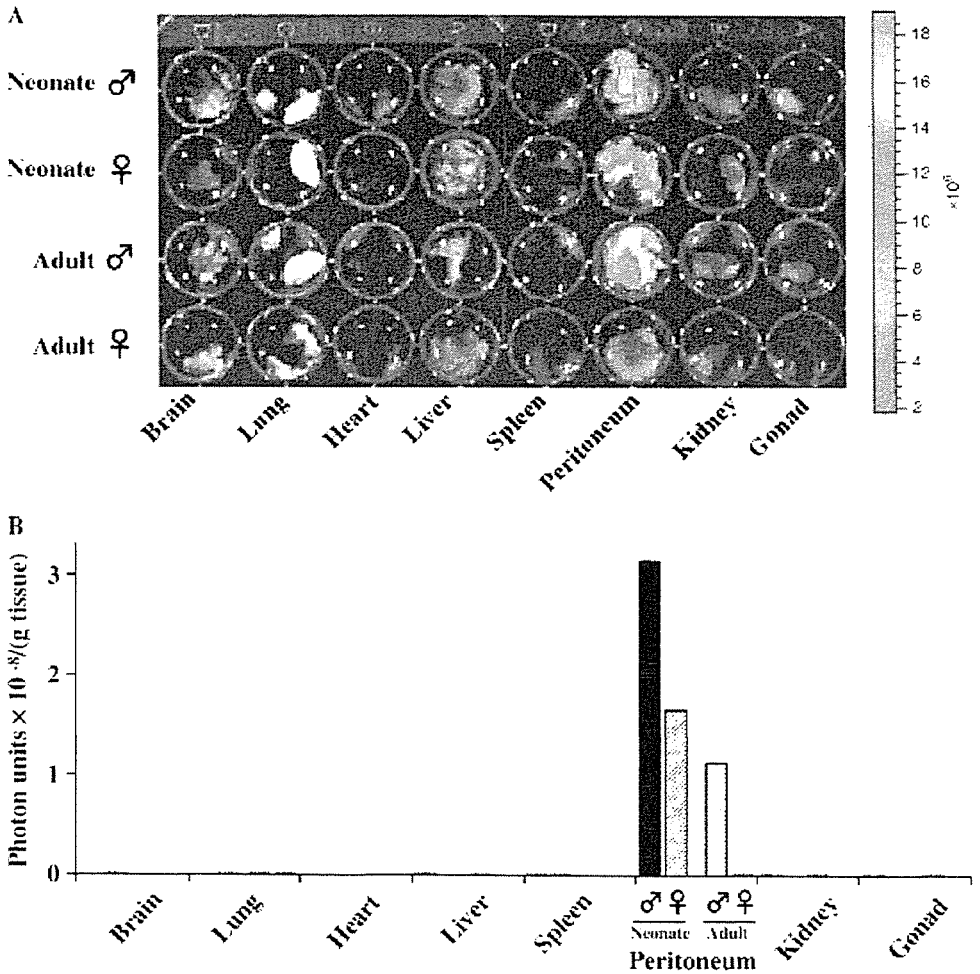

Figure 5. Analysis of tissue-specific expression after *ip* injection of the AAV5-CMV-Luc vector. (A) *Ex vivo* bioluminescence images of injected neonates and adults are shown. Mice were sacrificed at 10 weeks after vector injection and the major organs were extracted and placed into each well of a 24-well dish containing luciferin substrate solution in order to measure the individual bioluminescence. (B) Quantitative results of transgene expression are as indicated in (A). The ordinate shows the photon units (photons/s)

Discussion

In this study, we tested the utility of neonatal gene transfer by using AAV5-based vectors. All genes tested – *lacZ*, *hfIX*, and *luc* – demonstrated robust transgene expression after *ip* injection. The advantage of neonatal gene transfer was clearly demonstrated by the plasma hFIX levels after injecting both adult and neonatal mice with equivalent doses of the AAV-CMV-hFIX vector (3×10^{11} g.c./g). Throughout the observation period, a higher hFIX concentration was detected in neonates than in adults; therapeutic levels of hFIX were maintained even after maturation (Figure 2). Another comparison using vectors encoding luciferase at an equivalent vector dose also resulted in a higher transgene expression in neonates (Figure 4). These data support the advantages of neonatal gene transfer.

Neonatal gene delivery in mice is technically difficult due to their size. In this study, we demonstrated the usefulness of *ip* injections as a route of vector delivery. On the other hand, we did not include the *im* route in this series of experiments because the injection volume was strictly limited in neonates. However, this latter method is apparently an attractive route of administration in clinical applications. Therefore, the efficacy of *im* administration requires further analysis in larger animal models.

In this study, transgene expression was mostly confined to the peritoneum after *ip* injection into neonates. This was confirmed by different modes of detection. In addition, the vector genome distribution was mostly comparable to the level of transgene expression. However, in a previous report, transgene expression was also observed in tissues other than the peritoneum when fetuses were injected [17]. Since the vector system and the promoter were the same, the difference in tissue distribution may be related to the age at the time of injection, vector dose, technical details, or other unrecognized factors. At present, the mechanism responsible for tissue specificity is not clear. The abundance of receptor molecules, such as platelet-derived

growth factor (PDGF) receptors [27], may contribute to this phenomenon. Using other vector systems may result in different tissue specificity. Recently, transgene expression in the whole peritoneal cavity was observed by *ip* administration of polyethylenimine (PEI)/DNA complexes [28]. Further, in neonates, a long-term expression was observed in factor IX concentration, whereas in adult males a sharp decrease was observed at 12 weeks and later (Figure 3). When the peritoneum was analyzed, only the surface epithelium of the peritoneal tissue was transduced (Figure 4E), and it appeared to be responsible for continuously supplying the transgene product at a therapeutic level. These cells contain an extremely high copy number of transgenes even after a prolonged period of time (Figure 2C). The copy number of the vector genome within the peritoneum appears to be underestimated thus far because the whole peritoneal tissue was used for DNA extraction prior to Q-PCR. The presence of an extremely high copy number of vector genomes within the peritoneum is possibly related to the robust and persistent transgene expression in neonatal gene transfer. The mechanism for the persistence of high copy number and transgene expression is interesting and may offer important insights into the biology of the AAV vector.

Interestingly, a sex-related difference in transgene expression within the peritoneal tissues was observed after *ip* injection into adult mice regardless of the transgene. In a previous study, a sex-related difference in transgene expression was demonstrated in the liver, and an androgen-dependent pathway appeared to be involved [25,26]. We have also demonstrated an overwhelming sex-related difference in liver transduction efficiency in a mouse model [19]. Based on our knowledge, this is the first report that demonstrates a sex-related difference in transgene expression in tissues other than the liver. At present, it is not clear whether the same mechanism is involved in the peritoneal tissue. The difference may be a drawback when an attempt is made to transfer genes into females. However, our results indicate that this problem can be circumvented if neonates are targeted for gene therapy.

Neonatal gene transfer is also advantageous from an immunological point of view. Due to the immaturity of the neonatal immune system, tolerance to an 'immunogenic' transgene product can be induced. Recently, neonatal and fetal gene transfer experiments using adenoviral and retroviral vectors demonstrated the induction of tolerance to transgene products [14,15]. In our series of experiments, it is difficult to prove this point because all transgenes were expressed for a long period even in adults. Nonetheless, divergent levels of transgene expression between adults and neonates may reflect a difference in immunology, and needs to be analyzed in the future.

In conclusion, our findings support the efficacy of neonatal gene therapy and would help to design strategies for neonatal gene therapy using AAV vectors.

Acknowledgements

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Mechanisms of Plasmin-catalyzed Inactivation of Factor VIII

*A CRUCIAL ROLE FOR PROTEOLYTIC CLEAVAGE AT Arg^{336} RESPONSIBLE FOR PLASMIN-CATALYZED FACTOR VIII INACTIVATION**

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Plasmin not only functions as a key enzyme in the fibrinolytic system but also directly inactivates factor VIII and other clotting factors such as factor V. However, the mechanisms of plasmin-catalyzed factor VIII inactivation are poorly understood. In this study, levels of factor VIII activity increased ~2-fold within 3 min in the presence of plasmin, and subsequently decreased to undetectable levels within 45 min. This time-dependent reaction was not affected by von Willebrand factor and phospholipid. The rate constant of plasmin-catalyzed factor VIIIa inactivation was ~12- and ~3.7-fold greater than those mediated by factor Xa and activated protein C, respectively. SDS-PAGE analysis showed that plasmin cleaved the heavy chain of factor VIII into two terminal products, A1$^{37-336}$ and A2 subunits, by limited proteolysis at Lys^{36}, Arg^{336}, Arg^{372}, and Arg^{740}. The 80-kDa light chain was converted into a 67-kDa subunit by cleavage at Arg^{1689} and Arg^{1721}, identical to the pattern induced by factor Xa. Plasmin-catalyzed cleavage at Arg^{336} proceeded faster than that at Arg^{372}, in contrast to proteolysis by factor Xa. Furthermore, breakdown was faster than that in the presence of activated protein C, consistent with rapid inactivation of factor VIII. The cleavages at Arg^{336} and Lys^{36} occurred rapidly in the presence of A2 and A3-C1-C2 subunits, respectively. These results strongly indicated that cleavage at Arg^{336} was a central mechanism of plasmin-catalyzed factor VIII inactivation. Furthermore, the cleavages at Arg^{336} and Lys^{36} appeared to be selectively regulated by the A2 and A3-C1-C2 domains, respectively, interacting with plasmin.

Factor VIII, a plasma protein deficient or defective in individuals with the severe congenital bleeding disorder hemophilia A, functions as a cofactor in the tenase complex, which is responsible for anionic phospholipid surface-dependent conversion of factor X to Xa by factor IXa (1). Factor VIII circulates as a complex with VWF[2] that protects and stabilizes the cofactor. Factor VIII is synthesized as a single chain molecule consisting of 2,332 amino acid residues with a molecular mass of ~300 kDa (2, 3). The factor VIII molecule can be divided into three domains arranged in the order of A1-A2-B-A3-C1-C2 according to the amino acid content homology. It is processed into a series of metal ion-dependent heterodimers by cleavage at the B-A3 junction, generating a heavy chain consisting of the A1 and A2 domains, plus heterogeneous fragments of a partially proteolyzed B domain, linked to a light chain consisting of the A3, C1, and C2 domains (2–4).

The catalytic efficacy of factor VIII in the tenase complex is enhanced over 10^5 times by conversion into an active form, factor VIIIa, by limited proteolysis by either thrombin or factor Xa (5). Both enzymes cleave factor VIII at Arg^{372} and Arg^{740} of the heavy chain and produce 50-kDa A1 and 40-kDa A2 subunits. The 80-kDa light chain is also cleaved at Arg^{1689} generating a 70-kDa A3-C1-C2 subunit. Additionally, factor Xa cleaves at Arg^{1721} and produces a 67-kDa A3-C1-C2 subunit. Proteolysis at Arg^{372} and Arg^{1689} is essential for generating factor VIIIa cofactor activity (6). Cleavage at the former site exposes a functional factor IXa-interactive site within the A2 domain that is cryptic in the unactivated molecule (7). Cleavage at the latter site liberates the cofactor from its carrier protein, VWF (8), contributing to the overall specific activity of the cofactor (9, 10).

Factor VIIIa cofactor activity is down-regulated in the presence of serine proteases such as APC (5), factor Xa (5), and factor IXa (11) by proteolytic inactivation following cleavage at Arg^{336} within the A1 subunit. This inactivation appears to be the result of altered interaction with the A2 subunit and an increased K_m value of the truncated A1 for the substrate factor X (12, 13), the latter reaction reflecting loss of a factor X-interactive site within residues 337–372 (14). Factor Xa and APC also cleave at Lys^{36} (13) and at Arg^{562} (15), respectively. These events may alter the conformation of A1, limiting the productive interaction with the A2 subunit (13) and impairing the interaction with factor IXa in the tenase complex (16).

Hemostasis is further regulated by fibrinolysis. Proteolytic degradation of fibrinogen/fibrin by the serine protease, plasmin, occurs at the end stage of the blood coagulation cascade. Previous reports have suggested, however, that plasmin also regulates blood coagulation by proteolysis of several coagulation proteins, including factor Va (17, 18), IXa (19), X (20), and factor VIII (21, 22). These findings suggest that plasmin might down-regulate tenase activity by inactivating factor VIII(a), although the exact mechanism of this inactivation is poorly understood.

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[2] The abbreviations used are: VWF, von Willebrand factor; APC, activated protein C; MES, 4-morpholineethanesulfonic acid; CAPS, 3-(cyclohexylamino) propanesulfonic acid.

In this study, we report on the mechanism of plasmin-catalyzed factor VIII inactivation. Functional assays and SDS-PAGE analysis using isolated subunits of the cofactor demonstrated for the first time that inactivation was directly associated with unique, limited proteolysis that led initially to three terminal products, $A1^{37-336}$, A2, and A3-C1-$C2^{1722-2332}$, and subsequently to the crucial cleavage at Arg^{336}. Cleavage at Arg^{336} and Lys^{36} appeared to be selectively modulated following interaction of the protease with A2 and A3-C1-C2 subunits, respectively.

MATERIALS AND METHODS

Reagents—Purified recombinant factor VIII preparations were generous gifts from Bayer Corp. (Berkeley, CA). The monoclonal antibodies 58.12 (23) and C5 (24) recognizing the N- and C-terminal end of the A1 domain were gifts from Bayer Corp. and Dr. Carol Fulcher, respectively. The monoclonal antibody JR8 recognizing the A2 domain was obtained from J. R. Scientific Inc. (Woodland, CA). The monoclonal antibody NMC-VIII/10 recognizing the N-terminal end of the A3 domain was purified as described previously (25). VWF was purified from factor VIII/VWF concentrates using a gel filtration on a Sepharose CL-4B column (Amersham Biosciences) and immunobeads coated with immobilized factor VIII monoclonal antibody as reported previously (25). Enzyme-linked immunosorbent assays of factor VIII demonstrated greater than 95% purity of VWF. Phospholipid vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, and 30% phosphatidylethanolamine (Sigma) were prepared using *N*-octyl glucoside (26). Purified human Lys-plasmin was purchased from Sigma and was shown to be devoid of factor Xa and APC. Factor Xa, APC, and protein S were purchased from Hematologic Technologies Inc. (Essex Junction, VT).

Isolation of Factor VIIIa Subunits—Factor VIII (1.5 μM) was treated overnight at 4 °C in buffer containing 10 mM MES, pH 6.0, 0.25 M NaCl, 50 mM EDTA, and 0.01% Tween 20, and light and heavy chains were isolated following chromatography on SP- and Q-Sepharose columns (Amersham Biosciences) as described previously (7). Purified heavy chain was cleaved by thrombin, and the A2 and A1 subunits were purified by using Hi-Trap heparin column and Mono Q columns, respectively, as reported previously (12). A3-C1-C2 subunits were purified from thrombin-treated light chain by SP-Sepharose chromatography as described previously (27). Factor VIIIa was isolated from thrombin-treated factor VIII by CM-Sepharose chromatography (Amersham Biosciences) (28). A1/A3-C1-C2 dimers were prepared by reconstitution from isolated A1 and A3-C1-C2 subunits by incubation overnight at 4 °C in 20 mM HEPES, pH 7.2, 0.3 M NaCl, 25 mM $CaCl_2$, and 0.01% Tween 20 (13). SDS-PAGE of the isolated subunits followed by staining with GelCode Blue Stain Reagent (Pierce) showed >95% purity. Protein concentrations were determined by the method of Bradford (29).

Clotting Assay—Factor VIII(a) activity was measured in a one-stage clotting assay using factor VIII-deficient plasma (30). All reactions were performed at 22 °C. All factor VIII products were incubated in buffer (20 mM HEPES, pH 7.2, 0.1 M NaCl, 5 mM $CaCl_2$, 100 μg/ml bovine serum albumin, and 0.01% Tween 20) plus phospholipid vesicles (10 μM). Samples were removed from the mixtures at the indicated times, and plasmin reaction was immediately terminated by the addition of 0.5 mM Pefabloc SC (Roche Applied Science) and dilution. The presence of plasmin and Pefabloc in the 1000-fold diluted sample was shown not to affect factor VIII activity in the coagulation assay.

Cleavage of Factor VIII(a) and Its Subunits by Plasmin—Human plasmin was added to factor VIII(a) and its subunits at a 1:25 ratio (mol/mol) in the presence of phospholipid vesicles (10 μM) in buffer containing 20 mM HEPES, pH 7.2, 0.1 M NaCl, 5 mM $CaCl_2$, and 0.01% Tween 20 at 22 °C. Samples were taken at the indicated times, and the reactions were immediately terminated and prepared for SDS-PAGE by adding SDS and boiling for 3 min.

Electrophoresis and Western Blotting—SDS-PAGE was performed using 8% gels as described by Laemmli (31). Electrophoresis was carried out using a minigel apparatus (Bio-Rad) at 150 V for 1 h. Bands were visualized following staining with GelCode Blue (Pierce). For Western blotting, each protein sample was transferred to a polyvinylidene difluoride membrane using a Bio-Rad mini-transblot apparatus at 50 V for 2 h in buffer containing 10 mM CAPS, pH 11, and 10% (v/v) methanol. Protein bands were probed using the indicated anti-factor VIII monoclonal antibodies followed by goat anti-mouse peroxidase-linked secondary antibody (MP Biomedicals, Aurora, OH). Signals were detected using enhanced chemiluminescence (PerkinElmer Life Sciences). Densitometric scans were quantitated using Image J 1.34 (National Institute of Health, Bethesda).

N-terminal Sequence Analysis—N-terminal sequence analyses of the fragments following plasmin cleavage of factor VIII were performed using an Applied Biosystems model 491 sequencer (Foster City, CA). The plasmin-cleaved factor VIII fragments were recovered after electrophoresis and were subjected to 10 cycles of automated sequencing.

RESULTS

Factor VIII Activation and Inactivation by Plasmin—Earlier reports have described factor VIII inactivation by plasmin (21, 22); however, precise inactivation mechanisms remain unclear. We first examined plasmin-catalyzed factor VIII inactivation in the presence of phospholipid and Ca^{2+} using a one-stage clotting assay. Control experiments confirmed that the presence of either plasmin or Pefabloc did not affect these assays at ~1000-fold dilution of the reaction mixture. At concentrations of 100 nM factor VIII and 4 nM plasmin, maximum factor VIIIa activity was observed after 3 min and reflected an ~1.7-fold increase (Fig. 1). This peak activity was followed by a sharp decline to the initial level at 10 min, and finally to an undetectable level within 45 min. Similar results were observed in the absence of phospholipid and/or Ca^{2+}. Factor VIII circulates as a complex with VWF, which protects and stabilizes the cofactor from inactivation, for example, by APC (32, 33). In these experiments, however, the presence of VWF did not affect the reaction between plasmin and factor VIII.

Comparison of Factor VIII Inactivation by Plasmin, APC, and Factor Xa—The most potent known serine proteases responsible for factor VIII(a) inactivation are APC and factor Xa. There-

fore, we compared factor VIII inactivation by plasmin, APC, and factor Xa in a one-stage clotting assay. Each protease (4 nM) was incubated with factor VIII (100 nM) in the presence of phospholipid as described above. Control experiments showed that the presence of each protease alone did not affect the assays. APC mixed with protein S (1:10 molar ratio) inactivated factor VIII activity in a time-dependent manner with no initial elevation in activity (Fig. 2*A*). The activity of factor VIII incubated with factor Xa increased ~2-fold compared with that obtained after the addition of plasmin. However, the characteristic "spike" of factor VIIIa activity observed in the presence of plasmin was not observed with factor Xa. In contrast, a broad activation plateau was seen for ~15 min and was followed by a slower decrease in activity.

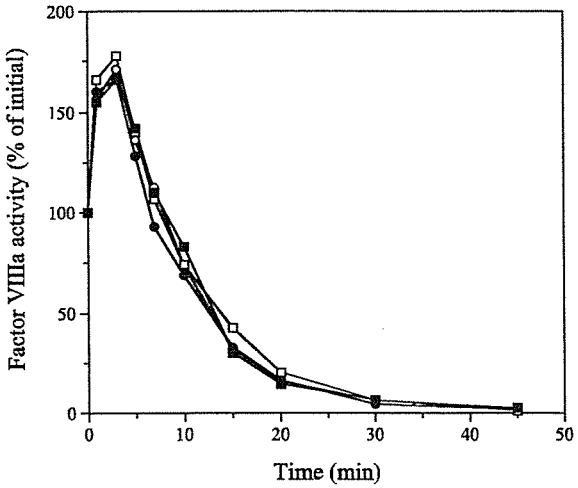

FIGURE 1. **Time course of activation of factor VIII following reaction with plasmin.** Factor VIII (100 nM) was incubated with plasmin (4 nM) in the presence (*open circles*) or absence (*closed circles*) of Ca^{2+} (5 mM) for the indicated times, after which the reaction was terminated by Pefabloc. Each sample was tested immediately for factor VIIIa activity in a one-stage clotting assay. In addition, factor VIII was preincubated with VWF (100 nM, *open squares*) or phospholipid vesicles (10 μM, *closed squares*) prior to addition of plasmin. The zero point was taken prior to addition of plasmin. The initial activity of factor VIII was ~50 units/ml and designated as 100%. Experiments were performed at least three separate times, and average values are shown.

The activity of factor VIIIa at any time point likely represents contributions from unactivated molecules, activated molecules, and activated molecules that have decayed following factor VIII subunit dissociation. To precisely evaluate the effect of inactivation of factor VIII by these enzymes, experiments were repeated using active factor VIIIa as a substrate. Each protease (4 nM) was incubated with factor VIIIa (25 nM) in the presence of phospholipid. The findings are illustrated in Fig. 2*B*. The data were fitted to a single exponential decay curve and showed that the inactivation rate of factor VIIIa activity in the absence of protease, reflecting A2 subunit dissociation from factor VIIIa trimer, was 0.015 ± 0.002 min^{-1}, similar to earlier reports (34). The rate constants of inactivation of factor VIIIa activity by plasmin, APC, and factor Xa under the same conditions were 1.11 ± 0.09, 0.30 ± 0.04, and 0.09 ± 0.01 min^{-1}, respectively, suggesting that the effect of factor VIIIa inactivation by plasmin was ~ 3.7- and ~12-fold greater than that with APC and factor Xa, respectively.

Cleavage of Factor VIII by Plasmin and Identification of Cleavage Sites—The factor VIII activation/inactivation patterns induced by plasmin that were characterized by initial mild elevation followed by a rapid reduction in activity, and the difference in inactivation potential between plasmin, APC, and factor Xa, strongly suggest that the mechanism of action of plasmin is different from those of APC and factor Xa. To provide direct evidence for this hypothesis, timed course changes in electrophoretic mobility of plasmin-treated factor VIII were studied using factor VIII (1.5 μM) and plasmin (20 nM) in the presence of phospholipid. SDS-PAGE showed that plasmin sequentially proteolyzed factor VIII at several cleavage sites (Fig. 3*A*). The ~180-kDa heavy chain fragments consisting of A1, A2, and full sequenced B domains completely disappeared at 1 min after the addition of plasmin. In contrast, the 90-kDa fragment appeared to increase initially, followed by limited proteolysis into several protein products of apparent 50, 48, 42, 40, and 38 kDa. The 50-, 48-, and 42-kDa products disappeared gradually with time. The 40- and 38-kDa fragments appeared to persist as terminal products at 30 min, in the absence of factor VIII activity. The 80-kDa light chain fragments were degraded sequentially to 70-kDa products and 67-kDa fragments within 45 min.

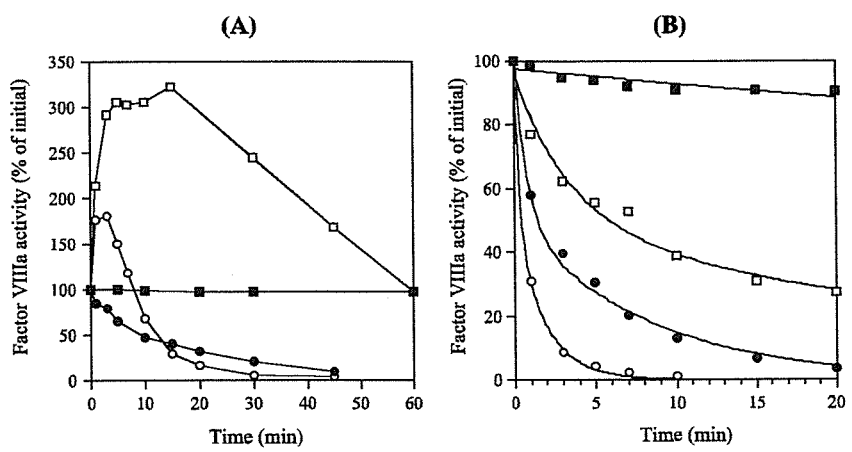

FIGURE 2. **Comparisons of the time course of factor VIII(a) inactivation by plasmin, APC, and factor Xa.** Factor VIII (*A*, 100 nM) or factor VIIIa (*B*, 25 nM) was incubated with 4 nM plasmin (*open circles*), APC (*closed circles*) together with protein S (40 nM), factor Xa (*open squares*), and buffer only (*closed squares*) in the presence of phospholipid vesicles (10 μM). Factor VIIIa activity was measured at the indicated times using a one-stage clotting assay. The initial activities of factor VIII or factor VIIIa (100% level) were ~50 or ~80 units/ml, respectively. The values of factor VIIIa activity were plotted as a function of incubation time, and the data in *B* were fitted to an equation of single exponential decay. Experiments were performed at least three separate times, and average values are shown.

To identify the sites of cleavage, all fragments were subjected to automated N-terminal sequence analysis and compared with the amino acid sequences derived from human factor VIII cDNA (2, 3). The results

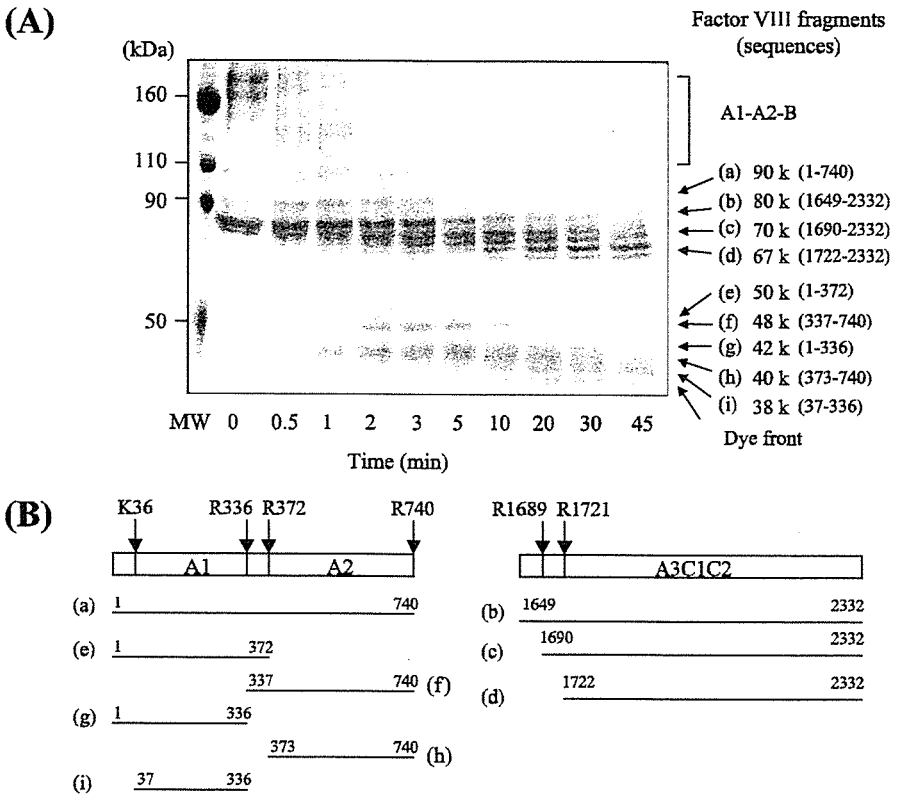

FIGURE 3. **Time course of plasmin-catalyzed proteolysis of factor VIII.** *A*, SDS-PAGE of factor VIII (1.5 μM) was incubated with plasmin (20 nM) in the presence of phospholipid vesicles (10 μM) as described under "Materials and Methods." At the indicated times, the reaction was terminated, and samples 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

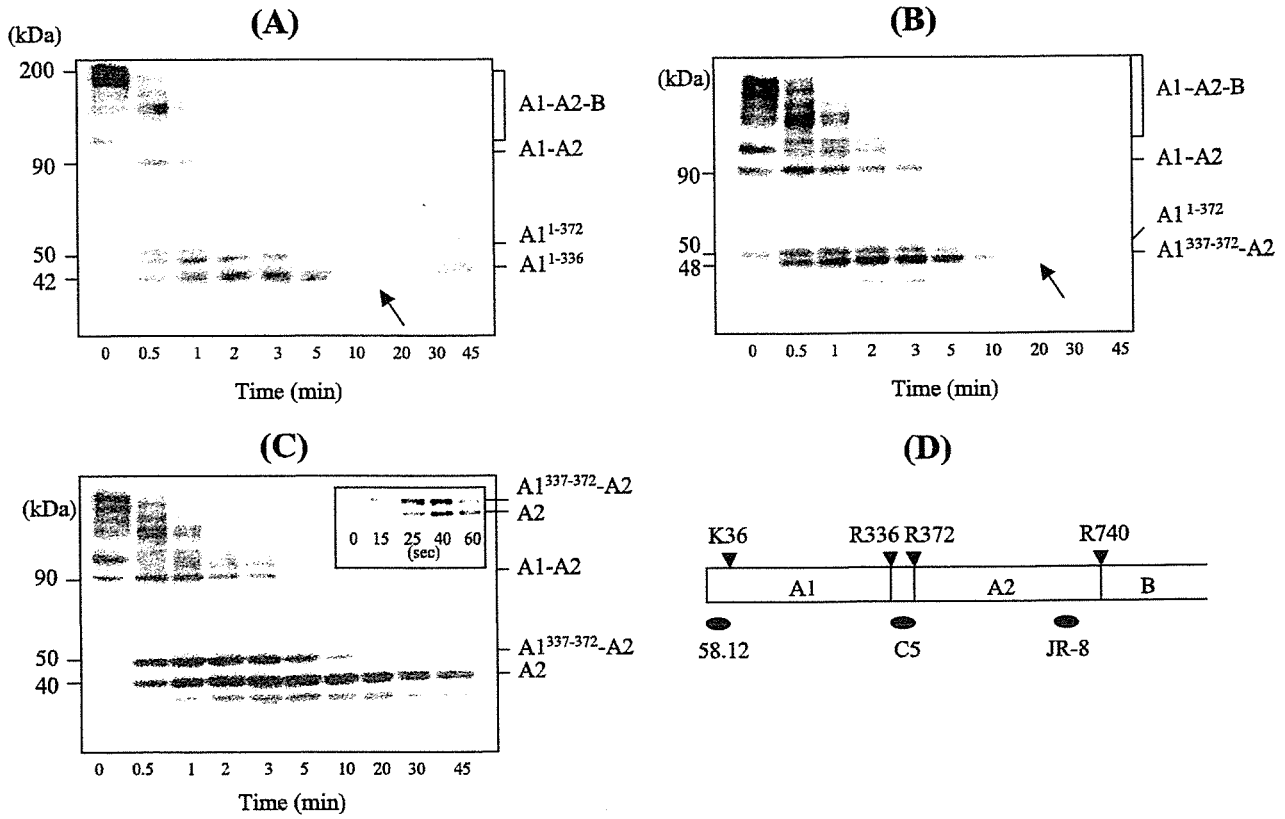

FIGURE 4. **Time course of plasmin-catalyzed cleavage of the heavy chain of factor VIII.** Factor VIII (500 nM) was incubated with plasmin (10 nM) in the presence of phospholipid vesicles (10 μM) for the indicated times as described under "Materials and Methods." Samples were run on an 8% gel followed 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

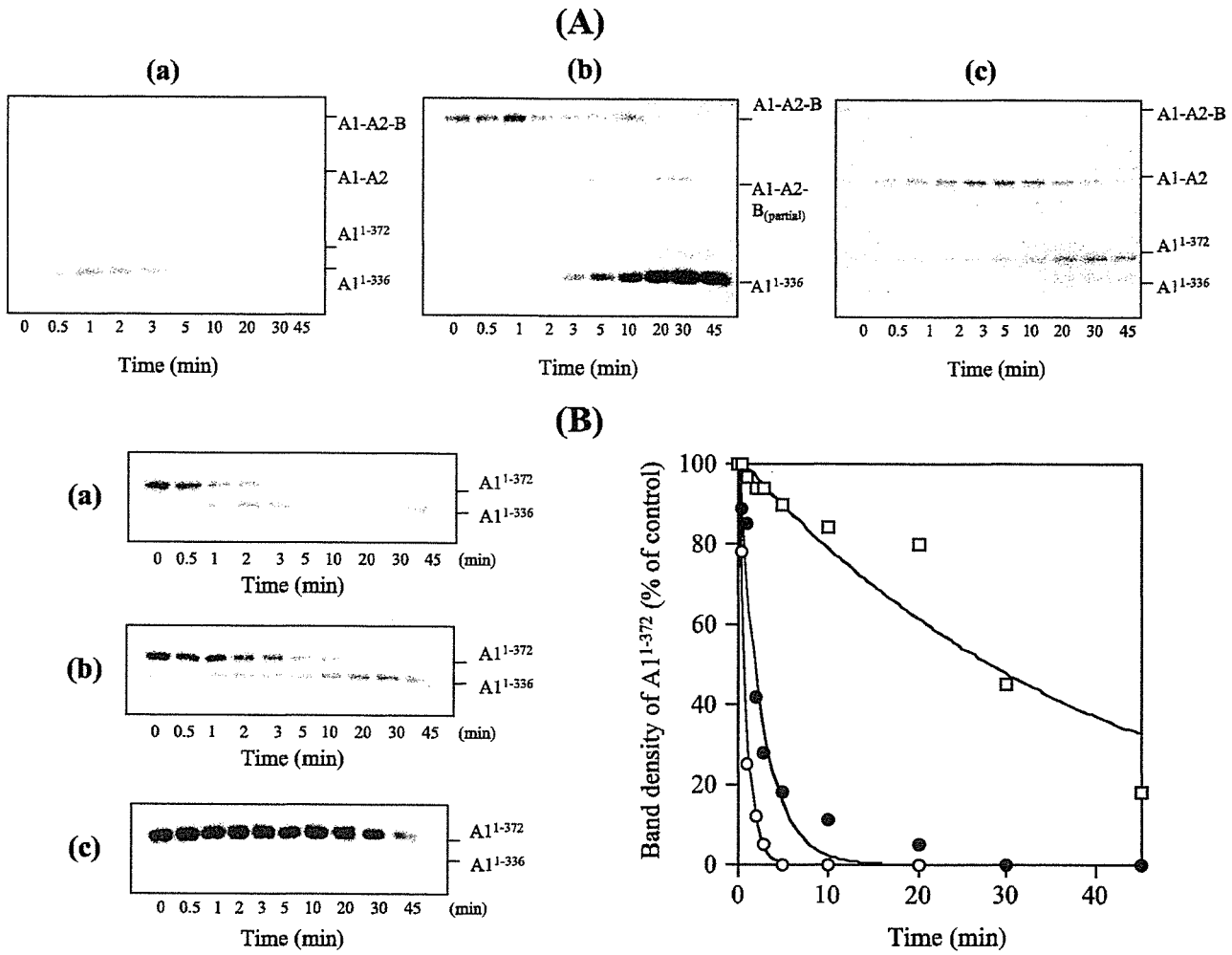

FIGURE 5. **Comparison with A1 cleavage in factor VIII(a) by plasmin, APC, and factor Xa.** Factor VIII (*A*, 100 nM) or factor VIIIa (*B*, 100 nM) was incubated with 4 nM plasmin (*panel a*), APC (*panel b*) together with protein S (40 nM), and factor Xa (*panel c*) in the presence of phospholipid vesicles (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

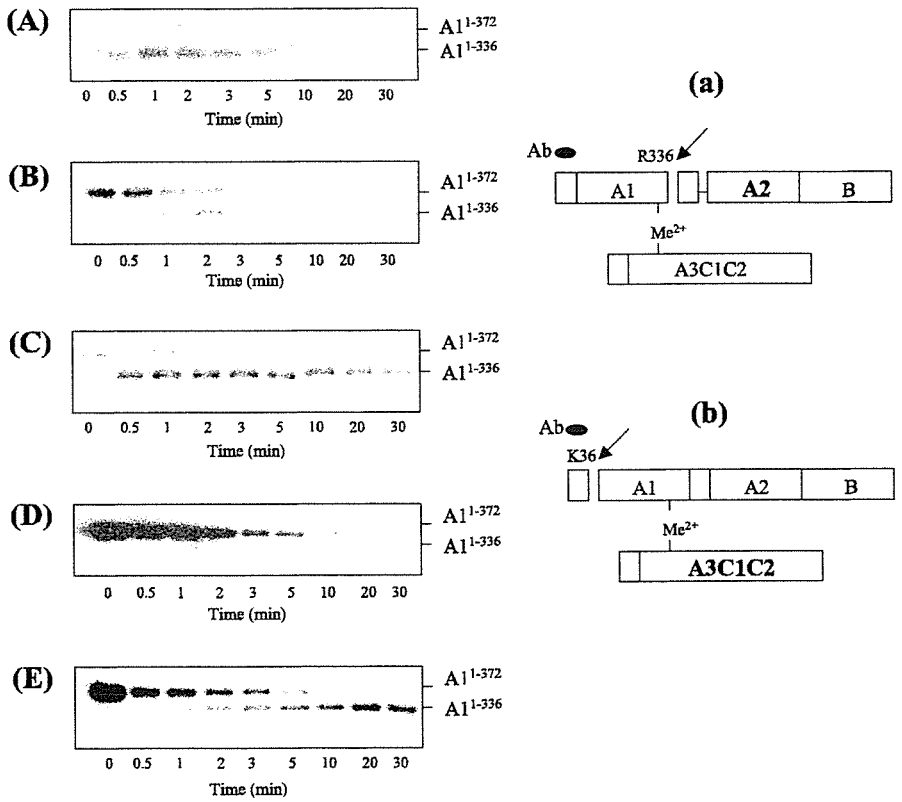

FIGURE 6. **Effects of factor VIII(a) subunit on Lys^{36} and Arg^{336} cleavages in A1 subunit by plasmin.** Equivalent concentrations (100 nM) of factor VIII (*A*), factor VIIIa (*B*), intact heavy chain (*C*), A1/A3-C1-C2 dimer (*D*), or A1 subunit (*E*) were incubated with plasmin (4 nM) in the presence 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.

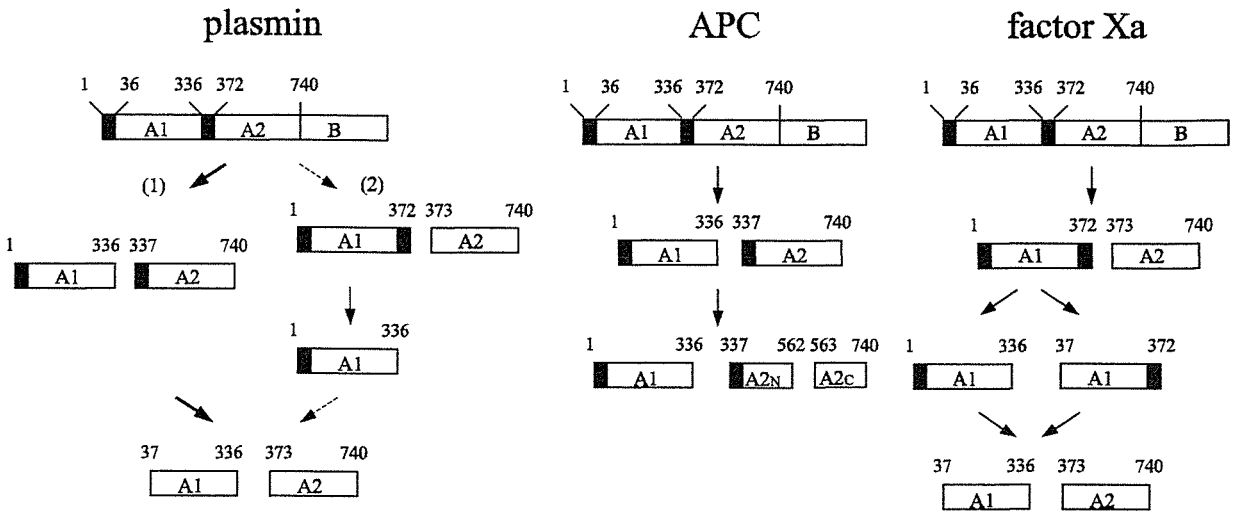

FIGURE 7. **A schematic comparison of the proposed pathways for the cleavage of factor VIII heavy chain by plasmin, APC, and factor Xa.**

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

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 ($\geq$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 $\geq$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

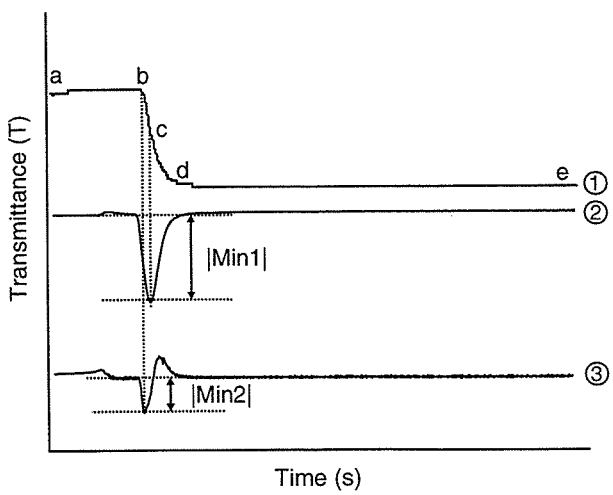

Fig. 1. Activated partial thromboplastin time (aPTT) clot waveform of normal plasma. The upper trace shows the recording of the changes in light transmittance (T) observed over time (t) during the performance of a normal diluted aPTT. The middle trace shows the first derivative (dT/dt: |Min1|) derived 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.

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.

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.

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).

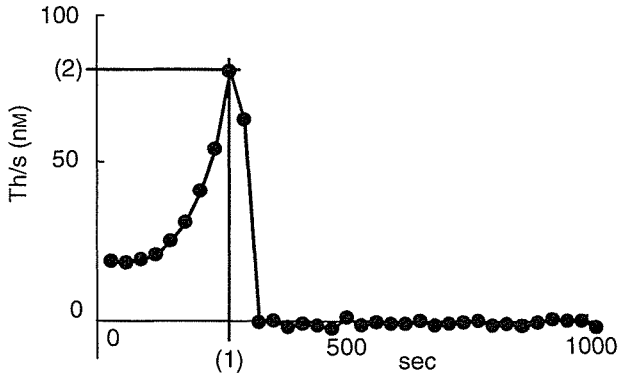

Fig. 2. Thrombin generation parameters of normal plasma. (1) Time to peak (s; TTP); (2) peak thrombin generation rate [nM; peak height (PH)].

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

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

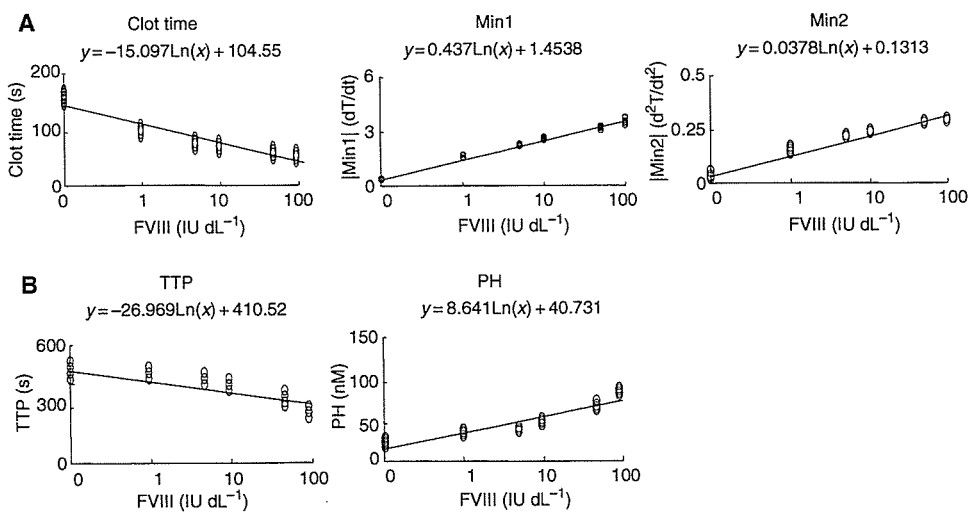

Fig. 3. Effects of addition of increasing concentrations of recombinant FVIII- to FVIII-deficient plasma on clot waveform and thrombin generation (FVIII 0–100 IU dL^{-1}). (A) Clot waveform parameters; (B) thrombin generation parameters.

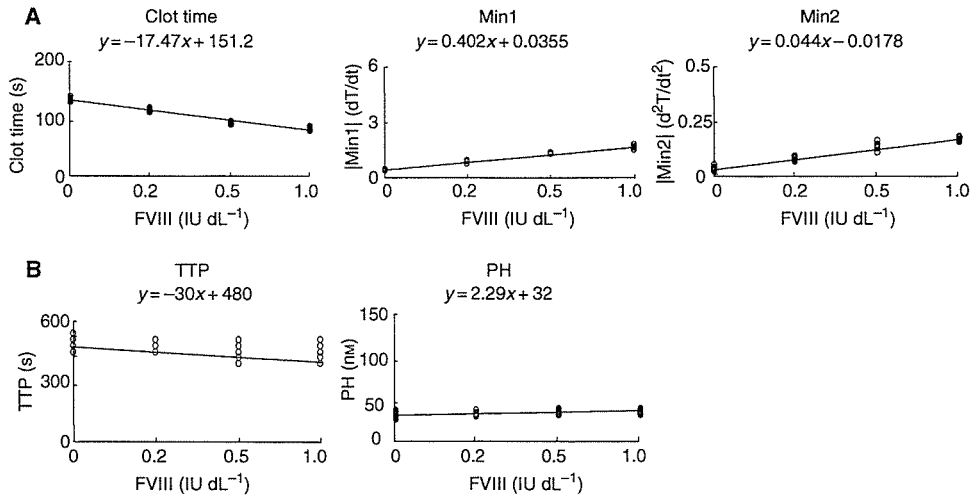

Fig. 4. Effects of addition of increasing concentrations of recombinant FVIII- to FVIII-deficient plasma on clot waveform and thrombin generation (FVIII 0–1.0 IU dL^{-1}). (A) Clot waveform parameters; (B) thrombin generation parameters.

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

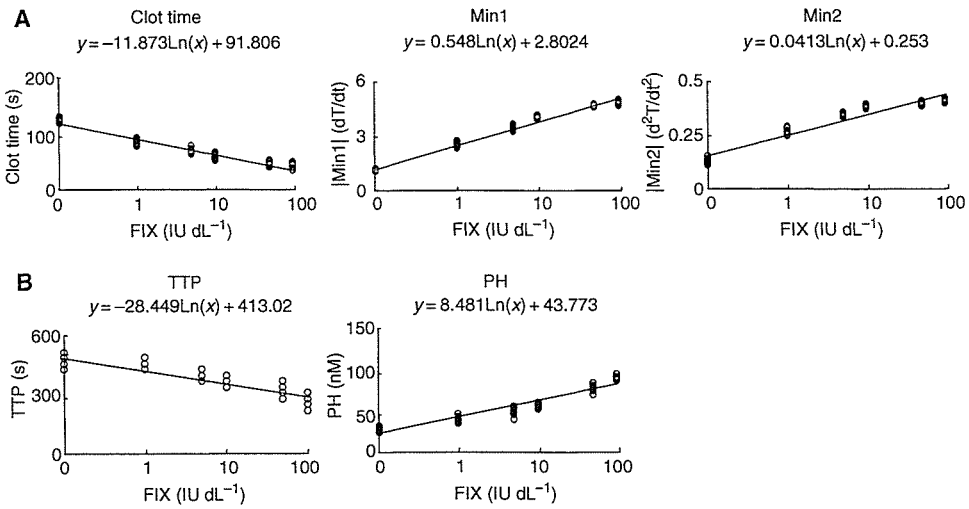

Fig. 5. Effects of addition of increasing concentrations of affinity purified FIX- to FIX-deficient plasma on clot waveform and thrombin generation (FIX 0–100 IU dL^{-1}). (A) Clot waveform parameters; (B) thrombin generation parameters.

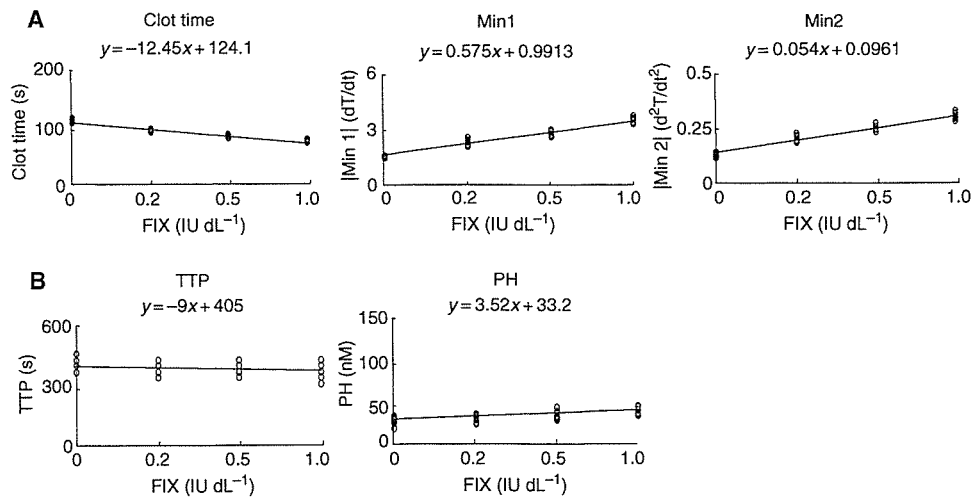

Fig. 6. Effects of addition of increasing concentrations of affinity-purified FIX- to FIX-deficient plasma on clot waveform and thrombin generation (FIX 0–1.0 IU dL^{-1}). (A) Clot waveform parameters; (B) thrombin generation parameters.

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

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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