use of recombinant FVIII alone. Treatment of this nature could be especially useful in patients requiring long-acting FVIII replacement therapy. Such therapy could result in a reduction in the total dose of recombinant FVIII concentrates and help to prevent the development of FVIII inhibitors. Furthermore, the enhancing effects of mAb216 on FVIII activity were evident even in the presence of common FVIII allo-inhibitors and mAbs (anti-A2 mAb413 and anti-C2 mAbNMC-VIII/5) that recognise A2 and/or C2 epitopes (5). It may be, therefore, that the administration of mAb216-related products could be a useful adjunct in protocols for replacement therapy in patients with either congenital haemophilia A with alloAb inhibitors or acquired haemophilia A with autoAbs. Further investigations on the effects *in vivo* of this unique antibody using animal models are proposed.

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ORIGINAL ARTICLE

Mechanisms of factor VIIa-catalyzed activation of factor VIII

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Summary. *Background:* Factor (F)VIIa, complexed with tissue factor (TF), is a primary trigger of blood coagulation, and has extremely restricted substrate specificity. The complex catalyzes limited proteolysis of FVIII, but these mechanisms are poorly understood. *Objectives:* In the present study, we investigated the precise mechanisms of FVIIa/TF-catalyzed FVIII activation. *Results:* FVIII activity increased ~4-fold within 30 s in the presence of FVIIa/TF, and then decreased to initial levels within 20 min. FVIIa (0.1 nM), at concentrations present physiologically in plasma, activated FVIII in the presence of TF, and this activation was more rapid than that induced by thrombin. The heavy chain (HCh) of FVIII was proteolyzed at Arg^{740} and Arg^{372} more rapidly by FVIIa/TF than by thrombin, consistent with the enhanced activation of FVIII. Cleavage at Arg^{336} was evident at ~1 min, whilst little cleavage of the light chain (LCh) was observed. Cleavage of the HCh by FVIIa/TF was governed by the presence of the LCh. FVIII bound to Glu-Gly-Arg-active-site-modified FVIIa (K_d, ~0.8 nM) with a higher affinity for the HCh than for the LCh (K_d, 5.9 and 18.9 nM). Binding to the A2 domain was particularly evident. Von Willebrand factor (VWF) modestly inhibited FVIIa/TF-catalyzed FVIII activation, in keeping with the concept that VWF could moderate FVIIa/TF-mediated reactions. *Conclusions:* The results demonstrated that this activation mechanism was distinct from those mediated by thrombin, and indicated that FVIIa/TF functions through a 'priming' mechanism for the activation of FVIII in the initiation phase of coagulation.

Keywords: factor VIIa, factor VIII, factor VIIIa, tissue factor.

Introduction

Factor (F)VIII, a plasma protein that is deficient or defective in individuals with the severe congenital bleeding disorder, hemophilia A, functions as a cofactor in the tenase complex, responsible for phospholipid (PL)-dependent conversion of FX to FXa by FIXa [1]. FVIII circulates as a complex with von Willebrand factor (VWF), a macromolecule that protects and stabilizes the cofactor. FVIII is synthesized as a single chain molecule consisting of 2332 amino acid residues with a molecular mass of ~300 kDa, and is arranged into three domains, A1-A2-B-A3-C1-C2, based on amino acid homology. FVIII is processed into a series of metal ion-dependent heterodimers by cleavage at the B-A3 junction, generating a heavy chain (HCh) comprising A1 and A2 domains together with heterogeneous fragments of partially proteolyzed B domain linked to a light chain (LCh) consisting of A3, C1 and C2 domains [2].

The catalytic efficiency of FVIII in the tenase complex is enhanced over 10^5-fold by conversion into an active form, FVIIIa, by limited proteolysis by either thrombin or FXa [3]. Both enzymes cleave native FVIII at Arg^{372} and Arg^{740} of the HCh and produce 50-kDa, A1 and 40-kDa, A2 subunits. The 80-kDa LCh, ^{1649}A3C1C2, is cleaved at Arg^{1689}, generating a 70-kDa, ^{1690}A3C1C2 subunit. Proteolysis at Arg^{372} and Arg^{1689} is essential for generating FVIIIa cofactor activity [4]. Cleavage at the former site exposes a functional FIXa-interactive site within the A2 domain that is cryptic in the unactivated molecule [5]. Cleavage at the latter site liberates FVIII from VWF [6], contributing to the overall specific activity of the cofactor [7]. FVIIIa activity is down-regulated by serine proteases including activated protein C (APC), FXa, FIXa and plasmin following cleavage at Arg^{336} within the A1 subunit [3,8,9]. This inactivation appears to reflect loss of a FX-interactive site, mediated by a modified interaction with the A2 subunit and an increased K_m of the truncated A1 for the substrate FX [10].

FVII is a single chain zymogen consisting of 406 amino acid residues with a molecular mass of 48 kDa [11]. The active form of FVII, FVIIa, forms a complex with tissue factor (TF), to generate a potent serine protease responsible for initiating and propagating the blood coagulation cascade in normal hemostasis [12]. The central role of FVIIa is the activation of FIX and FX. Following injury to the blood vessel wall, TF is exposed to circulating blood and forms the complex with FVIIa, resulting in initiation of hemostasis through activation of FX and the generation of a minimal amount of thrombin

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[13]. This trace amount of thrombin dissociates FVIII from VWF and promotes platelet activation. Following these 'priming' reactions, thrombin generation is accelerated through propagation of tenase and prothrombinase enzymes on negatively-charged PL exposed on platelet surfaces, synchronized with an increase in platelet procoagulant activity [14]. Moreover, a TF-independent cell-based FX activation mechanism has been identified involving direct binding to platelet membranes [15], and these concepts have been applied to hemostatic therapy for various bleeding disorders including hemophilia A and B.

FVIIa/TF has also been shown to proteolyze FV and FVIII [16,17]. Warren *et al.* [17] reported that FVIIa/TF induced FVIII cleavage at Lys^{36}, Arg^{336}, Arg^{372}, Arg^{562} and Arg^{740} in the HCh and at Arg^{1652} and Arg^{1689} in the LCh, over extended reaction times. In addition, FVIIa enhanced FVIII activity by ~2-fold by cleavage at Arg^{740}, Arg^{336} and Arg^{372}. These findings supported the concept that FVIIa might up-regulate tenase activity by activating FVIII. The exact mechanism of this activation is poorly understood, however. In the present study, we have investigated the mechanism of FVIIa/TF-catalyzed FVIII activation using a combination of functional assays, binding assays and SDS–PAGE analysis. We demonstrated that FVIIa/TF induced limited proteolysis of the HCh of FVIII at Arg^{740} and Arg^{372}, and appeared to function through a 'priming' mechanism to generate small amounts of FVIIIa in the initiation phase of coagulation. FVIIa specifically proteolyzed the HCh in a reaction that was governed by the LCh, and this activation mechanism appeared to be distinct from that mediated by thrombin or FXa.

Materials and methods

Reagents

Purified recombinant FVIII (Kogenate FS®) and FVIIa (Novoseven®) preparations were kindly provided by Bayer Corp. Japan (Osaka, Japan) and NovoNordisk (Bagsværd, Denmark). The monoclonal antibody (mAb) C5 [18], recognizing the C-terminal end of the A1 domain, was a generous gift from Dr Fulcher. The anti-C2 mAb, NMC-VIII/5, was prepared as previously reported [19]. VWF was purified from FVIII/VWF concentrates using gel filtration on a Sepharose CL-4B column (Amersham Bio-Science, Uppsala, Sweden) and immune-beads coated with immobilized FVIII mAb [19]. Human FXa (Enzyme Research Laboratories, Inc., South Bend, IN, USA), thrombin (Sigma, St. Louis, MO, USA), recombinant hirudin (Calbiochem, San Diego, CA, USA), FVIIa-specific inhibitor peptide E-76 (Bachem, Bubendorf, Switzerland), recombinant lipidated TF (Innovin®; Dade Behring, Marburg, Germany) and Glu-Gly-Arg-chloromethylketone (EGR-ck; Calbiochem) were purchased from the indicated vendors. Gla domainless (GDless)-FVIIa was prepared from FVIIa by limited chymotryptic digestion [20]. PL vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine and 30% phosphatidylethanolamine (Sigma) were prepared using *N*-octylglucoside [21].

Preparation of FVIIIa subunits

LCh (^{1649}A3C1C2 and ^{1690}A3C1C2), HCh (A1-A2-B), A1 and A2 subunits of FVIII were purified as previously reported [5,7,10]. FVIIIa was isolated from thrombin-treated FVIII by CM-Sepharose chromatography (Amersham) [22]. The A1/A3C1C2 dimer was prepared by reconstitution from isolated A1 and A3C1C2 subunits [10]. The recombinant (r)A3 domain of FVIII was expressed in *E. coli* using the pET expression system (Novagen, Madison, WI, USA) [23]. The protein was purified using His-Select affinity cartridges. A cDNA coding the C2 domain sequence of human FVIII was constructed, transformed into *Pichia pastoris* cells and expressed in a yeast secretion system [24]. The rC2 protein was purified by ammonium sulfate fractionation and cation-exchange high-performance liquid chromatography (HPLC). SDS–PAGE of the isolated subunits followed by staining with GelCode Blue Stain Reagent (Pierce, Rockford, IL, USA) showed >95% purity. Protein concentrations were determined by the method of Bradford.

Preparation of active-site modified EGR-FVIIa

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

Clotting assays

FVIII activity was measured in a one-stage clotting assay using FVIII-deficient plasma. All reactions were performed at 22 °C. FVIII products were incubated in HBS-buffer containing 5 mM $CaCl_2$ and 0.1% bovine serum albumin plus the indicated concentrations of PL and TF. Samples were removed from the mixtures at the indicated times, and FVIIa/TF reaction was immediately terminated by the addition of FVIIa-inhibitor E-76 (2.5 μM) and 1000-dilution. FVIII activity was converted from the obtained clotting times using a standard curve of FVIII in FVIII-deficient plasma. The presence of FVIIa/TF and E-76 in the diluted sample did not affect FVIII activity (<0.5%) in these coagulation assays.

Cleavage of FVIII(a) and its subunits by FVIIa

Various concentrations of FVIIa/TF were added to 10 nM FVIII(a) or subunits together with 20 μM PL in HBS-buffer containing 5 mM $CaCl_2$ at 22 °C. Aliquots were removed 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 at 150 V for 1 h, followed by Western blotting using a Bio-Rad mini-transblot apparatus at 50 V for 2 h. Protein bands were probed using anti-FVIII mAbs followed by goat anti-mouse peroxidase-linked secondary mAb (MP Biomedicals, Aurora, OH, USA). Signals were detected using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA, USA). Densitometric scans were quantitated using Image J 1.38 (National Institute of Health, Bethesda, MD, USA).

Surface plasmon resonance (SPR)-based assay

The kinetics of FVIII and FVIIa interaction were determined by SPR-based assays using a BIAcore X instrument (Biacore AB, Uppsala, Sweden). The reactions were run at 22 °C. EGR-FVIIa was covalently coupled to the CM5 sensor chip at a coupling density of 0.4 ng mm^{-2}. Binding (association) of the ligand was monitored in 10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$ and 0.005% surfactant P20, at a flow rate of 30 μL min^{-1} for 2 min. The dissociation of bound ligand was recorded over a 2-min period by replacing the ligand-containing buffer with buffer alone. The level of non-specific binding, corresponding to the ligand binding to the uncoated chip, was subtracted from the signal. The surface on the sensor chip was regenerated by washing in 1 M NaCl for 1 min. Rate constants for association (k_{ass}) and dissociation (k_{diss}) were determined by nonlinear regression analysis using the evaluation software provided by Biacore AB. Dissociation constants (K_d) were calculated as k_{diss}/k_{ass}.

Results

FVIII activation by FVIIa

To investigate the effect of FVIIa on FVIII activity, we first examined FVIIa-catalyzed FVIII activation in the presence of TF using a one-stage clotting assay. To preclude the influence of FVIIa, TF/PL and FVIIa-inhibitor in this assay, 1000-fold dilution of reactant mixtures were utilized. In addition, FVIII (10 nM) was used at ~10-fold higher than physiological concentrations, because the minimum level for measurement of FVIII activity was 0.01 nM. The results showed that in mixtures of FVIII (10 nM) and various concentrations of FVIIa with TF (1 nM), FVIII activity rapidly reached peak levels of ~4-fold of initial value within 30 s after adding FVIIa (1 nM)/TF. Procoagulant activity subsequently decreased to initial levels at ~20 min (Fig. 1). This FVIIa-catalyzed activation of FVIII was shown to be dose-dependent and saturable at 1 nM FVIIa. Even at 0.1 nM FVIIa, which was estimated to be ~1% of FVII (10 nM) physiologically present in normal plasma, FVIII activity was enhanced by ~2.4-fold and

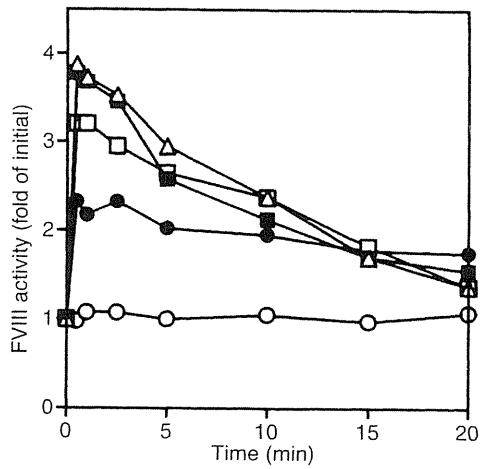

Fig. 1. Time course of activation of FVIII following reactions with FVIIa/TF. FVIII (10 nM) was incubated with various concentrations of FVIIa (○, 0 nM; ●, 0.1 nM; □, 0.25 nM; ■, 0.5 nM; △, 1 nM) in the presence of TF (1 nM) and PL (20 μM) at 22 °C. FVIII activity of the samples was measured at the indicated times in a one-stage clotting assay. The initial activity of FVIII was ~5 units mL^{-1}. Experiments were performed at least three separate times, and average values are shown.

persisted for ~20 min. FVIII activity was not appreciably increased by FVIIa in the absence of TF, indicating that the FVIIa association with FVIII was dependent on TF. The presence of hirudin had little effect on the FVIIa/TF-catalyzed reaction (data not shown), demonstrating no influence of contaminating thrombin.

We also examined whether Ca^{2+}-dependent PL-binding contributed significantly to the optimum catalytic function of FVIIa/TF on FVIII. FVIII activation by FVIIa/TF was enhanced by the presence of Ca^{2+} and PL in dose-dependent manners (data not shown). Furthermore, FVIII activation was markedly weaker with GDless-FVIIa than with intact FVIIa (data not shown), indicating that a functional Ca^{2+}-dependent PL-binding reaction, mediated by the Gla domain of FVIIa, regulated FVIIa/TF-catalyzed FVIII activation.

Comparison of FVIII activation by FVIIa/TF, thrombin and FXa

The most potent serine proteases responsible for FVIII activation are thrombin and FXa. We compared, therefore, the timed-course of FVIII activation by FVIIa/TF with that mediated by thrombin or FXa in a one-stage clotting assay. In the presence of 1 nM protease, the peak level of FVIII activity within 20 min after the addition of FVIIa/TF was ~5-fold lower than that of thrombin, and was similar to that of FXa (Fig. 2A, inset). In the early phases of activation, however, the increase in FVIII activity was greater with FVIIa/TF than with thrombin within 30 s after reaction, and was much greater than that with FXa within 2.5 min (Fig. 2A). Of note, in the presence of physiological levels of FVIIa (0.1 nM) with TF, the increase in FVIII activity was significantly greater within 2.5 min than that with thrombin at similar concentrations (Fig. 2B), although the peak level of FVIII activity was lower than with thrombin (Fig. 2B, inset). The results strongly

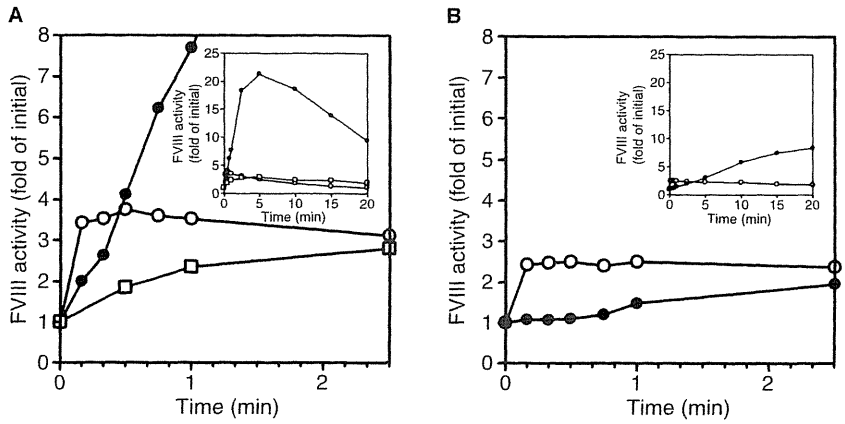

Fig. 2. Comparisons of the time course of activation of FVIII by FVIIa/TF, thrombin and FXa. FVIII (10 nM) was incubated with FVIIa (○, 1 nM (A), 0.1 nM (B)) with TF (1 nM), thrombin (●, 1 nM (A), 0.1 nM (B)), or FXa (□, 1 nM (A)) in the presence of PL (20 μM). The insets illustrate the time course for FVIII activation for incubation times of 0–20 min. FVIII activity of the samples was measured at the indicated times in a one-stage clotting assay. The initial activity of FVIII was ~5 units mL^{-1}. Experiments were performed at least three separate times, and average values are shown.

suggested that FVIIa/TF more effectively regulated the rapid generation of small amounts of FVIIIa in the early phases of interaction compared with thrombin or FXa.

Proteolytic cleavage of FVIII by FVIIa/TF

FVIIa/TF cleaves FVIII over an extended period of time at Lys^{36}, Arg^{336}, Arg^{372}, Arg^{562}, Arg^{740} in the HCh and at Arg^{1652} and Arg^{1689} in the LCh [17]. We focused on FVIIa/TF cleavage of both FVIII chains in early-timed reactions (within 30 min). Temporal changes in electrophoretic mobility of FVIIa/TF-treated FVIII were examined by SDS–PAGE and Western blotting using FVIII (10 nM) and FVIIa (1 nM)/TF (1 nM) in the presence of PL (Fig. 3). Products of proteolysis were visualized using an anti-A1 mAb recognizing the HCh (C5, panel A) and an anti-C2 mAb (NMC-VIII/5, panel B) recognizing the LCh. Proteolytic cleavage sites of FVIII fragments were determined using automated NH_2-terminal sequence analysis and were shown to be identical to those already reported (Arg^{336}, Arg^{372} and Arg^{740}; panel C) [17]. Incubation of FVIII with FVIIa/TF, demonstrated that the HCh (A1-A2-whole B) was rapidly degraded to A1-A2 fragments by cleavage at Arg^{740}, followed within 15 s by generation of $A1^{1-372}$ fragments by cleavage at Arg^{372} (panel A). This appeared to be consistent with the findings obtained by FVIIa/TF-catalyzed activation of FVIII in clotting assays. The bands above A1-A2 reflected fragments generated by partial cleavage of the B domain in the HCh (A1-A2-whole B). At subsequent time points, a $A1^{337-372}$-A2 fragment was apparent within 1 min, suggesting that cleavage at Arg^{336} in A1 was slower than that at Arg^{740} and Arg^{372}. The generation of this fragment appeared to coincide with the decrease in FVIII activity. The A1-A2-whole B, A1-A2, $A1^{1-372}$ and $A1^{337-372}$-A2 bands gradually disappeared over time (data not shown). HPLC-gel filtration was used to fractionate intact FVIII, but nevertheless, $A1^{1-372}$ fragments remained evident in the absence of FVIIa/TF, suggesting that mAbC5 was highly sensitive in

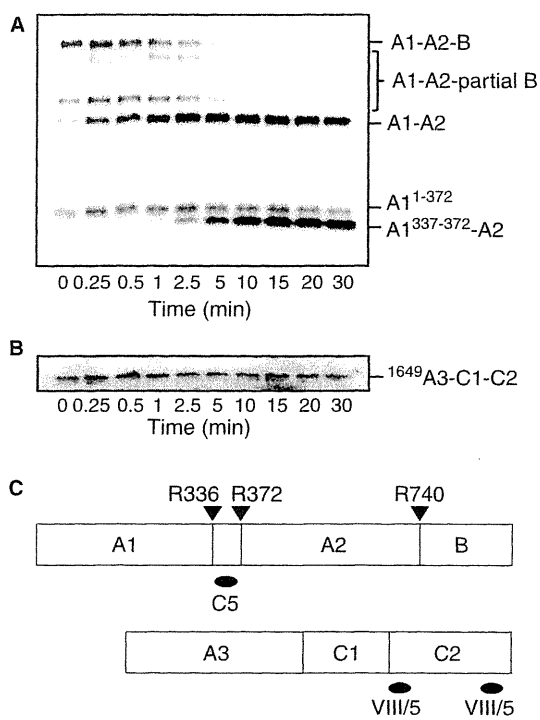

Fig. 3. Time course of FVIIa/TF-catalyzed cleavages of FVIII. FVIII (10 nM) was incubated with FVIIa (1 nM) in the presence of TF (1 nM) and PL (20 μM) for the indicated times. Samples were run on 8% gels followed by Western blotting using anti-A1 (C5, A) and anti-C2 (NMC-VIII/5, B) mAbs. Panel C shows a schematic presentation of the domain organization of both chains of FVIII, location of FVIIa/TF-catalyzed cleavage sites, and epitope regions of anti-FVIII mAbs.

these circumstances. Cleavages at Lys^{36} in A1 or at Arg^{562} in A2, however, were not a feature of reactions within 30 min identified using anti-A1 mAb58.12 recognizing the N-terminus and anti-A2 mAbJR8, respectively (data not shown), suggesting that the $A1^{1-336}$ and A2 fragments were terminal products. In addition, the $A1^{1-372}$ subunit, generated initially by cleavage

at Arg^{372}, was further proteolyzed at Arg^{336}, also resulting in the terminal product $A1^{1-336}$. Little cleavage of the LCh was observed within 30 min in these experiments (panel B), indicating that cleavage of LCh was not associated with FVIII activation by FVIIa/TF.

Comparison of FVIII cleavage by FVIIa/TF and thrombin

The contrasting temporal effects of FVIIa/TF and thrombin on FVIII activity might have been due to differences in cleavage patterns. We analyzed, therefore, FVIIa/TF- and thrombin-induced cleavage of HCh by Western blotting using anti-A1 mAbC5 (Fig. 4). $A1^{1-372}$ fragments, reflecting Arg^{372} cleavage, were further characterized using quantitative densitometry (panel C). In the presence of either protease (0.1 nM, *panel* A), FVIIa/TF converted the intact HCh (A1-A2-whole B) into the A1-A2 fragment by cleavage at Arg^{740} and the $A1^{1-372}$ fragment by cleavage at Arg^{372} more rapidly than thrombin. Cleavage at Arg^{372} by FVIIa/TF was more predominant than that by thrombin within 2.5 min, consistent with the results of FVIII activation. Relatively little cleavage by FVIIa/TF at Arg^{336} was observed, however, within 2.5 min. At 1 nM protease (panel B), cleavage at Arg^{740} or Arg^{372} by FVIIa/TF was slightly faster than that by thrombin within 10 s. At later time points, generation of the $A1^{1-372}$ fragment by thrombin continued in a time-dependent manner. In contrast, the concentration of this fragment gradually diminished in the presence of FVIIa/TF, indicating further cleavage at Arg^{336} and generation of $A1^{1-336}$. In addition, $A1^{337-372}$-A2 slowly appeared, possibly indicating cleavage at Arg^{372} prior to that at Arg^{336}. These data demonstrated distinct, rate-dependent cleavage patterns of FVIII at Arg^{740} and Arg^{372} induced by FVIIa/TF and thrombin.

Role of individual FVIII(a) subunits in HCh cleavage by FVIIa/TF

To further study the roles of each subunit in FVIII(a) for FVIIa/TF-mediated proteolysis, various FVIII(a) fragments (10 nM) were used as substrates for FVIIa (1 nM)/TF (1 nM), and analyzed by Western blotting using anti-A1 mAbC5 (Fig. 5A). Furthermore, cleavage at Arg^{336}, Arg^{372} and Arg^{740} was quantitatively estimated by band densitometry (Fig. 5B). Disappearance of A1-A2-B coincident with the appearance of A^{1-372} and $A1^{337-372}$-A2 fragments (panel A-a) was observed at an early time-point after the addition of FVIIa/TF to FVIII. Cleavage of isolated HCh by FVIIa/TF was not readily visible, however (panel A-b), suggesting that cleavage of HCh in the presence of LCh was markedly more rapid than that in its absence (panel B-a,b). Cleavage at Arg^{336} in FVIIIa, $A1^{1-372}/^{1690}$A3C1C2 dimer and isolated $A1^{1-372}$ was also examined. The $A1^{1-372}$ in FVIIIa and $A1^{1-372}/^{1690}$A3C1C2 diminished after the addition of FVIIa/TF in similar timed-dependent manners (panels A-c, d, respectively). No proteolysis of isolated A1 was evident under these conditions, however (panel A-e), in keeping with earlier findings that the LCh played an important role for cleavage at Arg^{336} in A1 (panel B-b). Taken together,

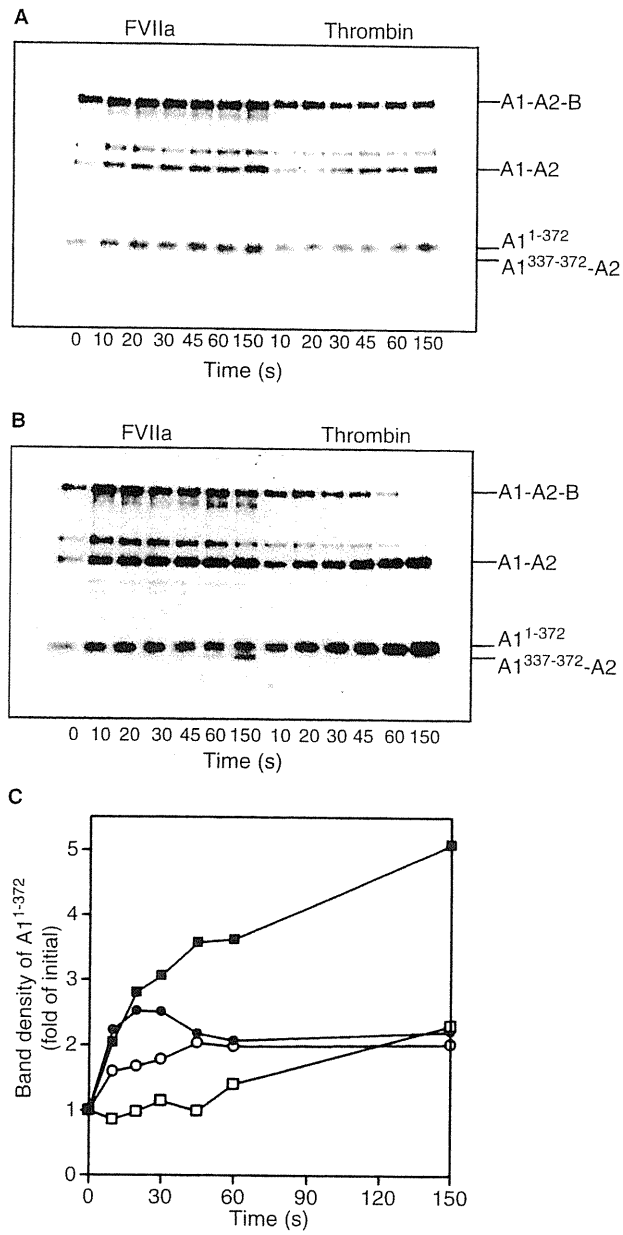

Fig. 4. Comparisons of cleavages of FVIII by FVIIa/TF and thrombin. FVIII (10 nM) was incubated with FVIIa (A, 0.1 nM; B, 1 nM) with TF (1 nM) and thrombin (A, 0.1 nM; B, 1 nM) for the indicated times. Samples were run on 8% gels followed by Western blotting using an anti-A1 mAbC5. Panel C shows the data obtained by quantitative densitometry of the $A1^{1-372}$ in A and B. The symbols used are as follows: 0.1 (○) and 1 nM (●) of FVIIa, 0.1 (□) and 1 nM (■) of thrombin.

these findings supported the concept that cleavages at Arg^{336}, Arg^{372} and Arg^{740} were predominantly regulated by the presence of the LCh.

Binding of FVIII(a) subunits to active-site modified EGR-FVIIa

To confirm interactions between FVIIa and FVIII(a), we examined direct binding of FVIII(a) and FVIIa in SPR-based assays. An active-site modified EGR-FVIIa preparation was

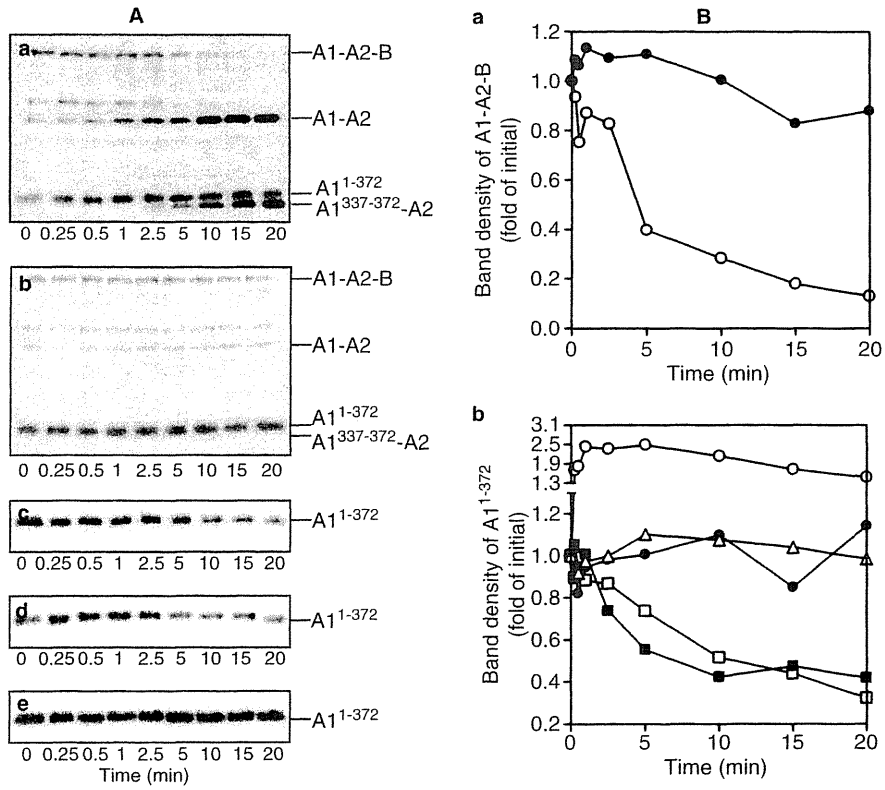

Fig. 5. Role of FVIII(a) subunit on FVIIa/TF-catalyzed FVIII cleavages at Arg^{740}, Arg^{372} and Arg^{336}. (A) Equivalent concentrations (10 nM) of FVIII (a), intact HCh (b), FVIIIa (c), A1/A3C1C2 dimer (d) and A1 (e) were incubated with FVIIa (1 nM)/TF (1 nM) and PL (20 μM) for the indicated times. Samples were run on 8% gels followed by Western blotting using an anti-A1 mAbC5. (B) Panels (a) and (b) show the data obtained by quantitative densitometry of the A1-A2-B and $A1^{1-372}$, respectively, in (A). The symbols used are as follows: ○, FVIII; ●, intact HCh; □, FVIIIa; ■, A1/A3C1C2; △, A1.

used in these experiments to eliminate difficulties of interpretation in the presence of enzymatically active FVIIa. In addition, because the PL contained in lipidated TF might bind to FVIII directly, experiments were repeated in the absence of TF. Figure 6 shows representative curves corresponding to the association/dissociation of FVIII to EGR-FVIIa immobilized on a sensor chip. The data could be comparatively well-fitted by nonlinear regression using a 1:1 binding model. Binding parameters are summarized in Table 1. Intact FVIII bound to EGR-FVIIa with high affinity (K_d, 0.82 nM) and the binding capacity of FVIII appeared to be higher (by ~10-fold) than that of FVIIIa (K_d,

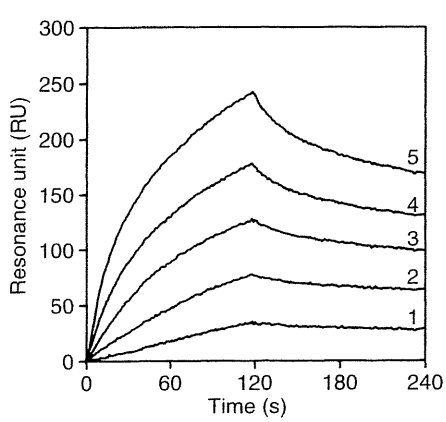

Fig. 6. Direct binding of FVIII to EGR-FVIIa. Various concentrations of the FVIII were injected into the EGR-FVIIa (0.4 ng mm^{-2}) immobilized onto the sensor chip at a flow rate of 30 μL min^{-1} for 2 min, followed by the change of running buffer for 2 min. The lines 1–5 show representative response curves for the different concentrations of FVIII (0.625, 1.25, 2.5, 5, 10 nM, respectively).

Table 1. Binding parameters for the interaction between FVIII(a) subunits and EGR-FVIIa in SPR-based assays

Ligands	$k_{ass} \times 10^4$ M^{-1} s^{-1}	$k_{diss} \times 10^{-3}$ s^{-1}	K_d (k_{diss}/k_{ass}) nM
FVIII	272 ± 2.0	2.23 ± 0.05	0.82
FVIIIa	34.2 ± 0.45	2.54 ± 0.05	7.4
A1-A2-B	11.1 ± 0.19	0.65 ± 0.06	5.9
^{1649}A3C1C2	17.8 ± 0.28	3.37 ± 0.04	18.9
^{1690}A3C1C2	15.2 ± 0.30	2.30 ± 0.06	15.1
A1	ND*	ND*	–
A2	10.0 ± 0.28	2.64 ± 0.09	26.4
A3	1.39 ± 0.17	3.80 ± 0.11	273
C2	1.20 ± 0.02	4.51 ± 0.06	376

Reactions were performed as described under 'Materials and Methods'. Results were calculated by nonlinear regression analysis using the evaluation software provided by Biacore AB. Experiments were performed at least five separate times, and average ± SE values are shown. *Not determined.

7.4 nM). Interestingly, the binding affinity for intact HCh, which did not directly regulate FVIII cleavage, was ~3-fold higher than that for the intact LCh (K_d, 5.9 and 18.9 nM, respectively). The ^{1690}A3C1C2 bound to EGR-FVIIa with similar affinity to ^{1649}A3C1C2, suggesting that the acidic region of A3 was not an essential component of the FVIIa-interactive site. The isolated A2 significantly bound to EGR-FVIIa with ~10-fold higher affinity than the A3 and C2 (K_d, 26.4, 273 and 376 nM, respectively). The isolated A1 failed to bind, however. These data demonstrated that not only the LCh but also the HCh of FVIII, especially the A2 domain, played significant roles in the interaction with FVIIa.

Effect of VWF on FVIII and FVIIa association

VWF binds to FVIII non-covalently and mediates a protective effect on the molecule in circulating plasma. A potential role of VWF in FVIIa/TF-catalyzed activation of FVIII was firstly examined in a one-stage clotting assay. FVIII (10 nM) was preincubated with VWF prior to reaction with FVIIa (1 nM)/TF (1 nM). The presence of VWF partially inhibited FVIII activation at the early stages of reaction in a dose-dependent manner. At physiological concentrations of VWF (10 μg mL^{-1}; ~40 nM) the peak level of FVIII activation was inhibited by only ~40%, and the peak activity persisted for ~20 min (Fig. 7A). The effect of VWF on FVIIa-catalyzed FVIII cleavage was also examined by Western blotting using anti-A1 mAbC5 (Fig. 7B). VWF significantly blocked cleavage at Arg^{740} and Arg^{372} in the HCh in a dose-dependent manner within 10 s of reaction (panel a). At 10 min in the presence of VWF (40 nM), however, cleavage at Arg^{740}, Arg^{372} and Arg^{336} (panel b) was partially inhibited compared with that in the absence of VWF, indicating that VWF modestly counteracted FVIIa-catalyzed FVIII activation. To further clarify the inhibitory mechanism of VWF, we examined the effect of VWF on FVIII and EGR-FVIIa interaction in an SPR-based assay. These experiments were performed in the absence of PL to eliminate the possibility that VWF competed with PL for FVIII binding [25]. Mixtures of FVIII (1 nM) and various concentrations of VWF were added to EGR-FVIIa immobilized on sensor chips. FVIII binds to VWF with high affinity (K_d: ~0.5 nM [26]), and under these conditions, therefore, >95% FVIII was considered to be present as the FVIII/VWF complex. The presence of VWF decreased maximum binding of FVIII to EGR-FVIIa by ~60% in a dose-dependent manner (Fig. 7C). Overall, these findings suggested that VWF partially modulated FVIIa/TF-mediated activation of FVIII by direct competition with the FVIII molecule.

Discussion

We have demonstrated for the first time that FVIIa/TF contributed to the up-regulation of procoagulant FVIII activity by limited proteolysis of the HCh at Arg^{372} and Arg^{740} in the early phases of coagulation, and that this reaction was regulated by the presence of the LCh.

The physiological significance of FVIIa-catalyzed FVIII activation remains to be fully determined. As noted above, however, in the presence of physiological concentrations (0.1 nM), FVIIa was shown to activate FVIII, and this activation was more rapid than with thrombin. Although concentrations of TF *in vivo* might not reach the level of 1 nM that we used in some experiments, FVIIa was shown to readily complex with TF, which could be expected to be exposed on injured blood vessel surfaces, possibly augmenting rapid activation of FVIII. The FVIIa/TF reactions appeared to be important, however, within the initial 30 s, with a ~4-fold increase in FVIII activity followed by rapid decline. In contrast, thrombin clearly appeared to be the most potent activator of FVIII (~5-fold more potent than FVIIa/TF) with a ~20-fold increase in activity, followed by slower decline in activity. This striking difference between thrombin and FVIIa/TF seemed likely to be due to both inactivation of FVIII by cleavage at Arg^{336} and failure to cleave the LCh in FVIII. These data indicated that FVIIa/TF principally mediates a 'priming' mechanism to generate trace amounts of FVIIIa in the initiation phase of coagulation.

FVIII and FV are structurally homologous and have similar functions [27]. Other studies have demonstrated that FVIIa mediated limited proteolysis of FV, leading to the generation of an intermediate that was resistant to full activation by thrombin and highly sensitive to complete inactivation by APC [16]. FV cofactor activity appeared unlikely, therefore, to be appreciably increased by FVIIa. Interactions between FVIIa and FV remain to be investigated, but it appears that the immediate effects of FVIIa on FVIII and FV are functionally opposite, reflecting up- and down-regulation, respectively.

An earlier investigation reported that FVIIa proteolyzed FVIII at Lys^{36}, Arg^{336}, Arg^{372}, Arg^{562} and Arg^{740} in the HCh, and at Arg^{1652} and Arg^{1689} in the LCh during over-extended reactions [17]. In the present study, both SDS–PAGE and N-terminal sequence analysis confirmed that limited proteolysis of FVIII by FVIIa/TF occurred within 5 min at only three locations in the HCh (Arg^{372}, Arg^{740} and Arg^{336}). No degradation of the LCh was observed. The HCh was immediately proteolyzed by FVIIa/TF at Arg^{372} and Arg^{740}, followed by Arg^{336}, and the former two cleavages were more rapid with FVIIa/TF than with thrombin. Mutational analysis and data reported in the Hemophilia A Database indicate that proteolysis at Arg^{372} is essential for generating the cofactor activity of FVIIIa. Newell *et al.* [28] reported that proteolysis at Arg^{740} facilitates subsequent bond cleavages during FVIII activation, indicating that cleavage at the A2-B junction was an essential step in the process of procofactor activation. It seems, therefore, that initial rapid activation of FVIII by FVIIa/TF at two cleavage sites is significantly associated with physiological hemostasis. Furthermore, the predominant cleavage observed at Arg^{372} rather than at Arg^{336} was similar to that reported for FXa.

We have developed a novel, direct binding assay to study FVIII interactions. Because the PL contained in lipidated TF might directly bind to FVIII, active-site modified EGR-FVIIa

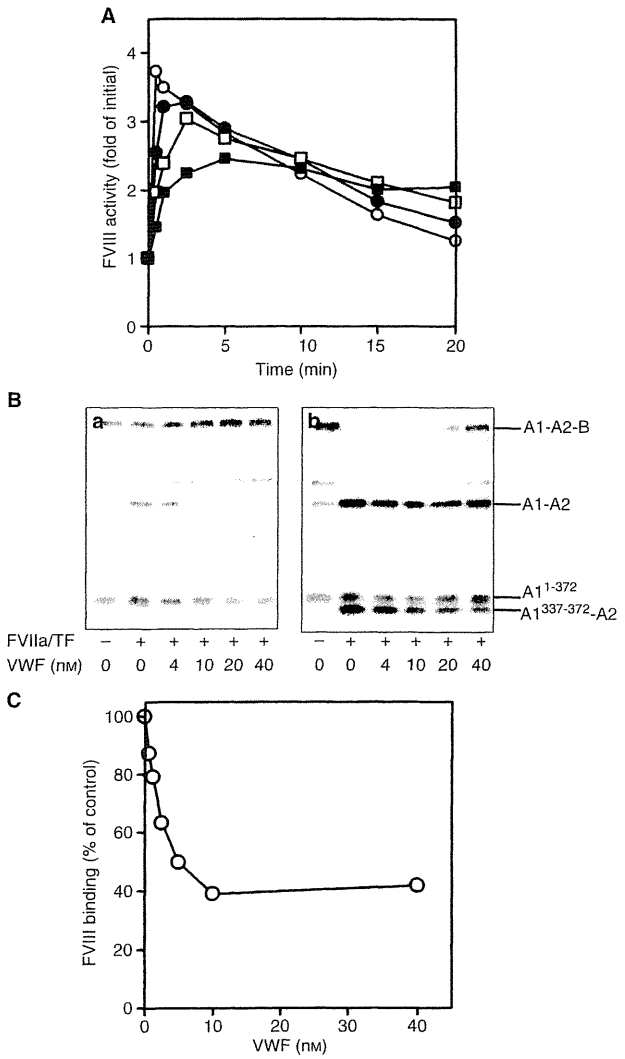

Fig. 7. Effect of VWF on FVIIa/TF-catalyzed reaction of FVIII. (A) Activation of FVIII. Mixtures of FVIII (10 nM) and various concentrations of VWF (○, 0 nM; ●, 10 nM; □, 20 nM; ■, 40 nM) were incubated with FVIIa (1 nM)/TF (1 nM) and PL (20 μM). FVIII activity was measured at the indicated times in a one-stage clotting assay. (B) Cleavage of FVIII. Mixtures of FVIII (10 nM) and various concentrations of VWF were incubated with FVIIa (1 nM)/TF (1 nM) and PL (20 μM) for 10 s (a) and 10 min (b). Samples were run on 8% gels followed by Western blotting using anti-A1 mAbC5. (C) FVIII/EGR-FVIIa binding. Mixtures of FVIII (1 nM) and various concentrations of VWF were added to EGR-FVIIa immobilized onto the sensor chip in an SPR-based assay. Maximum R_{max} value for FVIII binding to EGR-FVIIa in the absence of competitor was regarded as 100%. The percentage of FVIII binding was plotted as a function of VWF. Experiments were performed at least three separate times, and average values are shown.

was utilized in SPR-based assays in the absence of TF. This assay provided clear evidence that FVIIa did indeed interact with FVIII (K_d, ~0.8 nM) even in the absence of TF and PL. Some constructs of active-site-blocked FVIIa had been reported, however, to possess ~5-fold higher affinity for TF than native FVIIa [29], suggesting that the reactions between EGR-FVIIa and FVIII may be miscalculated. On the other hand, other studies have shown that the presence of TF increased the k_{cat} values for FVIIa-catalyzed the activation of FIX and FX by ~10^4-fold compared with those in its absence, whilst the K_m values were significantly unchanged [12]. Overall, therefore, it seems likely that TF significantly contributes to the functional interaction between FVIIa and FVIII, although FVIIa alone might interact directly with FVIII.

Although FVIIa bound to the HCh with higher affinity (K_d, ~6 nM) than with the LCh (~20 nM), cleavage at Arg^{372} and Arg^{740} (and Arg^{336}) was markedly accelerated by the presence of the LCh. It seemed, therefore, that proteolysis of the HCh was governed by the presence of the LCh. Precise mechanisms remain unclear, but it may be that reconstitution of the HCh and LCh might provide a more suitable conformation than the HCh alone for FVIIa/TF catalysis. Alternatively, the LCh might facilitate docking of the enzyme to promote HCh proteolysis.

Our observations indicated that the A2 domain in the HCh together with the A3 and C2 domains in the LCh bound to FVIIa. Our data further suggested, however, that these domains interacted with FVIIa in the order A2 > > A3 > C2. Binding sites in the FVIII molecule for several serine proteases have been previously reported. Thrombin- and FXa-interactive sites have been identified in the A2/C2 domains [30] and the A1/C2 domains [31,32], respectively. APC- and FIXa-interactive sites have been located in the A1/A3 domains [33,34] and the A2/A3 domains [35,36], respectively. We have recently demonstrated the presence of FIXa-interactive sites in the C2 domain [37] and the presence of plasmin-interactive sites in the A2/A3 domains [38,39]. The FVIIa-interactive sites were located in the A2/A3/C2 domains, showing that this binding pattern was similar to FIXa. The A3 domain binds to FIXa with ~20-fold higher affinity than the A2, however (K_d, ~15 and ~300 nM, respectively) [4,40], and it appears, therefore, that the contribution of each FVIII domain in FIXa and FVIIa binding reactions is different.

VWF binds to FVIII non-covalently in plasma, and protects FVIII from proteolysis by PL-dependent serine proteases including FXa, FIXa and APC [32,33,40]. Proteolysis by PL-independent proteases, thrombin and plasmin, is not affected by the presence of VWF [9]. Warren *et al.* [17] reported that VWF did not affect the activation of FVIII by PL-dependent FVIIa. The current findings, however, tended to support the general concept of the role of VWF in FVIII-related mechanisms, but activation of FVIII by FVIIa/TF was partially moderated by VWF at physiological concentrations (by ~40%). It might be that this protective action of VWF is associated with a range of mechanisms. Firstly, VWF is known to compete with PL binding to FVIII [25] and the catalytic activities of PL-dependent proteases are markedly reduced in the absence of PL. Secondly, VWF directly blocks the interaction between proteases and FVIII [32,40]. Thirdly, the affinity of FVIII for FVIIa might be altered allosterically by VWF binding. The second mechanism appeared to be implicated in our studies, but precise mechanisms by which VWF protects FVIII from serine proteases are unclear. Overall, it is noteworthy that, distinct from other PL-dependent serine proteases, FVIIa/TF activated FVIII to a significant extent in the presence of VWF.

In conclusion, we have shown that FVIIa/TF might function through a 'priming' mechanism for the FVIII activation in the initiation phase of blood coagulation. The physiological significance of these FVIIa-catalyzed events remains to be determined. We speculate, however, that FVIII, bound to VWF, could be partially activated by FVIIa/TF together with FIX and FX on the surface of injured blood vessel walls, participating in the initiation of hemostasis by enhancing the early formation of the intrinsic tenase complex and leading to the rapid local generation of thrombin. Further experiments are in progress to clarify these mechanisms.

Acknowledgements

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Disclosure of Conflict of Interests

T. Soeda is an employee of Chugai Pharmaceutical Company Ltd. The other authors state that they have no conflict of interest.

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Plasmin-induced procoagulant effects in the blood coagulation: a crucial role of coagulation factors V and VIII

Kenichi Ogiwara, Keiji Nogami, Katsumi Nishiya and Midori Shima

Plasminogen activators provide effective treatment for patients with acute myocardial infarction. However, paradoxical elevation of thrombin activity associated with failure of clot lysis and recurrent thrombosis has been reported. Generation of thrombin in these circumstances appears to be owing to plasmin (Plm)-induced activation of factor (F) XII. Plm catalyzes proteolysis of several coagulant factors, but the roles of these factors on Plm-mediated procoagulant activity remain to be determined. Recently developed global coagulation assays were used in this investigation. Rotational thromboelastometry using whole blood, clot waveform analysis and thrombin generation tests using plasma, showed that Plm (≥125 nmol/l) shortened the clotting times in similar dose-dependent manners. In particular, the thrombin generation test, which was unaffected by products of fibrinolysis, revealed the enhanced coagulation with a ~two-fold increase of peak level of thrombin generation. Studies using α_2-antiplasmin-deficient plasma revealed that much lower dose of Plm (≥16 nmol/l) actually contributed to enhancing thrombin generation. The shortening of clotting time could be observed even in the presence of corn trypsin inhibitor, supporting that Plm exerted the procoagulant activity independently of FXII. In addition, using specific coagulation-deficient plasmas, the clot waveform analysis showed that Plm did not shorten the clotting time in only FV-deficient or FVIII-deficient plasma in prothrombin time-based or activated partial thromboplastin time-based assay, respectively. Our results indicated that Plm did possess procoagulant activity in the blood coagulation, and this effect was likely attributed by multicoagulation factors, dependent on FV and/or FVIII. *Blood Coagul Fibrinolysis* **21:568–576 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: factor V, factor VIII, global coagulation assay, plasmin, procoagulant activity

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Introduction

Hemostasis is governed by the formation of the hemostatic plug and the limitation of plug development allowing reestablishment of normal blood flow. These physiological processes are generally termed the 'coagulation system' and the 'fibrinolytic system', respectively, and normal hemostasis depends on a controlled balance between these mechanisms. Plasmin (Plm) is the most potent fibrinolytic enzyme and is generated by activation of the zymogen, plasminogen (Plg), by tissue-type, and/or urinary-type Plg activators (tPA/uPA) [1]. It is a trypsin-like serine protease that binds to fibrin specifically via lysine binding sites resulting in fibrin degradation. The reaction develops only at the surface of the fibrin clot and free (unbound) Plm in plasma is rapidly neutralized by α_2-antiplasmin [1]. When excess Plm cannot be overcome by α_2-antiplasmin in plasma, however, Plm might react with other circulating proteins.

Previous reports indicated that such plasminemia caused the close interdependence of coagulation and fibrinolysis. For example, fibrinolytic therapy using Plg activators (tPA, uPA, and streptokinase) in patients with acute myocardial infarction (AMI) is well known, and sometimes result in increased thrombin activity and reocclusion of the coronary artery [2–4]. This paradoxical activation of coagulation is considered to be partly owing to elevated levels of Plm. Several reports also have suggested that Plm modulates blood coagulation by proteolysis of a number of circulating proteins. Plm has been shown *in vitro* to inactivate factor (F) IX [5] and FX [6], to activate FVII [7] and FXII [8], and to initially activate and subsequently inactivate FV [9] and FVIII [10]. Plm-catalyzed activation of FXII has been well studied and is considered to be a major feature responsible for increased procoagulant activity in patients treated with fibrinolytic agents [11,12]. Subsequently elevated thrombin levels by Plm-mediated activation of FXII promote activation of thrombin-activatable fibrinolysis inhibitor and attenuate fibrinolysis [13]. The roles of other coagulation factors on Plm-mediated procoagulant activity remain to be determined, however. Recently, we have demonstrated the precise mechanism of Plm-catalyzed proteolysis of FVIII, by which levels of FVIII activity were initially elevated ~two-fold in the presence of Plm and subsequently rapidly diminished [10,14,15]. We speculated, therefore, that, in addition to the activation of FXII, the action of Plm on FVIII and/or

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other coagulation factors might contribute to enhanced procoagulant activity and to Plm-induced generation of thrombin.

Assessment of blood coagulation *in vitro* is essential for precise clinical evaluation of clotting function. Conventional one-stage assays such as the prothrombin time (PT) and activated partial thromboplastin time (aPTT) are useful for routine laboratory examination, but they reflect only a part of coagulation in nonphysiological conditions and are based on the classical concepts of intrinsic and extrinsic enzyme cascade mechanisms. More recently, interest has been focused on global coagulation assays, developed from a better understanding of the coagulation reaction involving tissue factor (TF)-triggered, cell-based mechanisms generating thrombin on activated platelets [16]. In particular, three global tests of this nature have been established and systems for computerized thromboelastometry [17], clot waveform analysis [18,19], and thrombin generation tests [20] have become available commercially. These techniques provide a quantitative evaluation of clotting function using various parameters derived from continuous measurement of the overall coagulation process and the new data appear to correlate more closely with the clinical phenotype of patients [21].

In the present study, we monitored Plm-induced changes in blood coagulation using these global coagulation assays and demonstrated that accelerated procoagulant activity in the presence of high concentrations of Plm (≥125 nmol/l) was dependent on FV and/or FVIII.

Materials and methods

Reagents

Purified human plasma-derived Lys-Plm was purchased from Sigma (St Louis, Missouri, USA) and its specific activity was 4.8 unit/mg. We confirmed that Plm contained any little amounts of thrombin, FXa, activated protein C, and other relevant coagulation factors (data not shown). In addition, the chromogenic method and SDS-PAGE showed that Plm activity was stable during each assay (data not shown). Phospholipid vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, and 30% phosphatidylethanolamine were prepared using *N*-octyl glucoside [22]. APTT reagent (12 μmol/l phosphatidylserine, 76 μmol/l phosphatidylcholine, 40 μmol/l phosphatidylethanolamine, and 0.1 mmol/l ellagic acid) and PT reagent (0.5 ml/ml rabbit brain thromboplastin, 12.5 mmol/l calcium lactate) were purchased from Sysmex Corporation (Kobe, Japan). Recombinant human TF (Innovin, Dade Behring, Marburg, Germany), fluorogenic specific-substrate for thrombin, Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland), thrombin calibrator (Thrombinoscope, Maastricht, Netherlands), α_2-antiplasmin (Biodesign International, Saco, Maine, USA), and corn trypsin inhibitor (CTI; Haematologic Technologies Inc, Essex, Vermont, USA) were purchased from the indicated vendors. HEPES-buffered saline (20 mmol/l HEPES pH 7.2, 100 mmol/l NaCl, and 0.01% Tween 20) was used for dilution of reagents.

Blood samples

Whole blood was obtained by venepuncture from healthy volunteers into tubes containing a 1:9 volume of 3.8% (w/v) trisodium citrate. Pooled normal plasma from 10 healthy volunteers were prepared from platelet poor plasma (PPP) obtained by centrifugation of citrated whole blood for 10 min at 1500*g*. FV, FVII, FVIII, FIX, FXI, and FXII-deficient plasmas derived from patients with individual coagulant factor-deficiency were purchased from George King Bio-Medical Inc. (Overland Park, Kansas, USA) and α_2-antiplasmin immune deficient plasma was purchased from Affinity Biologicals Inc. (Ontario, Canada).

Rotational thromboelastometry

Rotational thromboelastometry (ROTEM) was performed using the whole blood hemostasis analyzer (Pentapharm, Munich, Germany). After drawing, citrated whole blood was kept at rest for 30 min at room temperature and was used within 2 h. Various concentrations of Plm (20 μl; final concentration up to 1500 nmol/l) were mixed with whole blood (280 μl) at the start of measurement with 20 μl of $CaCl_2$ (final concentration 12.5 mmol/l). In some experiments, CTI (final concentration 25 μg/ml) was added just after drawing whole blood and measurements were started when Plm and $CaCl_2$ were added with or without 10 μl of TF final concentration 0.5 pmol/l). Clot formation was assessed using clotting time (CT; the time from the start of measurement until detection of clot firmness of 2 mm amplitude), clot formation time (CFT; the time from the initiation of clotting until detection of clot firmness of 20 mm amplitude), and maximum clot firmness (MCF; the maximum amplitude indicating the clot stabilization).

Clot waveform analysis

Clot waveform analysis was performed on the MDA-II system (Trinity Biotech plc, Dublin, Ireland) using APTT and PT reagents as previously described [18,19]. In an APTT-based assay, plasma (50 μl) was mixed with APTT reagent (50 μl) for 4 min, followed by $CaCl_2$ (50 μl; final concentration 6.7 mmol/l) and various concentrations of Plm. In a PT-based assay, plasma (50 μl) was mixed with PT reagent (100 μl) containing $CaCl_2$ (final concentration 8.3 mmol/l) and various concentrations of Plm simultaneously. The clot waveform obtained was computer-processed using the commercial kinetic algorithm. The rate of clotting and acceleration of the clotting rate were computed respectively from the first-order and second-order differentials of transmittance. The minimum absolute value of the first-order differential, |min 1|, represents the maximum coagulation rate and |min 2|, the minimum absolute value of the second-order differential, represents the maximum acceleration of coagulation rate.

The clot time was defined as the time until the start of coagulation.

Thrombin generation test

The calibrated automated thrombin generation test (TGT) (Thrombinoscope, Maastricht, Netherlands) was performed according to the method of Hemker with minor modifications [20]. In this assay, polystyrene microplates (Immulon 2HB 'U' bottom microtiter plates; Thermo, Milford, Massachusetts, USA) were used. Sample plasma (PPP, 75 μl) was incubated for 10 min with 20 μl of reagent containing phospholipid and TF (final concentration 4 μmol/l and 0.5 pmol/l, respectively). In FV-deficient or FIX-deficient plasma, as a very low value of thrombin generation was observed at 0.5 pmol/l of TF, 5 pmol/l of TF was used. Various concentrations of Plm (5 μl) were added to samples prior to the addition of $CaCl_2$ (final concentration 16.7 mmol/l) and fluorogenic substrate (final concentration 2.5 mmol/l). Standard parameters including lag time, peak height, time to peak, and endogenous thrombin potential (ETP) were estimated.

Data analyses

All experiments were performed at least several separate times, and the average values and standard errors were calculated. Significant differences were determined by the paired Student's *t*-test. *P* value (<0.05) was considered as statistically significant.

Results

Effect of plasmin on whole-blood clotting in rotational thromboelastometry

The influence of Plm on whole-blood clotting was initially examined using ROTEM. Various concentrations of Plm (up to 1500 nmol/l) were mixed with individual normal whole blood obtained from healthy volunteers at the same time of the addition of $CaCl_2$. Representative thromboelastograms and the parameters obtained from these data are illustrated in Fig. 1. The well known Plm-catalyzed fibrinolysis in whole blood clot was demonstrated in the thromboelastogram by a decrease in clot firmness in a dose-dependent manner after reaching MCF (Fig. 1a). As the presence of Plm at at least 750 nmol/l revealed significant fibrinolytic reaction, the other parameters were difficult to be calculated. Therefore, these parameters were evaluated under the conditions of Plm at 500 nmol/l or less. CFT values, likely reflecting the velocity of fibrin formation, were also prolonged in a dose-dependent manner, and this effect was significant ($P < 0.05$) by the addition of Plm at at least 250 nmol/l. In contrast, CT values were shortened in a dose-dependent manner. In particular, CT values were significantly shortened ($P < 0.05$) by addition of Plm at 250 nmol/l at least illustrating a procoagulant effect of Plm (Fig. 1b–d). These data supported that Plm affected both the procoagulant and fibrinolytic (and/or anticoagulant) functions.

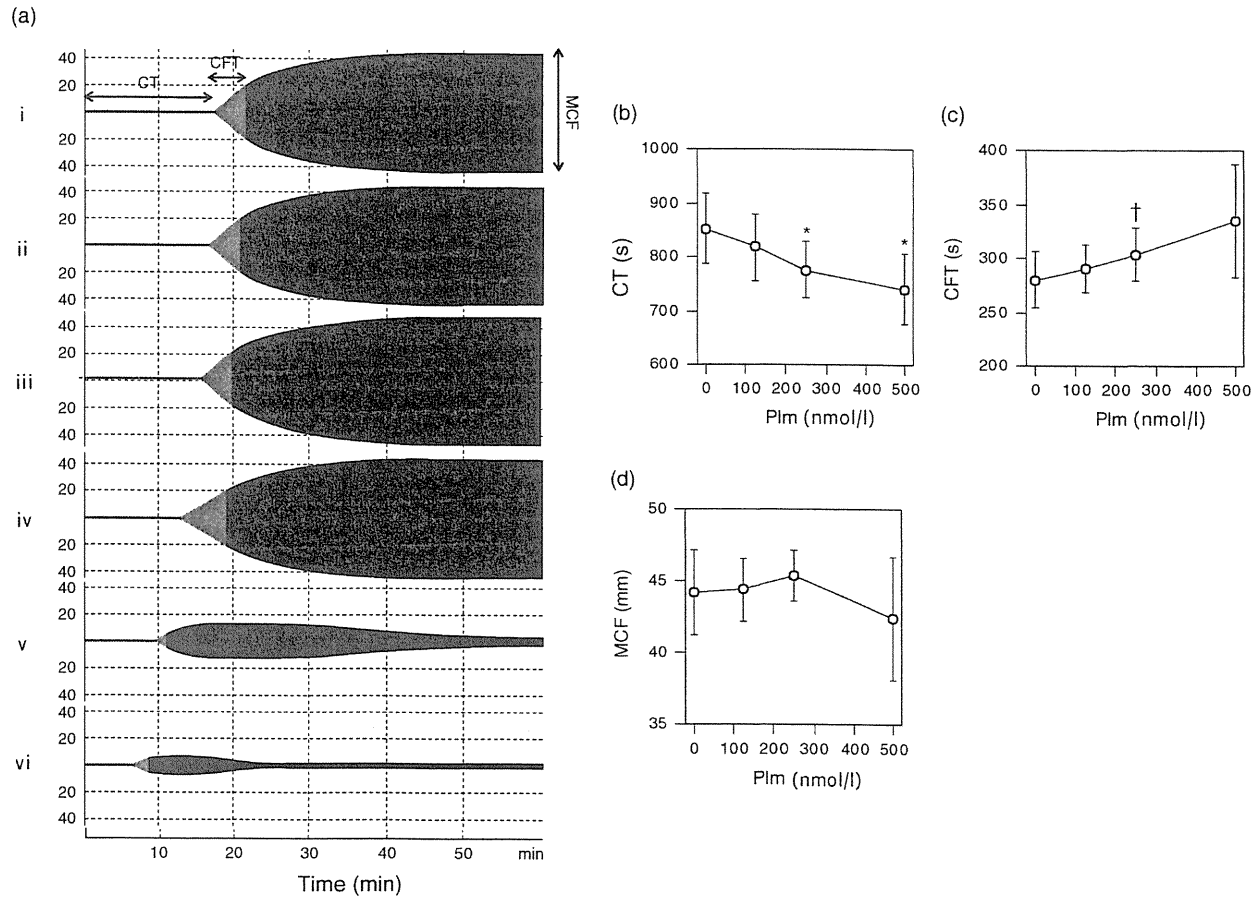
Evaluation of plasmin reaction by clot waveform analysis

Citrated normal pooled plasma was mixed with APTT reagent and incubated together with $CaCl_2$ and Plm. APTT reagent was diluted by four-fold to measure within adequate ranges of APTT. Representative clot waveforms and clot waveform parameters are illustrated in Fig. 2. As well as shortened CT values in ROTEM, the clot times in this assay were significantly shortened even by the presence of Plm at 125 nmol/l ($P < 0.01$), and this effect was dose dependent (Fig. 2a and b). This finding indeed suggested the Plm-induced procoagulant activity. However, both |min 1| and |min 2| values, representing maximum coagulation velocity and acceleration respectively, were decreased in similar dose-dependent manners unexpectedly (Fig. 2c and d). Furthermore, the transmittance curves reascended after descending to the plateau in a Plm dose-dependent manner (Fig. 2a). These results appeared to represent the fibrinolytic action of Plm, different with the clot time. Similar results were observed when plasmas were mixed with PT reagent described in Materials and Methods (data not shown).

Thrombin generation test in the presence of plasmin

The TGT has been utilized recently to focus on global coagulation function based on cell-based clotting mechanisms. Differed with ROTEM and clot waveform analysis, TGT has an advantage that the data are unaffected by fibrin generation or degradation. Therefore, the procoagulant effect of Plm was examined using normal plasma in TGT. Citrated normal plasma was mixed with TF (0.5 pmol/l) and phospholipid (4 μmol/l), and incubated for 3 min with Plm prior to addition of fluorogenic substrate and $CaCl_2$. Representative thrombograms and TGT parameters are illustrated in Fig. 3. A control experiment confirmed that Plm alone little directly reacted with the fluorogenic substrate (by 1–2%, data not shown). The lag time and time to peak values were shortened in dose-dependent manners (Fig. 3b and c), and the peak height and ETP values were increased (Fig. 3d and e), indicating that all parameters were affected by Plm-induced procoagulant activity. In the presence of Plm at 500 nmol/l, the lag time and time to peak shortened by 28 and 35%, respectively, and the peak height and ETP increased by 99 and 47%, respectively, compared to its absence. All parameters, demonstrating accelerated thrombin generation, were significantly moderated at the presence of 250 nmol/l Plm ($P < 0.05$ or $P < 0.01$), and the peak height was particularly increased even at 125 nmol/l Plm ($P < 0.05$). Of note was that the parameters of TGT were not affected by Plm-induced fibrinolysis, whilst as expected, the results of both the ROTEM and clot waveform analysis were affected by the fibrinolytic reaction.

Fig. 1



Effects of plasmin on rotational thromboelastometry.(a) Plasmin (Plm) (i–vi; 0, 125, 250, 500, 750, and 1500 nmol/l, respectively) was added to citrated whole blood of a healthy volunteer with $CaCl_2$ at the start of the assay. The thromboelastograms were visualized using rotational thromboelastometry as described in Methods. Representative data were illustrated in (a). (b–d) Parameters obtained from (a) are shown (clotting time, clot formation time, and maximum clot firmness, respectively). All experiments were performed at least five separate times, and the average values and standard errors were calculated. Significant differences for Plm (0 nmol/l) were expressed as *; $P < 0.05$ and †; $P < 0.01$. CFT, clot formation time; CT,clotting time; MCF, maximum clot firmness; Plm, plasmin.

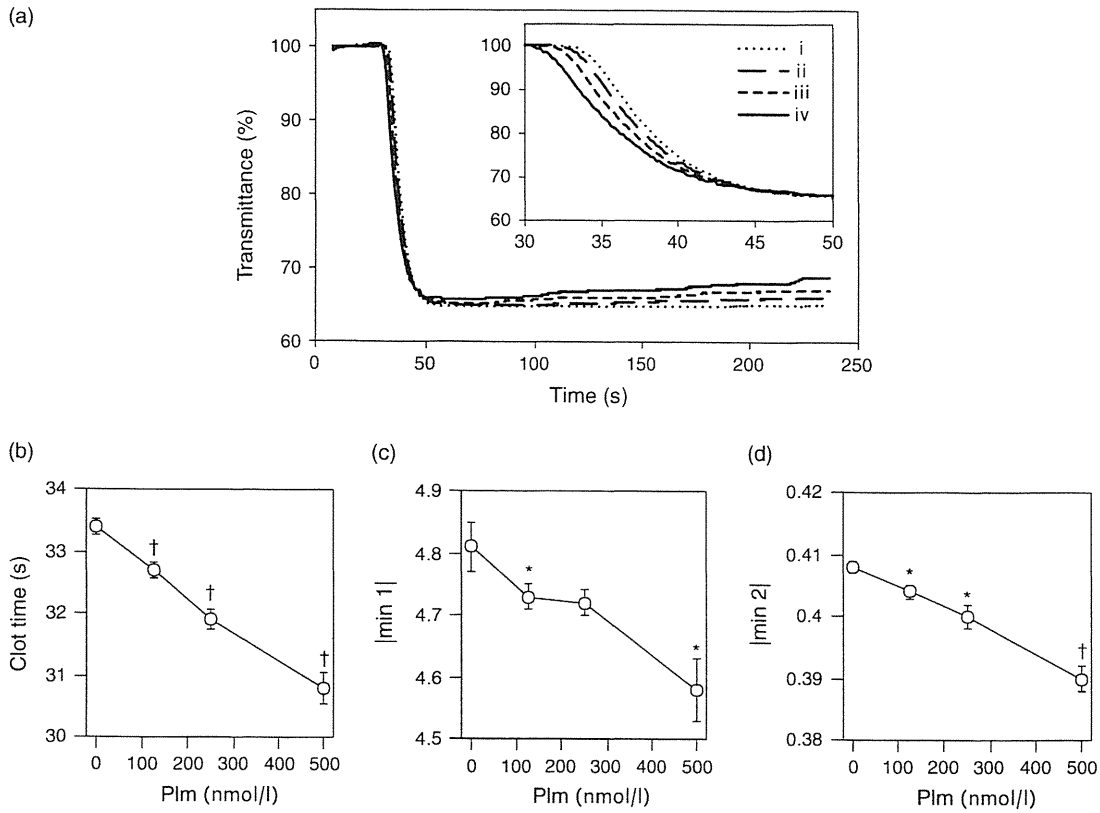
Role of α_2-antiplasmin on plasmin-induced procoagulant activity

Free Plm is rapidly neutralized by α_2-antiplasmin, a Plm inhibitor present physiologically in plasma [1]. We investigated using TGT whether the Plm-induced procoagulant activity was neutralized by α_2-antiplasmin. By the addition of 0.5 μmol/l α_2-antiplasmin in normal plasma, the procoagulant effect of Plm (250 nmol/l) was significantly diminished (data not shown). In order to further confirm that Plm-induced procoagulant effect was modulated by α_2-antiplasmin, we evaluated the Plm effect using α_2-antiplasmin-deficient plasma, instead of normal plasma. The thrombograms obtained by the addition of Plm (0–256 nmol/l) in α_2-antiplasmin-deficient plasma are shown in Fig. 4. The peak height value was significantly increased even at lower concentration of 16 nmol/l Plm ($P < 0.05$, *inset*), and its increase showed the similar level to that by addition of Plm (125 nmol/l) in normal plasma (Fig. 3). This effective concentration was ~10-fold lower than that obtained in normal plasma. Of interest, the addition of Plm (250 Nmol/l) in α_2-antiplasmin-deficient plasma with exogenous α_2-antiplasmin (0.5 μmol/l) showed the similar level of peak height, compared with that of Plm (16 nmol/l) in α_2-antiplasmin-deficient plasma alone (Fig. 4 *line* vi). These findings supported that Plm-induced procoagulant effect likely occurred functionally even at much lower dose of Plm, which could not be overcome by α_2-antiplasmin in plasma.

Corn trypsin inhibitor does not affect the procoagulant effect of plasmin

Several studies have demonstrated that Plm possesses broad-spectrum effects on several coagulation factors [5–10,23]. Therefore, we attempted to determine whether specific coagulation factor(s) attributed to Plm-induced procoagulant effect. The effect of the contact pathway was firstly examined, because some researchers had

Fig. 2



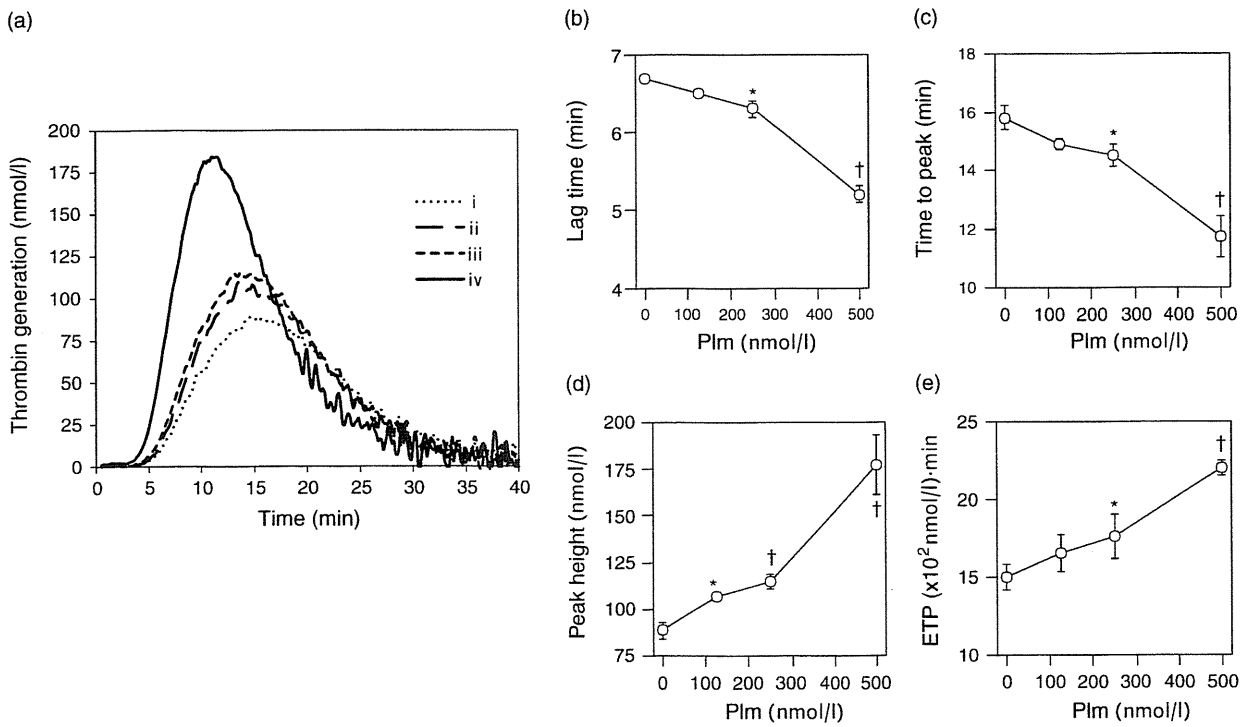
Effects of plasmin on clot waveform analysis.(a) Normal pooled plasma was incubated with activated partial thromboplastin time reagent for 4 min and then plasmin (Plm) (*lines* i–iv; 0, 125, 250 and 500 nmol/l, respectively) was added with $CaCl_2$ at the start of the assay as described in Methods. Representative data were illustrated in (a). *Inset* shows enlarged figure around the point of clot time. (*panels* b–d) Parameters obtained from clot waveform in (a) are shown (clot time, |min 1|, and |min 2|, respectively). All experiments were performed at least five separate times, and the average values and standard errors were calculated. Significant differences for Plm (0 nmol/l) were expressed as *; $P < 0.05$ and †; $P < 0.01$. Plm, plasmin, |min 1|; the minimum absolute value of the first order differential, represents the maximum coagulation rate, |min 2|; the minimum absolute value of the second order differential, represents the maximum acceleration of coagulation rate.

reported that FXII appeared to participate in Plm induced its effect clinically [11–13]. The ROTEM was performed using whole blood in which CTI (25 μg/ml), a specific inhibitor of contact pathway, was added immediately after venepuncture as described in Method. In control experiment in the absence of Plm, the clotting time in the presence of CTI was prolonged by 2.8-fold compared to that in its absence (2412 ± 176 and 852 ± 65 s, respectively), confirming the evident inhibition of contact pathway (Table 1). In the presence of CTI, the clotting time by addition of Plm was shortened dose-dependently, and even at 125 nmol/l Plm showed the significant difference ($P < 0.05$). Furthermore, when TF (0.5 pmol/l) was added to whole blood together with $CaCl_2$ as trigger of coagulation, the clotting time was significantly shortened even in the presence of CTI. These results demonstrated that the contact pathway was not essential to Plm-induced procoagulant effect in this assay.

Procoagulant effect of plasmin using plasmas lacking specific coagulation factors

Next, to determine which coagulation factor(s) were specific for procoagulant activity of Plm, we used several coagulation factor-deficient plasmas (FV, FVII, FVIII, FIX, FXI, and FXII). FII and FX were excluded from these experiments because the techniques are totally dependent on these factors for thrombin generation and fibrin clot formation. Because fresh whole blood samples completely lacking the various coagulation factors were not available, the ROTEM could not be used. Thrombin generation was inconsistent in the TGT using TF (0.5 pmol/l) with FV-deficient or FIX-deficient plasma. Therefore, TF (5 pmol/l) was used in TGT assay, and the results using the individual of deficient plasmas showed reliable thrombin generation. However, the peak height values in all deficient plasmas used were increased by the addition of Plm (250 nmol/l, data not shown), likely supporting the difficulty of this study by TGT.

Fig. 3



Plasmin-induced procoagulant effect in thrombin generation test.(*panel* a) Normal pooled plasma was incubated with tissue factor (0.5 pmol/l) and phospholipid vesicles (4 μmol/l). Plasmin (Plm) (*lines* i–iv; 0, 125, 250 and 500 nmol/l, respectively) was added prior to the addition of fluorogenic substrate and $CaCl_2$ as described in Methods. Representative data were illustrated in (a). (*panels* b–e) Parameters obtained from thrombin generation test in (a) are shown (lag time, time to peak, peak height, and endogenous thrombin potential, respectively). All experiments were performed at least five separate times, and the average values and standard errors were calculated. Significant differences for plasmin (0 nmol/l) were expressed as *; $P<0.05$ and †; $P<0.01$. ETP, endogenous thrombin potential; Plm, plasmin.

The clot waveform analysis using both the APTT-based and PT-based assays utilizing these plasmas were further investigated. In this technique, the clot time parameter reflects the procoagulant activity (Fig. 2). Unlikely the data shown in Fig. 2, APTT reagent was not diluted in this APTT assay, because some of coagulation factor-deficient plasmas prolonged the APTT over the limit of measurement. With normal plasma, the clot time was shortened by the addition of Plm (500 nmol/l) in both the APTT and PT assays (Table 2). Similarly, with FVII-deficient, FIX-deficient, FXI-deficient, and FXII-deficient plasmas, the clot times were shortened by Plm in both assays. However, only FV-deficient and FVIII-deficient plasmas showed distinct results from other plasmas. PT assay with FV-deficient plasma and APTT

Table 1 Effect of corn trypsin inhibitor on clotting time in rotational thromboelastometry in the presence of plasmin

		Plm			
CTI	TF	0 nmol/l	125 nmol/l	250 nmol/l	500 nmol/l
		s			
(–)	(–)	852 ± 65	818 ± 62	776 ± 52*	740 ± 65*
(+)	(–)	2412 ± 176	2212 ± 127*	1964 ± 68*	1576 ± 90†
(+)	(+)	580 ± 32	473 ± 17†	481 ± 27*	433 ± 17†

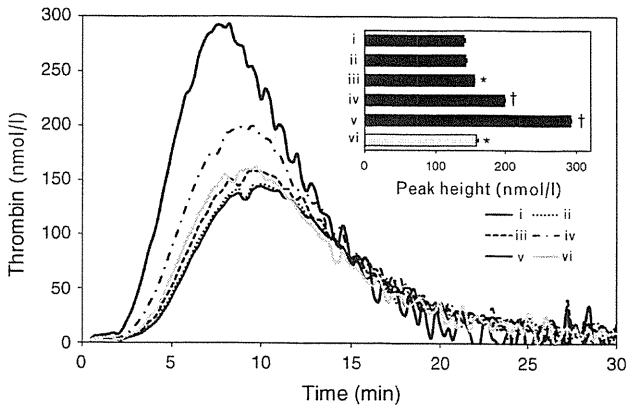
The rotational thromboelastometry was performed using whole blood in the presence of corn trypsin inhibitor (25 μg/ml) with or without tissue factor (0.5 pmol/l). All experiments were performed at least five separate times, and the average values and standard errors were calculated. Significant differences for plasmin (0 nmol/l) were expressed as *; $P<0.05$ and †; $P<0.01$. CTI, corn trypsin inhibitor; Plm, plasmin; TF, tissue factor.

Table 2 Clot time of clot waveform analysis on plasmas lacking of coagulation factors by the addition of plasmin

	APTT-based assay		PT-based assay	
	Plm		Plm	
Deficient plasma	0 nmol/l	500 nmol/l	0 nmol/l	500 nmol/l
	s		*s*	
Normal	27.0 ± 1.0	23.0 ± 0.7†	10.9 ± 0.2	10.4 ± 0.2†
FV	82.9 ± 0.7	56.6 ± 0.4†	41.6 ± 0.3	***49.1 ± 1.2***†
FVII	27.0 ± 0.1	20.6 ± 0.1†	68.3 ± 1.3	58.4 ± 0.4†
FVIII	90.2 ± 7.5	***103 ± 7***†	13.8 ± 0.0	12.7 ± 0.0†
FIX	74.5 ± 0.9	73.0 ± 1.2*	13.5 ± 0.1	12.5 ± 0.1†
FXI	77.7 ± 5.1	57.4 ± 0.4†	11.7 ± 0.2	11.1 ± 0.1†
FXII	212 ± 14	100 ± 4†	14.3 ± 0.0	13.4 ± 0.0†

All experiments were performed at least five separate times, and the average values and standard errors were calculated. Significant differences for plasmin (Plm) (0 nmol/l) were expressed as *; $P<0.05$ and †; $P<0.01$. Bold shows expanding of clot time by Plm. Activated partial thromboplastin time (APTT) in normal plasma was different from that obtained in Fig. 2 because APTT reagent was not diluted in this assay. APTT, activated partial thromboplastin time; FV, factor V; Plm, plasmin; PT, prothrombin time.

Fig. 4



Effect of α_2-antiplasmin on plasmin-induced procoagulant activity in thrombin generation test. The α_2-antiplasmin-deficient plasma was incubated with tissue factor (0.5 pmol/l) and phospholipid vesicles (4 μmol/l), and then plasmin (Plm) (*lines* i–v; 0, 4, 16, 64 and 256 nmol/l, respectively) was added prior to the addition of fluorogenic substrate and $CaCl_2$ as described in Methods on thrombin generation test. *Line* vi shows the addition of Plm (256 nmol/l) to the α_2-antiplasmin-deficient plasma with the exogenous α_2-antiplasmin (0.5 μmol/l). (*Inset*) The peak height values obtained in lines i–vi are shown. All experiments were performed at least five separate times, and the average values and standard errors were calculated. Significant differences compared to the absence of Plm (*line* i) were expressed as *; $P < 0.05$ and †; $P < 0.01$.

assay with FVIII-deficient plasma demonstrated no shortening but rather prolongation of the clot time. Nevertheless, each paired assay (APTT assay with FV-deficient plasma and PT assay with FVIII-deficient plasma) showed shortening of the clot times. This discrepancy between PT and APTT assays might be explained by the reason that the raised levels of other coagulation factors, including FVIII in FV-deficient plasma and FV in FVIII-deficient plasma, influence these assays. Taken together, these findings supported that Plm accelerated the blood coagulation reaction in association with FV and/or FVIII.

Discussion

We have demonstrated, using the combination of global hemostasis assays of ROTEM, clot waveform analysis, and TGT, reflecting real-time monitoring of whole-blood clotting, fibrin clot formation and thrombin generation, respectively, that Plm at relatively high concentrations (≥125 nmol/l) significantly accelerated the blood coagulation. Furthermore, the clot waveform analysis using FV-deficient and FVIII-deficient plasmas indicated that these Plm-induced procoagulant activities could be linked to an association with the cofactor activities of FV and/or FVIII.

Plg circulates at a concentration of ~2.0 μmol/l in plasma [1] and high concentrations of Plm (≥125 nmol/l) may be unlikely in physiological coagulant and/or fibrinolytic states. Some pharmacological (therapeutic) interventions, however, could be associated with plasminemia. Eisenberg *et al.* [2] reported that in patients with AMI where coronary occlusion persisted and/or recurred after administration of Plg activators, an increase in thrombin activity was paradoxically observed. Several other clinical studies also indicated that pharmacological Plg activation induced an elevation of thrombin generation [3,4]. We also observed that the addition of uPA (Plg activator), instead of Plm, gave similar ROTEM patterns obtained by adding Plm (unpublished data). Circulating free Plm is neutralized immediately by α_2-antiplasmin (~1 μmol/l at plasma concentration), and the actual functional concentration of Plm available to mediate a procoagulant effect would likely be much less than 125 nmol/l used in the present study. Indeed, examinations using α_2-antiplasmin-deficient plasma showed that elevation of thrombin generation was observed in the presence of Plm even at ~10 nmol/l. Our earlier studies also demonstrated that even very low concentrations (1~4 nmol/l) of Plm were sufficient to promote catalytic activation and inactivation of FVIII *in vitro* [10], suggesting that small amounts of Plm generated physiologically in the fibrinolytic response might contribute to the upregulation and downregulation of blood coagulation.

The procoagulant effects of fibrinolytic therapy may be assessed by measuring fibrinopepitide A, thrombin-antithrombin complex, and prothrombin fragment 1+2, but these techniques are complex and provide limited information. In comparison, the global coagulation assays used in the present study offer the advantages of being relatively straightforward to perform and provide multiple parameters obtained during the real-time course of clot formation and fibrinolysis. Such parameters, the clotting time in clot waveform analysis and ROTEM were significantly shortened by the addition of Plm, indicative of a procoagulant effect. Plm-induced fibrinolysis suppresses the development of the fibrin clot, however, and this could have moderated the measurements of other parameters in these assays (CFT and MCF in ROTEM, and |min 1| and |min 2| in clot waveform analysis). In contrast, the TGT monitors thrombin generation directly and is independent of fibrin formation. Our results demonstrated procoagulant patterns with all TGT parameters and these data provided strong evidence that Plm was associated with procoagulant activity that could be masked in assays based on fibrin formation. It seemed highly likely, therefore, that the Plm-induced procoagulant effect occurred simultaneously with fibrinolysis.

Mechanisms responsible for the procoagulant effect of Plm have been previously investigated and activation of FXII by Plm appeared to be involved [11–13]. In the present study, of surprise, analyses using whole blood with CTI and using plasmas devoid of specific coagulation factors (FV, FVII, FVIII, FIX, FXI, and FXII) clearly showed, however, that Plm-induced procoagulant activity was exerted even in the presence of CTI and in the absence of either FXI or FXII. We also observed the

similar level of Plm-induced increase of thrombin generation even in the presence of CTI compared with in its absence using our TGT (data not shown). Furthermore, the clot times obtained in PT-based and APTT-based assays were not shortened in FV-deficient and FVIII-deficient plasma, respectively, even in the presence of FXI and FXII. These results suggested that FV and FVIII rather than FXII play significant roles in Plm-induced procoagulant effect, showing our findings appear to contradict earlier reports. Although the reasons for it remain unclear, the presence of TF might affect. In the earlier reports showing that Plm-mediated procoagulant activity was dependent on FXII activation, only recalcification as trigger of blood coagulation was used [11], which assay was more sensitive to contact pathway compared with TF-triggered assay. In our investigations, calcium-triggered TGT demonstrated a significant inhibitory effect of CTI on Plm-mediated increase of thrombin generation when TF was absent. When TF was present, however, Plm increased thrombin generation sufficiently even in the presence of CTI (data not shown). In these days, a concept of cell-based blood coagulation mechanism is widely accepted, which requires a small amount of TF as trigger of blood coagulation [16]. More recently, it has been reported that fibrinolysis failure in AMI was related to higher TF levels associated with microparticles and higher thrombin generation [23], which might support TF-triggered model in Plm-mediated procoagulant activity. In addition, our data might be supported by an earlier study that the intrinsic tenase complex (FVIIIa/FIXa/FX) was essential for Plm-mediated thrombin generation [24]. Furthermore, although we have utilized novel techniques of global hemostasis analysis, studies are in progress to resolve potential discrepancies.

Earlier basic studies using purified systems demonstrated that Plm initially activated FV and FVIII followed by rapid inactivation [9,10]. However, Plm-induced procoagulant activity was maintained during greater than 30 min in plasma (data not shown). This reason remains to be known at present and further investigations would be required. Nevertheless, such biphasic effects of Plm on FV and FVIII appear to be unique and are different from effects of Plm on other coagulation factors. FV and FVIII are structurally homologous and have similar functions [25]. FVa is a cofactor in the prothrombinase complex and FVIIIa functions as a cofactor for intrinsic tenase complex and enhance the activation of prothrombin or FX by ~240-fold [26] or ~170 000-fold [27], respectively. The cofactors are widely considered to play important roles not only for the blood coagulation system, but also for the interaction between coagulation and fibrinolysis.

Our present data may show indirect evidence of a crucial role of FV and/or FVIII for Plm-induced procoagulant activity. Because Plm possesses multieffects on several coagulant factors, it is very difficult to confirm direct evidence using plasma or whole blood. However, based on our present data and previous basic studies that Plm initially activates FVIII and FV, followed by inactivation through FVIII(a)-Plm and FV(a)-Plm direct interaction [9,10,14,15,28], we suggest that the procoagulant activity of Plm in the blood coagulation appeared to be attributed by FV and/or FVIII.

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