transfer was elucidated as the membrane defect or the hydropore induced by hydropressure.[8,9] However, the hydrodynamic method has a major side effect of heart failure because of the large volume of solution. Therefore, many studies have examined rodents, but only a few have been carried out on animals larger than rodents, such as rabbits.[10] One report described dogs,[11] but it concentrated mainly on rodent data and dogs were not discussed in detail. There has been no effective report discussing animals larger than rabbits, so the hydrodynamic method has been considered only as an experimental method. Recently, in an electronic journal, Herreo *et al.*[12] reported hydrodynamic gene transfer to the pig whole liver. This study was very interesting as it was the first report of hydrodynamic gene transfer to a large animal like a pig, but the efficiency of the gene transfer was three orders lower than their study in mice. Therefore, the problem of effective gene transfer to pigs using hydrodynamic gene delivery still remained.

In previous studies, we reported that we used a catheter to reduce the volume of solution in the rat hydrodynamic procedure; we transferred cytotoxic T-lymphocyte antigen 4-immunoglobulin (*CTLA4-Ig*) gene to the rat liver and showed the transplantation data;[13] and we transferred some genes to the rat limb and performed limb transplantation.[14] Furthermore, we successfully transferred the small interfering RNA to the transgenic rat liver and limbs.[15] The hydrodynamic method has been a useful technique for experimental gene transfer, but it has not been accommodated to clinical gene transfer. In this study, we tried to transfer plasmid DNA to the pig liver using the hydrodynamic method with some additional techniques.

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Results

The conditions of injection

The green fluorescent protein (GFP) gene was used as a reporter gene to confirm the conditions for the hydrodynamic method in pigs. The gene expression of the hydrodynamic gene transfer method depended on the gene quantity, the solution volume and the injection speeds.[4,9] In our rat study, the best conditions for the quantity of naked gene was 125 μg with solution (2.5% of the body weight) per rat.[13] However, the partial liver (half of the left outer lobe) in pigs was 8–12 times heavier than the whole liver in rats; therefore, for the same condition in pigs the quantity of naked gene was about 1–1.5 mg and the volume of solution was 60–90 ml per pig. However, in the pig experiment, balloon occlusion was not stanched completely; therefore, the solution and the naked gene were expanded about two times, so that we used 150 ml solution and 3 mg plasmid DNA per pig, respectively, in this study. One hundred and fifty milliliters of normal saline containing 3 mg GFP-expressing gene was injected rapidly by hand or with a power injector. We occluded vessels such as the hepatic artery and the portal vein. Additionally, we infused 200 ml of normal saline to flush out the liver blood. These occlusion of the hepatic artery and the portal vein and the flushing out of the blood from the liver were important techniques and are talked about in detail in the next section.

In injecting the solution by hand within a maximum of 60–80 s, only a little GFP expression was observed. Therefore, the power injector was an essential tool. Injection speeds of 20, 15, 10, 5 and 3 ml/s were tested, and the injection times were 10, 16, 17.5, 32.5 and 52.5 s, respectively, because in the first 5 s we set the 'rising time' so that the injection speed gradually increased. At 20 ml/s, the pig liver burst upon injection. At 15 and 10 ml/s, the livers were broken, with bleeding under the liver capsula and detachment of the vascular endothelium, respectively. At 3 ml/s, GFP expression was not found. However, at 5 ml/s, GFP expression was observed. Therefore, the best injection speed for pig liver was 5 ml/s.

In hydrodynamic gene delivery, a suitable gene quantity was present. With less than the suitable gene quantity, we found little or no gene expression. With more than the suitable gene quantity, gene expression was almost identical. We compared the delivery of 3 mg of plasmid DNA and 1 mg of plasmid DNA. In the 3 mg study, GFP expression was well observed but in the 1 mg study, only limited GFP expression was observed. And also, we compared the delivery of more than 3 mg of plasmid DNA, but gene expression was almost identical to that in the 3 mg study. Therefore, the gene quantity we decided to use was 3 mg.

The solution volume was also important in hydrodynamic gene transfer. We tried 100 and 150 ml solutions containing 3 mg plasmid DNA of GFP, respectively. In the examination of 100 ml solution, little GFP was observed, but in the 150 ml solution, GFP expression was well defined. We could not try a higher volume of solution because our power injector was limited to a maximum of 150 ml.

We decided that the condition for the hydrodynamic method in pigs was 3 mg plasmid DNA containing 150 ml normal saline and 5 ml/s high-speed injection with a power injector. Additionally, occlusion of the hepatic artery and the portal vein and flushing out of the blood from the liver were performed in this experiment. The GFP gene expression was good and identically observed in almost all of the targeted area of the pig liver lobe (Figure 1).

Surgical preparation of the pig

In rodent studies, only plasmid DNA and solution were injected via the tail vein or using a catheter with the hydrodynamic method. However, in the pig study, blood flow occlusion was necessary. We tried to inject plasmid DNA using a catheter-based hydrodynamic method similar to that in rat[13] or rabbit,[10] but no GFP expression was observed in the pig liver.

In the pig hydrodynamic gene transfer method, the blood flow of the liver was crucial. Therefore, we tested to occlude or wash out the blood of the liver. We divided the four groups as follows: 'only the portal vein clamp', 'only the left hepatic artery clamp', 'portal vein and left hepatic artery clamp' and 'both vessels clamp and wash out blood flow by 200 ml lactate linger solution from the portal vein' (Table 1). Also, the conditions of the hydrodynamic gene transfer to the pig liver were 3 mg of plasmid DNA, 5 ml/s speed injection and 150 ml solution volume. 'Only the portal vein clamp' group and 'only the hepatic artery clamp' group showed no GFP gene expression, but in the group of 'both vessels clamp' GFP gene expression was observed. However, in this group, which did not wash out the portal blood flow, GFP gene expression was limited to being around central veins (Figure 2a). And in the 'wash out the portal blood flow and clamp both vessels' group, GFP expression was observed in a greater quantity and area (Figure 2b). Therefore, the best conditions for hydrodynamic gene transfer to the pig liver were that the portal flow was occluded and the portal blood was washed out, and the left hepatic artery was also clamped (Figure 3).

Wedge pressure of the portal vein

In hydrodynamic gene transfer to the liver method, it was well recognized that the pressure of the hepatic vein was important,[9,13] so we examined to monitor the venous pressure. However, in this study, the hepatic vein of the outer left branch was not measured directly because of the intravessel turbulent flow, so we monitored the wedge pressure of the portal vein of the left outer branch as the approximate pressure (Figure 4). At 3 ml/s of injection speed, the peak pressure was 44 mm Hg (Figure 5a), and at 5 ml/s, the peak pressure was 58 mm Hg (Figure 5b).

CTLA4Ig expression in pigs

Next, we examined the serum level of gene expression using pCAG-CTLA4Ig. The conditions for hydrodynamic injection were the same as in the experiment of GFP gene transfer, the solution volume was 150 ml, CTLA4Ig gene was 3 mg and injection speed was 5 ml/s (32.5 s), and with clamping the hepatic artery and the portal vein and washing out the liver blood. The serum CTLA4Ig level in the pig peripheral blood obtained on days 0, 1, 2, 4, 7, 14 and 21 thereafter was measured by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 6, in No. 1 a remarkably high level of CTLA4Ig