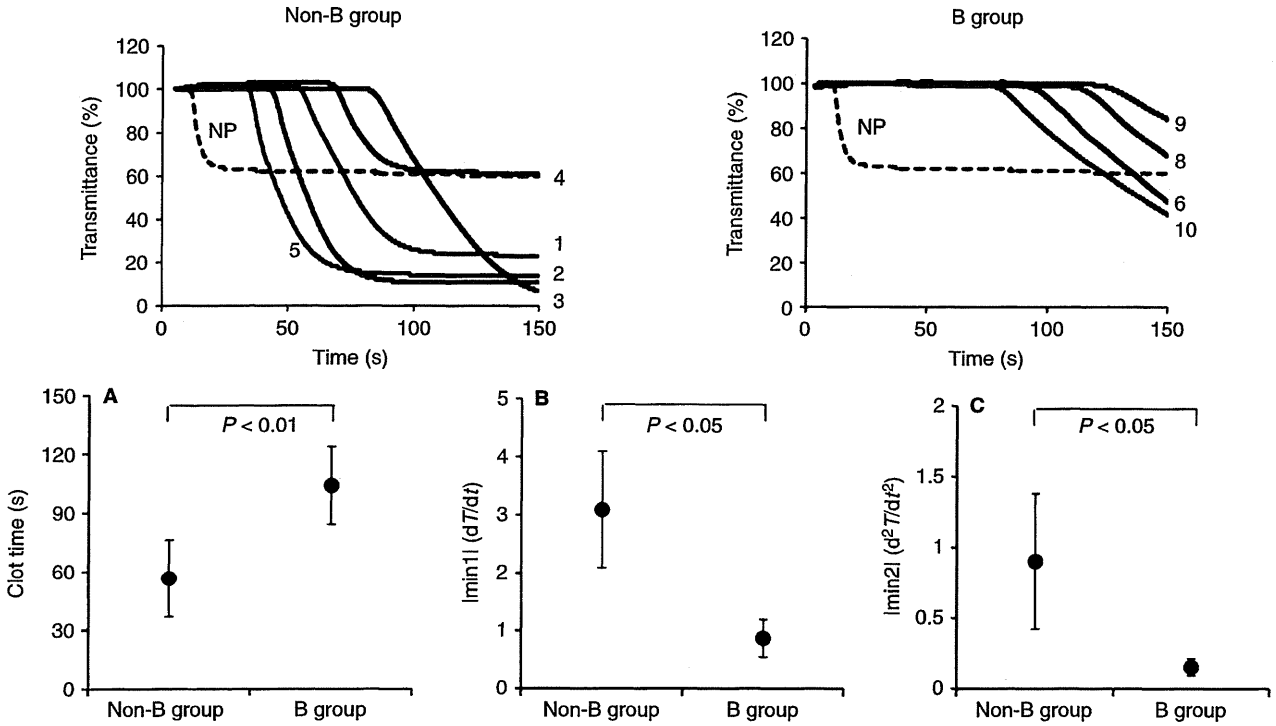

Fig. 1. Clot waveform analysis in plasmas from patients with acquired factor V inhibitors. The prothrombin times in patients' plasmas in both groups were measured with the MDA-II system. The parameters (lower panels) clot time (A), min1 (B) and min2 (C) were calculated from the clot waveform (upper panels) observed, as described in Materials and methods. In all instances, results are shown as mean ± standard deviation from at least five separate experiments. NP and lines 1–10 refer to normal plasma and cases 1–10. Because of the small volume of the sample from case 7, this case failed to show the curve of clot waveform.

the B group inhibited this binding dose-dependently (by 60–90%; IC_{50}, 10–30 μg mL^{-1}), whereas those from the non-B group did not significantly inhibit binding (i.e. by < 15%), even at the maximum concentration employed (Fig. 3). These findings strongly suggested that the severe hemorrhagic tendency in the B group was associated with significant inhibition of FV binding to PL by the anti-FV inhibitors.

Effects of anti-FV autoAbs on prothrombinase activity

The effects of anti-FV autoAbs on the activity of the prothrombinase complex were examined in a purified assay, even though thrombin generation appeared to be equally depressed in all patients. All of the available IgGs from the B group significantly inhibited prothrombinase activity dose-dependently, by > 90% at the maximum concentration, probably because of failure of FV(a)–PL binding. Similarly, all IgG preparations from the non-B group also depressed prothrombinase activity dose-dependently, but this inhibition (by 50–60%) at the maximum concentration was comparatively modest (Fig. 4). The autoAbs from the non-B group had little effect on FV(a)–PL binding, and it might be that inhibition of prothrombinase activity in these circumstance reflected interactions of FVa with other components of the complex (FXa and/or prothrombin).

APC sensitivity in the presence of A-FV inhibitors

An alternative function of FV is as an anticoagulant cofactor of APC in FVIIIa inactivation [6], and it seemed possible that the asymptomatic phenotype in some patients with FV autoAbs might be attributable to some effect of the acquired inhibitor on these secondary properties of FV. To investigate this, APC-mediated inactivation of FVIIIa with anti-FV inhibitor plasmas was examined in an intrinsic FXa generation assay. FXa generation was determined with a plasma-based assay with exogenous APC. Figure 5A shows absorbance readings in this assay in the absence or presence of exogenous APC, and Fig. 5B shows the calculated APCsr. The results indicated that the APCsr in the B group (2.05 ± 0.08) was lower than that in normal plasma (2.45 ± 0.03), but was markedly decreased in the non-B group (1.45 ± 0.13). The APCsr in the non-B group appeared to be similar to that observed with APCR plasmas with FV Leiden or FV Nara (~ 1.5) [27,28].

Effects of anti-FV autoAbs on the APC cofactor activity of FV

The APC cofactor function of FV in FVIIIa inactivation was further examined in a purified assay. The initial rates

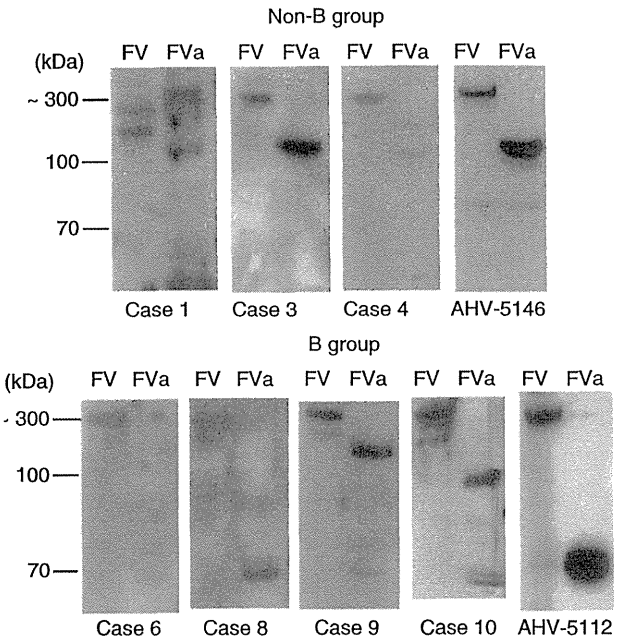

Fig. 2. Identification of epitope(s) of acquired factor V inhibitors. Samples of FV and thrombin-cleaved FVa (100 nM) were analyzed by 8% gel SDS-PAGE, and this was followed by transfer to poly (vinylidene difluoride) membranes. The membranes were incubated with patients' plasmas, and binding to FV(a) fragments was detected by further incubation with anti-human peroxidase-linked secondary antibody. As a positive control, detection with AHV-5146 (heavy chain) and AHV-5112 (light chain) are shown.

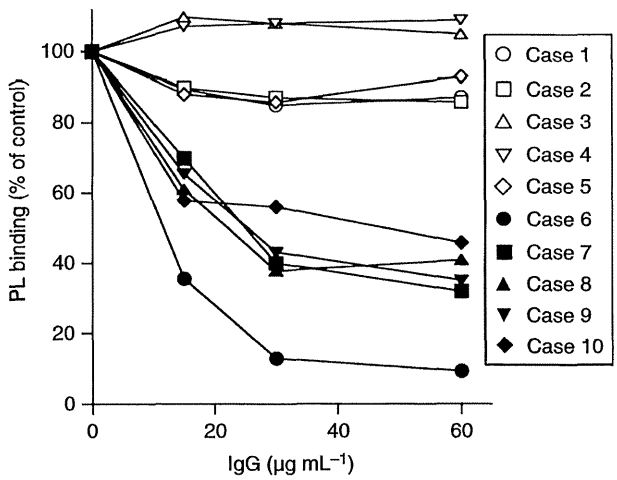

Fig. 3. Effects of anti-FV autoantibodies (autoAbs) on FV binding to phospholipid (PL) in solid-phase-based ELISA. α-Phosphatidyl-L-serine (5 μg mL^{-1}) in methanol was added to microtiter wells, and air-dried at 4 °C overnight. After blocking with gelatin solution, the reactant mixtures with FV (1 nM) and various concentrations of anti-FV autoAbs were added to the PL-coated well. Bound FV was quantified with anti-FV mAb as described in Materials and methods. The absorbance of FV binding to PL without anti-FV autoAb represents 100%. Binding to FV in the presence of normal IgG was used as a control. The percentage of FV binding was plotted as a function of the anti-FV autoAb concentration. All experiments were performed at least three separate times, and the average values are shown.

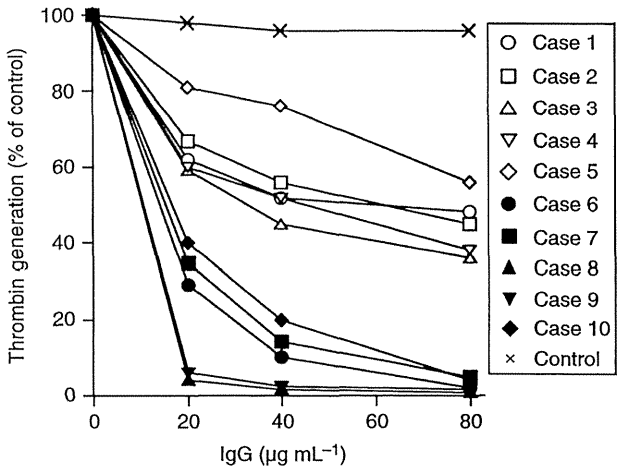

Fig. 4. Prothrombinase activity in the presence of anti-FV autoantibodies (autoAbs). Mixtures of FVa (2 nM) and anti-FV autoAbs were added to prothrombin (1.4 μM), phospholipid vesicles (20 μM), and 5-dimethylamino-naphthalene-1-sulfonylarginine-*N*-(3-ethyl-1,5-pentanediyl)-amide (30 μM). Thrombin generation was initiated by the addition of FXa (10 pM) as described in Materials and methods. Rates of thrombin generation were determined at 405 nm, after the addition of S-2238. The initial rate of thrombin generation without anti-FV autoAb represents 100%. The percentage of prothrombinase activity was plotted as a function of the anti-FV autoAb concentration. All experiments were performed at least three separate times, and the average values are shown.

of FXa generation are shown in Fig. 6. Control experiments demonstrated that FXa generation in the absence of APC (~ 205 nM) was reduced by the presence of APC (~ 175 nM), and was further reduced when both APC and FV were present (~ 145 nM), again confirming FV cofactor activity in the APC-mediated inactivation of FVIIIa. Mixtures of FV with anti-FV autoAbs from all patients in the non-B group enhanced FXa generation dose-dependently, whereas the results obtained with similar mixtures containing anti-FV autoAbs from the B group were not significantly different from those obtained with normal IgG. These results strongly indicated that anti-FV autoAbs from the non-B group impaired the APC cofactor activity of FV, and inhibitors from the B group had little effect on this function of FV.

Effects of anti-FV autoAbs on APC-catalyzed cleavage of FVa HCh

FV/FVa-related APCR is governed by reduced sensitivity of FVa to APC-mediated inactivation and/or impairment of the APC cofactor activity of FV in FVIIIa inactivation. Experiments were therefore designed to investigate the effects of anti-FV autoAbs on APC-mediated proteolytic inactivation of FVa (Fig. 7). The time-related cleavage of HCh was analyzed by SDS-PAGE/western blotting with an anti-FV HCh mAb recognizing residues 307–506. When FVa and normal IgG were used, the band representing residues 1–506 rapidly appeared within

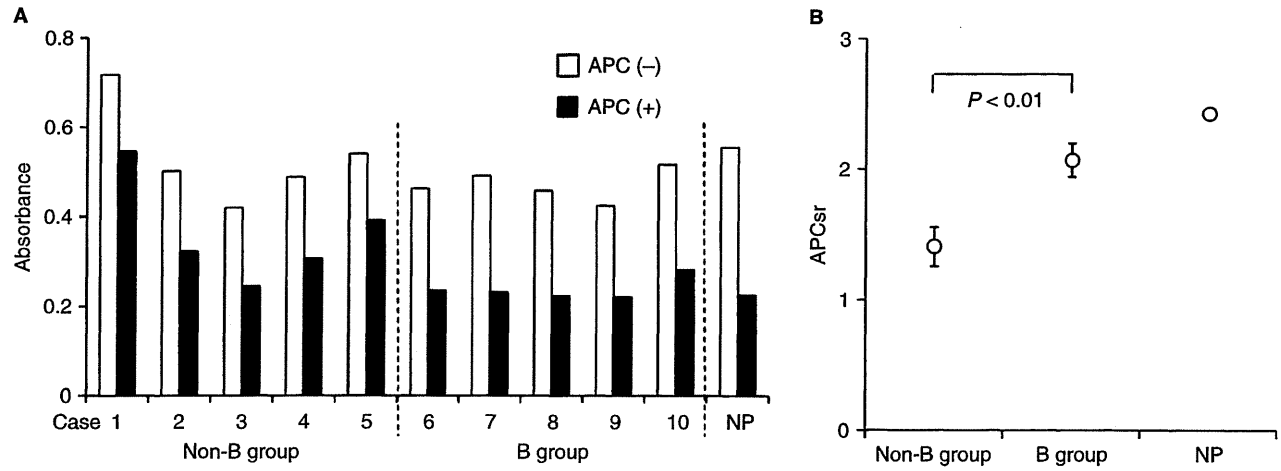

Fig. 5. Activated protein C (APC) sensitivity ratio (APCsr) in the non-B group or B group with acquired factor V inhibitors. FXa generation assays in the presence of APC with patients' plasmas or normal plasma (NP) were performed with COATEST SP FVIII as described in Materials and methods. (A) The absorbance readings for FXa generation obtained in the absence (open bars) or presence (solid bars) of exogenous APC (40 nM) in both groups. The APCsr shown in (B) was expressed as the absorbance obtained in the absence of APC divided by that in its presence. Results are shown as mean ± standard deviation from at least five separate experiments.

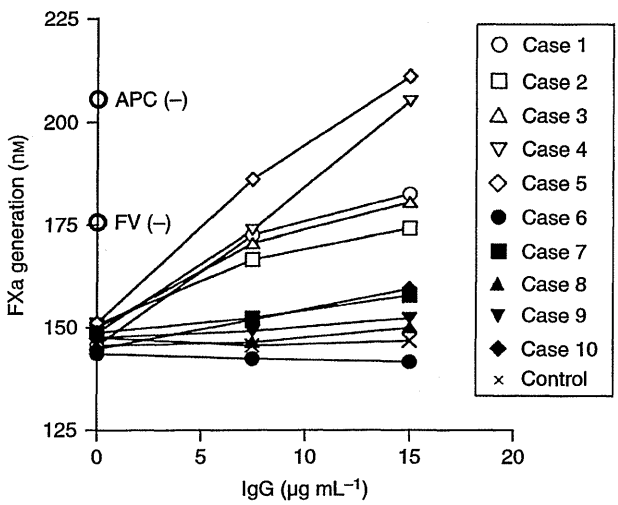

Fig. 6. Effects of anti-FV autoantibodies (autoAbs) on the activated protein C (APC) cofactor activity of FV assessed by FVIIIa degradation. FVIII (10 nM) and phospholipid (20 μM) were activated by thrombin (5 nM), and this was followed by the addition of hirudin. Generated FVIIIa was incubated with APC (1 nM) and protein S (4 nM) together with mixtures of FV (1 nM) and anti-FV inhibitor autoAbs (7.5 and 15 μg mL^{-1}) for 20 min. FXa generation was initiated by the addition of FIXa (2 nM) and FX (200 nM) for 1 min, and the generated FXa was measured by the addition of S-2222. The initial rates of FXa generation were expressed as the FXa generation calculated from the standard reference curve for FXa. Control experiments were performed in the absence of either APC or FV. All experiments were performed at least three separate times, and the average values are shown.

2 min after the addition of APC, and this was followed sequentially by strong band representing residues 307–506, and a faint band representing residues 307–709. These patterns were consistent with rapid, consecutive cleavage of FVa at Arg506 and Arg306. The cleavage patterns of FVa with anti-FV autoAbs in the non-B group demonstrated the appearance of bands representing residues 1–506 from all cases, except for case 5, at a similar velocity to that obtained with control IgG, but the appearance of bands representing residues 307–506 from all cases appeared to be markedly delayed. In all instances, densitometry analysis also demonstrated a 50–90% reduction relative to control in the intensity of bands representing residues 307–506, suggesting inhibition of cleavage at Arg306 but not at Arg506. In all B group cases, however, APC cleavage of HCh was significantly inhibited or completely abolished at both Arg306 and Arg506, owing to the inhibition of FVa–PL binding by anti-FV autoAbs (data not shown). Taken together, these findings suggest that the APCR in the non-B patients could be attributable not only to impairment of the APC cofactor activity of FV in FVIIIa inactivation, but also to reduced APC-mediated inactivation of FVa, related to significant delay in cleavage at Arg306.

Discussion

We investigated coagulation activity and functional mechanisms, associated with either asymptomatic or hemorrhagic symptoms, in patients with A-FV inhibitors. The results indicated that the clinical phenotype in these patients was dependent on specific epitopes of the anti-FV autoAbs. Our evidence is based on the following findings: (i) the PT-based CWA showed lower hemostatic function in the B group than in the non-B group; (ii) anti-FV autoAbs from the B group reacted predominantly with the LCh, whereas those from the non-B group reacted more with the HCh; (iii) anti-FV autoAbs from the B group

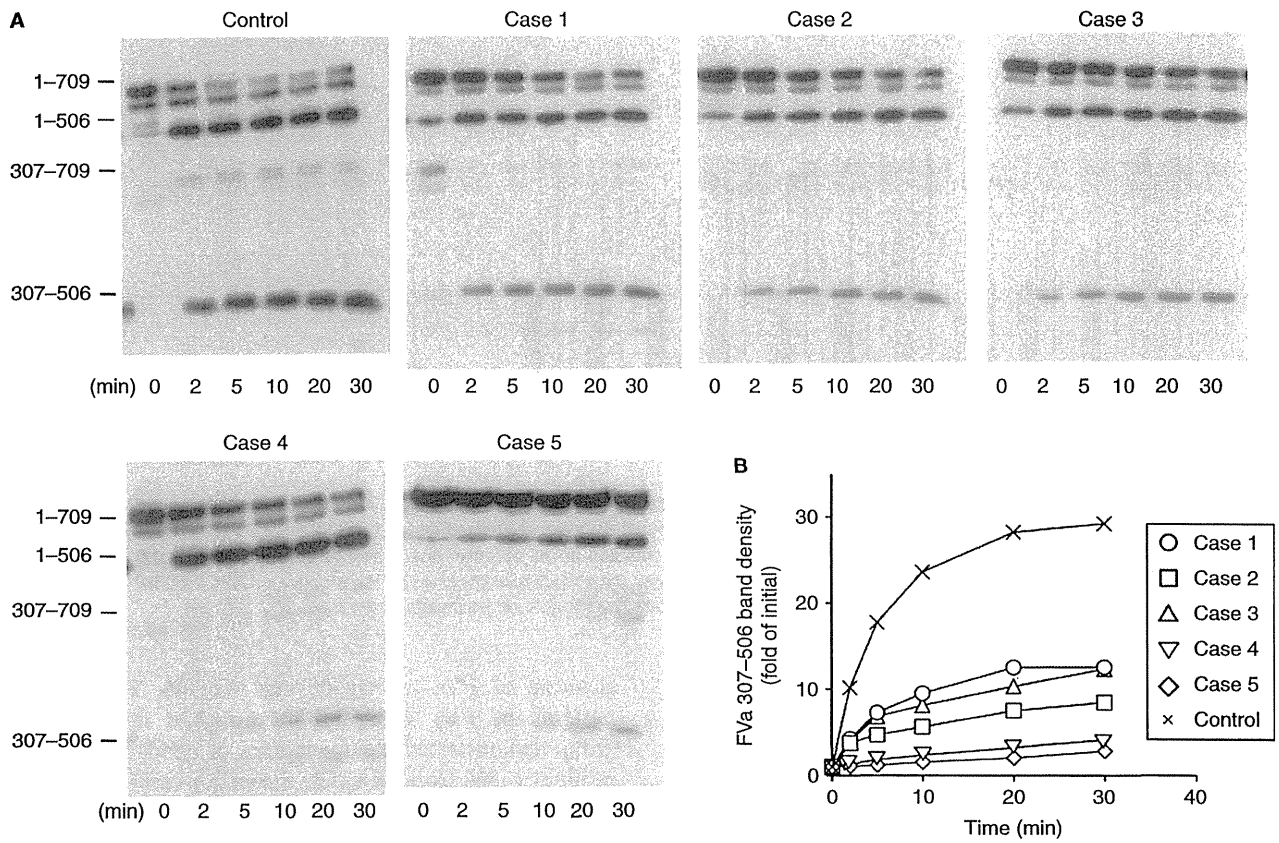

Fig. 7. Activated protein C (APC)-catalyzed proteolytic cleavage of the FVa heavy chain in the presence of anti-FV autoantibodies (autoAbs) from the non-B group. Mixtures of FV (8 nM) and anti-FV autoAbs (30 μg mL^{-1}) from the non-B group were incubated with thrombin (30 nM) for 5 min, and the reaction was terminated by the addition of hirudin. FVa (0.5 nM) from these mixtures was incubated with APC (0.7 nM), protein S (30 nM) and phospholipid (20 μM) for the indicated times. Samples were analyzed on 8% gels, and this was followed by western blotting with an anti-FV mAb. Band densities of the 307–506 fragments were measured by quantitative densitometry. Individual band densities at time zero were regarded as initial, and those obtained at the indicated times were expressed as fold of initial. All experiments were performed at least three separate times, and representative data are shown.

significantly inhibited FV–PL binding, whereas this was unaffected by non-B inhibitors – consequently, prothrombinase activity was completely depressed in the presence of B group antibodies, but was only modestly depressed in the presence of non-B inhibitors; and (iv) anti-FV autoAbs from the non-B group inhibited APC-mediated cleavage of FVa at Arg306, but not at Arg506, and also impaired the APC cofactor activity of FV, consistent with APCR in the non-B group. Overall, the findings demonstrated that negligible or severely reduced prothrombinase activity in the B group, as a result of the inhibition of FV(a)–PL binding by anti-FV autoAbs, caused the severe hemorrhagic symptoms in these patients, whereas the modestly reduced prothrombinase activity together with APCR in the non-B group caused the asymptomatic phenotype.

A one-stage clotting assay is commonly used for the measurement of clotting factors, including FV, and activity levels generally correlate with clinical presentation. Earlier reports have shown, however, that this type of assay has limitations for the assessment of coagulation function in patients with acquired coagulation inhibitors. In consequence, a number of global coagulation functional assays have been established and have been utilized for clinical diagnosis [29,30]. We recently reported that CWA and the TGT identified more disordered coagulation function in patients with acquired hemophilia A than in those with congenital severe hemophilia A (FVIII:C of < 1%) [31]. The present data complement those findings, and show that CWA could help to distinguish between the bleeding and non-bleeding phenotypes in patients with A-FV inhibitors. The results highlight the possibility that this technique could provide valuable data for predicting and/or monitoring hemorrhagic symptoms in patients of this type.

The PL-binding site(s) of FV(a) is located on the C1 and C2 domains within the LCh [26,32], and this binding is associated with the procoagulant action of FV as a cofactor for FXa in the prothrombinase complex. We found that the anti-FV autoAbs in our B group reacted with the LCh, although the precise C2 epitope remains to be identified. Furthermore, these autoAbs inhibited

FV–PL binding by 60–90%, and markedly depressed prothrombinase activity. Ortel *et al.* [33] reported that their anti-FV inhibitors in patients with hemorrhagic symptoms bound to the C2 domain, and resulted in markedly reduced prothrombinase activity, owing to interference with FV–PL binding. The findings strongly suggest the presence of C2 epitopes in our anti-FV autoAbs.

A degradation assay demonstrated that the effects of anti-FV autoAbs on the APC cofactor activity of FV were relevant *in vivo*, as there was FV expressing APC cofactor activity in both groups. Also, as these FV inhibitors fully blocked the APC cofactor activity in the non-B group, FXa generation increased to the levels observed without FV, such as in cases 1–3, whereas in cases 4 and 5 it increased to the levels obtained without APC. As two autoAbs contained neither anti-PC nor anti-PS autoAbs (data not shown), the FVa–anti-FV autoAb complexes might indirectly affect the association of FVIIIa with APC/PS, but the precise reason is unclear. Although the APC cofactor activity of FV is PL-dependent [34], the autoAbs in the B group that impaired PL binding did not significantly affect the APC cofactor activity. This contradiction may raise the possibility that, because the autoAbs fully block the FV-related procoagulant process, the action of APC cofactor activity may be unlikely to be relevant.

The anti-FV autoAbs in the non-B group significantly delayed APC-mediated cleavage of FVa at Arg306 alone. In addition, these antibodies impaired the APC cofactor activity of FV in FVIIIa inactivation. Many studies on the R506Q mutation (FV Leiden) [13,14] have shown that defective APC cofactor activity in these instances is related to the inhibition of APC-mediated cleavage of FV at Arg506. In the non-B group, APC-mediated proteolytic patterns were different between FV and FVa. Inhibition of cleavage of FV at Arg506 was evident, whereas proteolysis of FVa at Arg306 was delayed with inhibitors. The APC-binding site(s) on the FVa HCh remains to be identified, however, and studies to localize these molecular interactions are now in progress.

Kalafatis *et al.* [19] reported severe thrombotic manifestations in one patient with an A-FV inhibitor. This autoAb recognized a conformational epitope on the entire FV molecule, and, as in our present study, the inhibitor was associated with APCR, owing to impaired APC cofactor activity and restricted APC-mediated cleavage at Arg506 and Arg306 in FVa. However, inhibition of APC-mediated cleavage of FVa in our patients was related to impaired cleavage at Arg306. Unlike the individual described by Kalafatis [19], our patients were asymptomatic, and it might be that inhibition of APC-mediated cleavage at both Arg506 and Arg306 of FVa has a more detrimental effect on the normal hemostatic balance, resulting in thrombotic complications.

The role of FV in hemostasis involves activity present in both plasma and platelets [35], and it might be that platelet FV:C is more important than plasma FV:C in physiologic mechanisms. We were unable, however, to examine platelet FV with the available frozen-stored plasma samples. Further investigations would be required, using platelet-rich plasma, to identify the potential importance of platelets in the phenotypic differences observed. A report has recently demonstrated that the FV LCh mediates FV uptake by megakaryocytes [36], and this may offer an alternative/additional explanation for the fact that hemorrhagic patients have anti-LCh antibodies.

Addendum

T. Matsumoto performed experiments, analyzed the data, and produced the figures. K. Nogami designed the research, analyzed and interpreted the data, and wrote the paper. M. Shima edited the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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ORIGINAL ARTICLE

Tissue factor pathway inhibitor in activated prothrombin complex concentrates (aPCC) moderates the effectiveness of therapy in some severe hemophilia A patients with inhibitor

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Abstract Some hemophilia A patients who have developed inhibitors are poorly responsive to activated prothrombin complex concentrates (aPCC) after daily dosage, but the mechanism(s) underlying this remain unknown. We examined two representative cases. In case 1, we found that changing to recombinant factor VIIa (rFVIIa) therapy was more effective, and the response to aPCC was restored within ~2 weeks. Tissue factor (TF)-triggered thrombin generation demonstrated a prolonged lag-time and decreased peak thrombin, and this impairment was focused on TF pathway inhibitor (TFPI). Plasma-free TFPI was elevated post-infusion of aPCC, while this was unaffected by rFVIIa. TFPI returned to normal range within 2–3 weeks. Plasmas obtained from patients with poor or good response to aPCC (aPCC-poor or aPCC-good), and good response to rFVIIa (FVIIa-good) demonstrated that free TFPI levels are increased in both aPCC groups, but not in FVIIa-good. TFPI levels pre- and post-infusion in aPCC-poor were significantly higher than those in aPCC-good. Addition of anti-TFPI antibody to the reaction samples demonstrated a greater increase of peak thrombin in aPCC-poor compared to aPCC-good, showing the higher TFPI activity in aPCC-poor. Free TFPI contained in aPCC corresponded to the increasing levels in plasma. In conclusion, TFPI in aPCC attenuated thrombin generation, and the reduced effectiveness of therapy in these circumstances appeared to be related to TFPI activity.

Presented in abstract form at the 52nd annual meeting of the American Society of Hematology, Orlando, FL, December 6, 2010.

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Keywords Hemophilia A · Inhibitor · aPCC · TFPI · Thrombin generation

Introduction

Treatment of patients with hemophilia A (HA) has improved markedly in recent years, but the development of factor (F)VIII inhibitor alloantibodies (alloAbs) after multi-transfusions of FVIII concentrates remains a serious clinical problem. Bypassing therapy, utilizing activated prothrombin complex concentrates (aPCC; FEIBA®: Baxter) and recombinant FVIIa (rFVIIa; NovoSeven®: Novo Nordisk), is now widely available, however, and is used especially in patients classified as high responders [1, 2]. A randomized study comparing aPCC and rFVIIa (FENOC study) demonstrated that both forms of treatment had similar effects on joint bleeding [3]. The therapeutic effectiveness of these products appears to be inconsistent in individual patients, and a number of factors appear to contribute to this variable response. Berntorp [4] suggested that this heterogeneous response to bypassing agents resulted from some patient-specific factors, including tissue factor (TF) activity, natural anticoagulant factors, inherited prothrombotic factors, fibrinolytic parameters, the presence of cross-reactive antibodies to other coagulation factors, and prothrombin concentration. Precise mechanisms remain unclear, however.

Characteristically, some patients appear to be unresponsive to bypassing agents after repeated infusions on consecutive days, although single infusions remain effective. In this context, Stenbjerg and Jorgensen [5] speculated that increases in natural coagulation inhibitors such as antithrombin resulted in the lack of response to aPCC in their patients who did not respond to consecutive aPCC

treatment. In our two earlier reports, one case suggested that a decrease in antithrombin led to disseminated intravascular coagulation (DIC) in a HA patient with high-titer inhibitor treated with repeated infusions of aPCC for intracranial bleeding [6]. The other case also described life-threatening episodes in a HA patient who was unresponsive, alternately, to aPCC and rFVIIa during specific therapy [7]. Although mechanisms were not fully clarified, DIC was not evident and consumption of coagulation factors and enhanced fibrinolysis were not observed. We now have investigated two new HA patients with inhibitors who were unresponsive to aPCC on consecutive days. We have shown for the first time that plasma samples from some HA patients with poor response to aPCC contained increased levels of TF pathway inhibitor (TFPI). The findings indicated that the moderated response could have resulted from the accumulation of TFPI that was contained in the aPCC itself.

Materials and methods

Reagents

Phospholipid vesicles (PL) containing 10 % phosphatidylserine, 60 % phosphatidylcholine, and 30 % phosphatidylethanolamine were prepared using *N*-octyl glucoside [8]. The aPTT and PT reagents were purchased from Sysmex Corp. (Kobe, Japan). Recombinant human TF (Innovin®, Dade Behring, Marburg, Germany), FVIII-deficient plasma (George King, Overland Park, KS), fluorogenic specific-substrate for thrombin, Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland), thrombin calibrator (Thrombinoscope, Maastricht, Netherlands), and corn trypsin inhibitor (CTI; Haematologic Technologies Inc, Essex, VT) were purchased from the indicated vendors. Recombinant TFPI [9] was a generous gift from Dr. Tsutomu Hamuro. Anti-human TFPI (Kunitz-2 domain) monoclonal antibody (mAb) was purchased from Sanquin Reagents (Amsterdam, Netherlands). An anti-FVIII alloAb was purified from the plasma of a HA patient with inhibitor. HEPES-buffered saline (20 mM HEPES pH 7.2, 100 mM NaCl, containing 0.01 % Tween 20) was used for dilution.

Blood samples

Whole blood was obtained by venipuncture from HA patients with inhibitor and healthy volunteers after informed consent. Blood was taken into tubes containing one-tenth volume of 3.8 % (w/v) trisodium citrate. Samples were obtained just before infusions of bypassing agents (pre-infusion) and at 30 min after infusions (post-infusion). Plasma samples were recovered after centrifugation for 10 min at 1,500*g*.

Measurement of TFPI level in plasma

Free TFPI was determined by ELISA using two different anti-TFPI mAbs (KAKETSUKEN, Kumamoto, Japan) [10]. Briefly, the primary mAb recognizing the Kunitz-3 domain in TFPI was immobilized on microtiter wells, and free TFPI was determined with the other mAb conjugated with horseradish peroxidase, recognizing the specific conformation formed between the Kunitz-1 and Kunitz-2 domains. Total TFPI was also determined by ELISA in aPCC and rFVIIa using the same procedure except for the primary Ab (rabbit anti-TFPI polyclonal Ab) [10].

Rotational thromboelastometry (ROTEM)

ROTEM was performed 30 min after venipuncture using the Whole Blood Hemostasis Analyser® (Pentapharm, Munich, Germany). Blood (300 µl) was mixed with 20 µl $CaCl_2$ (100 mM), placed in the plastic cup and the viscosity of clot formation was monitored. The coagulation process was assessed using the clotting time (CT; the time from the start of measurement until detection of clot firmness at 2 mm amplitude), the clot formation time (CFT; the time from the initiation of clotting until detection of clot firmness of 20 mm amplitude), and the maximum clot firmness (MCF; the maximum amplitude, indicating clot stabilization).

Thrombin generation test (TGT)

The calibrated automated TGT (Thrombinoscope) was performed using a method of Hemker with minor modifications [11]. Plasma samples (80 µl) were mixed with CTI (30 µg/ml) and incubated for 10 min with 20 µl PL (60 µM) containing the indicated concentrations of TF. Measurements were recorded after the addition of 20 µl $CaCl_2$ (100 mM) containing 2.5 mM fluorogenic substrate. The standard parameters including lagtime and peak thrombin level were derived, and the average values and standard error of the mean (SEM) were calculated. TFPI activity was determined in the TGT by assessing peak thrombin in the presence of anti-TFPI mAb [12].

Data analyses

All experiments were performed in triplicate, and the average values were determined. Significant differences were determined by the Wilcoxon *t* test and the Mann–Whitney *U* test. *P* values <0.05 were considered as statistically significant.

Patient profiles

A "poor response to aPCC" was defined when bleeding symptoms in patients were not resolved and the ROTEM parameters were not improved as expected. Either non-steroidal anti-inflammatory drugs or tranexamic acid had not been administered during the aPCC (and rFVIIa) therapy.

Case 1

A 5-year-old boy with severe HA with inhibitor (148 BU/ml) attended our hospital with an occipital hematoma. He had been effectively treated previously with aPCC (90 unit/kg; 1–2 times/day, 1–3 days) for every bleeding episode. The present hematoma was also effectively treated with aPCC once a day for 2 days, but he developed a hemorrhage into the left knee joint on day 2, and this did not improve despite further daily infusions of aPCC. ROTEM measurements suggested unresponsiveness to aPCC on day 6 (Fig. 1A-a). He was admitted on day 7 and treated with rFVIIa (137 μg/kg, 3 times/day). The effectiveness of this treatment was confirmed by ROTEM (Fig. 1A-b) and he had fine-needle aspiration of the intra-articular hematoma. On day 18 (12 days after discontinuing aPCC infusions), aPCC was re-administered and shown to be effective in ROTEM (Fig. 1A-c). He was discharged on day 20. ROTEM on day 37 confirmed that aPCC remained effective, and aPCC was continued for on-demand therapy. On day 247, he was successfully treated with aPCC (twice a day) for endoscopic synovectomy on his knee arthropathy. Four days later (day 250), unresponsiveness to aPCC was again observed, however, and rFVIIa was required for clinical effect.

Case 2

A 22-year-old man with severe HA with inhibitor (54 BU/ml) had been treated with aPCC or rFVIIa for on-demand therapy. He had hemarthrosis into his right elbow and was treated with aPCC (75 unit/kg, twice a day) for 3 days. He had another hemarthrosis on day 4, and the treatment of aPCC was continued. ROTEM demonstrated a reduced effect of aPCC on day 6 (Fig. 1C-a) compared to previous occasions, and we considered the development of diminished responsiveness after repeated infusions of aPCC. Infusions were reduced to once a day on day 8 and discontinued on day 12 after improvement in symptoms. On day 32, aPCC (75 unit/kg) was infused for hemorrhage into the hip joint, and effectiveness was evident on ROTEM (Fig. 1C-b). Since then, he had repeated bleeding episodes, and infusions of aPCC once a day for 4–5 days have remained effective both clinically and in ROTEM. After 5 months, further infusions of rFVIIa (90 μg/kg, twice a day) were clinically effective and were associated with a good response in ROTEM (data not shown).

Results

Evaluation of blood coagulation after aPCC infusions

Case 1 demonstrated a poor clinical response to aPCC on day 6, a good response to rFVIIa on day 7, and a good response to aPCC on day 18. The results of ROTEM after infusion of aPCC or rFVIIa reflected the clinical symptoms (Fig. 1Aa-c; Table 1). The plasmas were further examined using the TGT triggered with TF (5 pM) and PL. The lagtime was not prolonged in control tests using commercial FVIII-deficient plasma, in keeping with earlier results in HA patients without inhibitor [13]. In patient's plasma obtained on day 6, however, the lagtime pre-infusion of aPCC was prolonged relative to normal plasma, and was not significantly shortened post-infusion (Fig. 1B-a; Table 1). Surprisingly, the peak thrombin level post-infusion was lower than that pre-infusion. A prolonged lagtime was again observed pre-infusion of rFVIIa on day 7. After infusion of rFVIIa the lagtime was shortened, although peak thrombin level showed little change (Fig. 1B-b). Daily infusions of rFVIIa clinically improved the hemarthrosis of his knee. In addition, prior to further infusions of aPCC on day 18 the lagtime was not prolonged, and the peak thrombin level post-infusion showed a significant increase (Fig. 1B-c).

ROTEM in Case 2 also reflected the clinical symptoms post-infusion of aPCC (Fig. 1C:a-b; Table 1). The TGT triggered with TF (1 pM) and PL again demonstrated that in plasma obtained at the time of a poor response to aPCC on day 6 (Fig. 1D-a) the prolonged lagtime was not significantly shortened post-infusion, and the peak thrombin level post-infusion was slightly lower than that seen pre-infusion. The lagtime pre-infusion of aPCC on day 32 (after 3 days without treatment) was not prolonged, however, and peak thrombin post-infusion was elevated (Fig. 1D-b).

TFPI antigen levels

Standard laboratory tests of hemostasis were undertaken to investigate possible factor(s) associated with the unresponsiveness to aPCC. Blood counts, levels of activity and antigen of both pro- and anticoagulant factors failed to provide evidence associated with unresponsiveness to aPCC in either case, however (data not shown). Levels of coagulation factors including prothrombin, FVII, FIX, FX, and protein C contained in aPCC were elevated as expected after infusion [14]. Moreover, in vitro experiments using

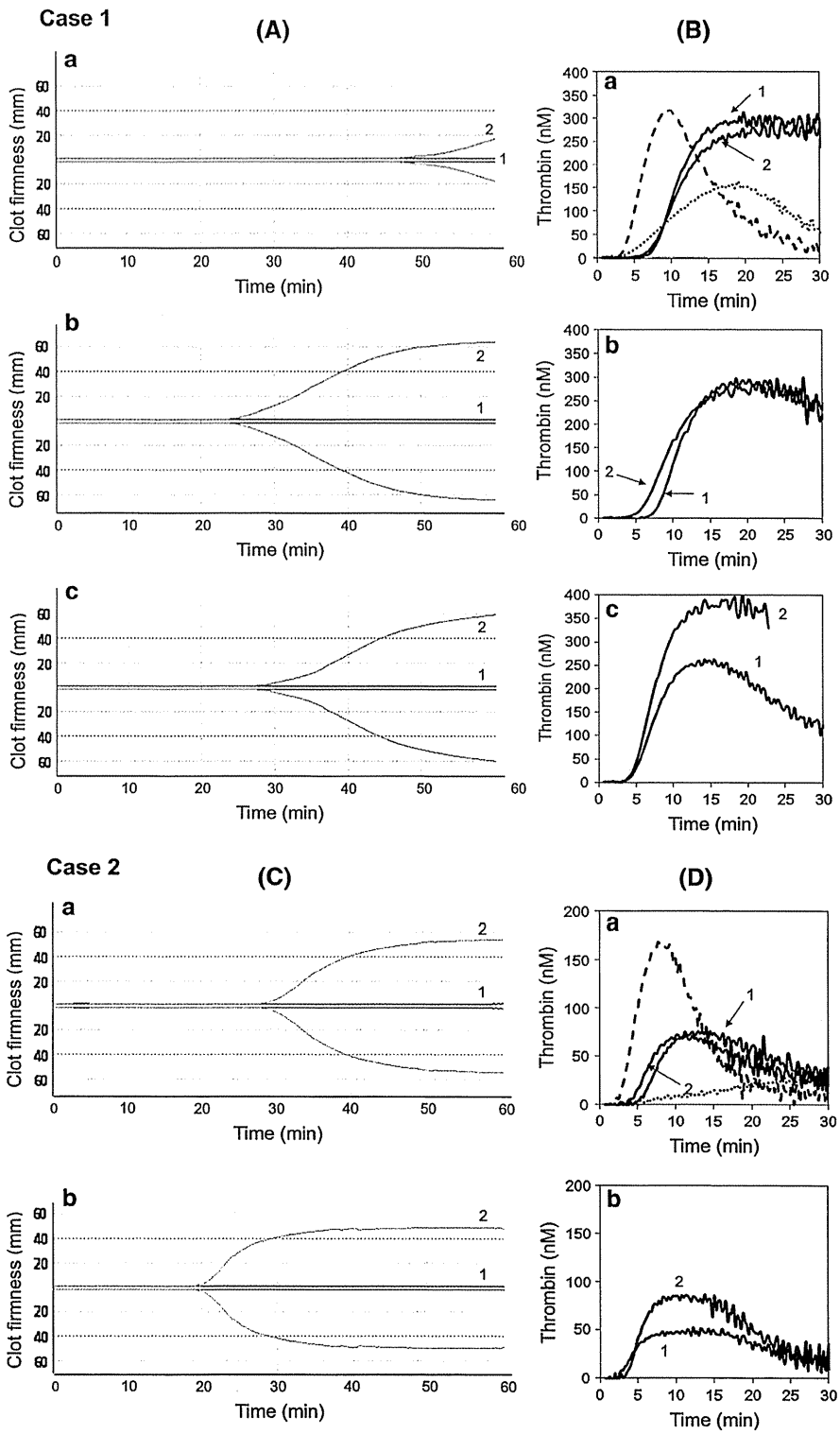

Fig. 1 Effects of aPCC and rFVIIa on global blood coagulation. ROTEM: $CaCl_2$ was added to whole blood obtained in case 1 (**A**-*a* pre/post-aPCC infusion on day 6, *b* pre/post-rFVIIa on day 7, *c* pre/post-aPCC on day 18) and in case 2 (**C**-*a* pre/post-aPCC on day 7, *b* pre/post-rFVIIa on day 32). Representative thrombograms are illustrated. *Solid black* (*line 1*) and *gray* (*line 2*) *lines* show the results on samples pre/post-infusion, respectively. **B** TGT: Plasma samples obtained in case 1 (**B**-*a* pre/post-aPCC on day 6, *b* pre/post-rFVIIa on day 7, *c* pre/post-aPCC on day 18) and case 2 (**D**-*a* pre/post-aPCC on day 6, *b* pre/post-rFVIIa on day 32), were mixed with TF (*case 1* 5 pM, *case 2* 1 pM) and PL (10 μM), followed by TGT. Representative thrombograms are illustrated. *Solid lines 1–2* show the results on samples pre/post-infusion, respectively. *Dashed* and *dotted lines* illustrate normal and congenital FVIII-deficient plasma, respectively

TGT confirmed that the elevated levels of the individual coagulant factors had little effect on the thrombogram patterns (in Fig. 1) associated with unresponsiveness to aPCC (data not shown).

Unexpected increases in free TFPI antigen levels were observed post-infusion of aPCC in both cases. The assessment of free TFPI following long-term aPCC therapy is illustrated in Fig. 2. In case 1, the free TFPI levels post-

Table 1 ROTEM and TGT parameters

(i) Case 1

Parameters	Day 6 (poor) pre/post-aPCC	Day 7 (good) pre/post-rFVIIa	Day 18 (good) pre/post-aPCC	Control	FVIII-def
ROTEM					
CT (s)	>6,000/2,816	>6,000/1,419	>6,000/1,641	852 ± 65	>6,000
CFT (s)	n.d./862	n.d./535	n.d./609	280 ± 26	n.d.
MCF (mm)	n.d./44	n.d./64	n.d./65	44 ± 3	n.d.
TGT					
Lagtime (min)	8.5 ± 0.3/ 8.1 ± 0.4	8.5 ± 0.1/ 6.8 ± 0.1	5.4 ± 0.1/ 5.1 ± 0.1	4.4 ± 0.1	5.6 ± 0.1
Peak Th (nM)	296 ± 5/273 ± 9	290 ± 1/276 ± 1	257 ± 2/382 ± 4	315 ± 1	155 ± 7

(ii) Case 2

Parameters	Day 6 (poor) pre/post-aPCC	Day 32 (good) pre/post-aPCC	Control	FVIII-def
ROTEM				
CT (s)	3,589/1,668	4,710/1,181	852 ± 65	>6,000
CFT (s)	1,133/332	2,267/198	280 ± 26	n.d.
MCF (mm)	29/55	21/50	44 ± 3	n.d.
TGT				
Lagtime (min)	5.5 ± 0.2/ 4.5 ± 0.1	3.0 ± 0.1/ 3.5 ± 0.2	3.5 ± 0.1	6.6 ± 0.2
Peak Th (nM)	77 ± 2/72 ± 3	48 ± 3/86 ± 4	185 ± 18	21 ± 0.7

TGT was initiated using TF (case 1; 5 pM, case 2; 1 pM). Control and FVIII-def; normal plasma and FVIII-deficient plasma. Poor and good; poor and good response of aPCC

ND not determined, *CT* clot time, *CFT* clot formation time, *MCF* maximum clot firmness, *Peak Th* peak thrombin

infusion were elevated on days 6 and 18 (pre-/post-; 41/51 and 30/44 ng/ml, respectively). When the aPCC infusions were discontinued for more than 2 weeks (days 34 and 247), free TFPI levels appeared to be less than 20 ng/ml. Consecutive aPCC infusions, however, appeared to mediate gradual increases in free TFPI, and the level reached to 59 ng/ml on day 250 when unresponsiveness was again observed, suggesting that TFPI was accumulated by aPCC infusions, and returned to lower (or normal) levels when aPCC was not given. The increased amounts of free TFPI on days 6 and 18 were similar (10 and 14 ng/ml, respectively), independently of the response to aPCC. Nevertheless, free TFPI post-infusion at the time of poor response (day 6, 51 ng/ml) seemed to be modestly greater than that seen during a good response (day 18; 44 ng/ml). The prolonged lagtime in TGT associated with a poor response appeared likely to be linked to a failure in the initiation phase of blood coagulation involving the FVIIa/TF pathway.

Also in case 2, all free TFPI levels post-infusion of aPCC were elevated compared to those pre-infusion, and the increase levels ranged from 6.4 to 15.2 ng/ml. The free TFPI reached a maximum value of 66.0 ng/ml post-infusion on day 7. Subsequently, free TFPI gradually decreased after infusions were reduced from twice a day to once a day from day 8. On day 154, 8 days after discontinuation of aPCC, free TFPI was 19.4 ng/ml pre-infusion of rFVIIa, and interestingly, did not change (19.0 ng/ml) post-infusion.

These findings prompted further investigations on the role of TFPI in the reduced effectiveness of aPCC.

Effect of full-length TFPI on the TGT in HA plasmas

A previous report described that the addition of TFPI (free TFPI) dose-dependently inhibited thrombin generation [15]. We examined, therefore, whether the TGT using congenital FVIII-deficient plasmas in the presence of aPCC was affected by increases in free TFPI. Recombinant full-length TFPI, with similar properties to free TFPI [9, 16], was added to aPCC, and we initially confirmed that this product inhibited the TGT using normal plasma (data not shown). Constant amounts of aPCC (2 unit/ml) were incubated with dilutions of full-length TFPI and FVIII-deficient plasmas without or with anti-FVIII inhibitor alloAb (10 BU/ml). Representative TGT curves on the effects of full-length TFPI without (Fig. 3a) or with (Fig. 3b) anti-FVIII alloAb are illustrated. The lagtime was prolonged (Fig. 3c) and the peak thrombin levels were reduced (Fig. 3d) in the presence of TFPI in dose-dependent manners in each instance. The addition of full-length TFPI (16–32 ng/ml) prolonged the lagtime by 1.15–1.28-fold and decreased the peak thrombin levels by 0.6–0.75-fold, relative to controls. These findings appeared to be

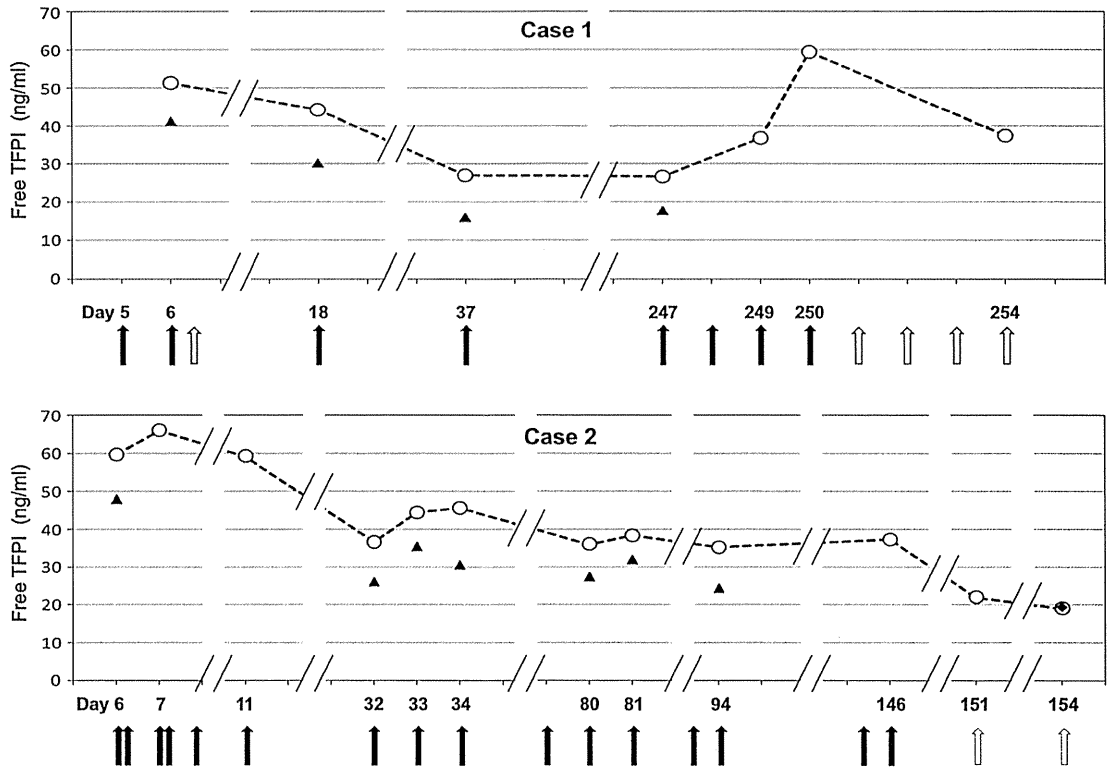

Fig. 2 Changes in free TFPI antigen levels. Plasma-free TFPI antigen in both cases were measured by ELISA. The TFPI antigen levels pre- (*filled triangle*) and post- (*circle*) infusion of aPCC or rFVIIa are shown. The *filled* and *open arrows* represent the infusion of aPCC (90 unit/kg) and rFVIIa (90 μg/kg), respectively

similar to the results of thrombin generation during periods of apparent resistance to aPCC, and supported, therefore, that free TFPI might have contributed to the poor response to aPCC.

Effect of anti-TFPI mAb on the moderated response to aPCC

Although the addition of TFPI was shown to attenuate thrombin generation in experiments in vitro, it remained uncertain that the moderated response to aPCC could be attributed to TFPI concentration. We attempted to confirm this, therefore, using anti-TFPI mAb. Insufficient plasma was available from days 6 and 18 in case 1, and samples from day 37 with good response to aPCC and from day 250 with poor response were utilized as alternatives. In the absence of anti-TFPI mAb (line 1), plasma from day 250 (Fig. 4A-b) illustrated a longer lagtime (6.8 ± 0.2 min) and a lower peak thrombin level (114 ± 2 nM) than that from day 37 (Fig. 4A-a, 5.0 ± 0.1 min, 195 ± 2 nM), similar to the thrombogram shown in Fig. 1B. The addition of anti-TFPI mAb (16 μg/ml, line 2) shortened the lagtime, and elevated the peak thrombin in both samples, but the peak thrombin level in the sample from day 250, however, increased significantly (459 ± 8 nM, ~4.0-fold of control) relative to that obtained from day 37 (377 ± 4 nM, ~1.9-fold of control). The shortened lagtime appeared to be insufficient to counteract the overall inhibition of thrombin generation mediated by TFPI in plasma from day 250. Similarly the presence of anti-TFPI mAb in case 2 shortened the lagtime and elevated peak thrombin activity more significantly in plasma at the time poor response (day 6, Fig. 4B-b) compared with that during good response (day 32, Fig. 4B-a) (see the Table 2). These results suggested that the effects of TFPI on inhibiting peak thrombin generation were greater at the time of poor response to aPCC, in keeping with more elevated TFPI antigen in samples at this time.

TGT parameters and TFPI concentrations in patients with different responses to aPCC and rFVIIa

The relationship between TFPI and response to aPCC (or rFVIIa) was further investigated by TGT using plasma samples obtained from another four HA patients with inhibitor treated with bypassing therapy (cases 3–6). Sixteen plasma samples (pre/post-infusion) obtained from all cases were classified into three groups; poor response to

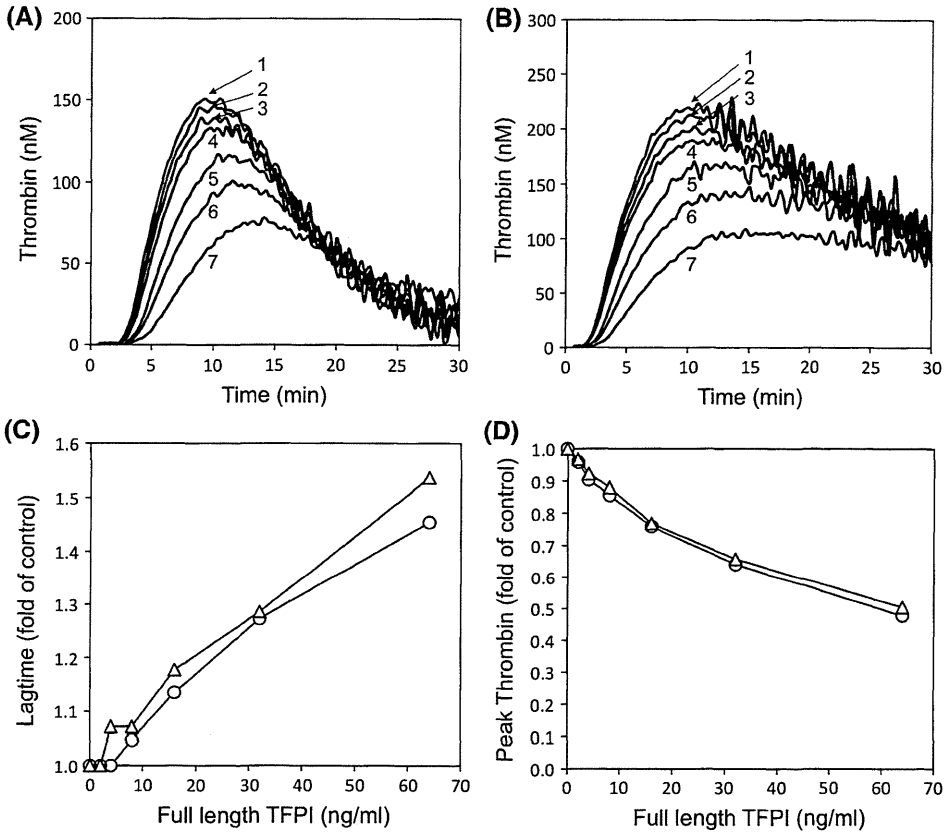

Fig. 3 Effects of TFPI on the TGT in FVIII-deficient plasmas with aPCC. FVIII-deficient plasma in the absence (**a**) or presence (**b**) of anti-FVIII inhibitor alloAb (10 BU/ml) was mixed with full-length TFPI, followed by the addition of aPCC (2 unit/ml) and the measurement of TG. *Lines 1–7* represent thrombogram curves with full-length TFPI at 0, 2, 4, 8, 16, 32, and 64 ng/ml, respectively. Lower panels illustrate the results of lagtime (**c**) and peak thrombin levels (**d**) obtained in (**a–b**). The symbols used are, the absence (*triangle*) and presence (*circle*) of FVIII inhibitor

aPCC (aPCC-poor), good response to aPCC (aPCC-good), and good response to rFVIIa (FVIIa-good) as defined above. All aPCC-poor patients had been treated with aPCC with every 12 h consecutive infusions, whilst all in aPCC-good and FVIIa-good the respective bypassing agent had been given at intervals of more than 2 days. The TGT in cases 2–6 was performed using TF at 1 pM (TF 5 pM in case 1). The results are summarized in Table 2. Similar TGT patterns were observed in all aPCC-poor samples (i.e. the lower peak thrombin levels and little or slightly prolonged lagtime post-infusion than those pre-infusion). Furthermore, the addition of anti-TFPI mAb modified the TGT patterns, and in the presence of antibody the peak thrombin levels post-infusion were greater than those pre-infusion. Hence, the peak thrombin ratios in the aPCC-poor patients, represented by the increase in peak thrombin in the presence of anti-TFPI mAb [12], were much greater post-infusion. In contrast, these ratios were similar or lower post-infusion in those classified as aPCC-good and FVIIa-good.

Free TFPI antigen levels were compared in these patients (Fig. 5). The levels were significantly elevated after aPCC infusion in the aPCC-poor samples [(A) before/after; 39.8 ± 3.6/52.8 ± 3.0 ng/ml, $p = 0.0015$] and in the aPCC-good plasmas [(B) 27.6 ± 3.3/36.8 ± 2.7 ng/ml, $p = 0.005$], but were unchanged in those defined as FVIIa-good [(C) 22.2 ± 2.3/20.4 ± 2.4 ng/ml, $p = 0.097$]. Moreover, free TFPI levels before or after aPCC infusion were significantly greater in aPCC-poor patients than in the aPCC-good group ($p = 0.018$ and 0.0021, respectively).

TFPI antigen in aPCC and rFVIIa

The increases in free TFPI post-infusion of aPCC indicated the likelihood that the aPCC therapeutic materials contained significant concentrations of TFPI. We used, therefore, the same ELISA method to measure free TFPI antigens in five individual batches of aPCC and a batch of rFVIIa. Table 3 shows that on average, one unit of aPCC contained 12.4 ± 2.1 ng of free TFPI, confirming that aPCC contained considerable amounts of free TFPI. We also measured total TFPI using same ELISA except for the primary antibody (alternatively using anti-TFPI polyclonal). The results indicated that the TFPI in aPCC was almost all free form (free/total 1.05). No TFPI was detected in rFVIIa.

Discussion

The ROTEM technique has provided useful data for monitoring the clinical effect of bypassing agents in

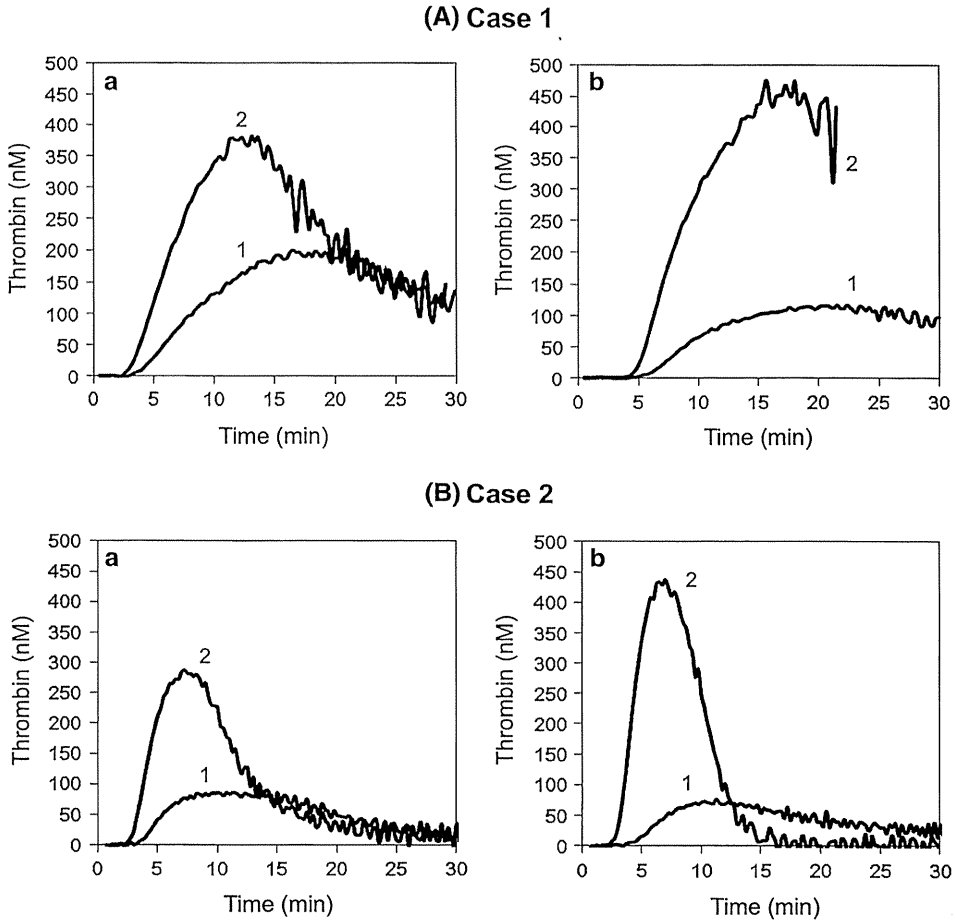

Fig. 4 Effects of anti-TFPI mAb on plasma samples. Plasmas obtained at good response (*a* day 37) and poor response (*b* day 250) in *case 1* (**A**), and plasmas at good response (*a* day 32) and poor response (*b* day 6) in *case 2* (**B**) were incubated with anti-TFPI mAb (16 μg/ml), prior to TGT. *Lines 1–2* illustrate the results in the absence and presence of anti-TFPI mAb, respectively

hemostasis [17]. However, the TF-triggered TGT has been recently developed and appears to provide additional information [18]. In this study, we have utilized the TGT to examine the defective response to aPCC. A prolonged lagtime (not observed in HA) and decreased peak thrombin levels were observed after aPCC infusions in poorly responsive patients. Further investigations suggested a possible association between aPCC-resistance and the FVIIa/TF-dependent anticoagulant factor, TFPI. This conclusion was supported by the following observations, (i) free TFPI levels were elevated in all cases after aPCC infusions, (ii) free TFPI gradually increased after consecutive infusions of aPCC, (iii) free TFPI levels were significantly greater in aPCC-poor than in aPCC-good patients, (iv) the addition of exogenous TFPI to HA plasma with inhibitor decreased the effect of aPCC dose-dependently, (v) the decreased peak thrombin observed in the presence of anti-TFPI mAb, improved significantly in the aPCC-poor group to a greater extent than in aPCC-good patients and (vi) relatively high concentrations of TFPI, especially the functional free form, were contained in aPCC (FEIBA®).

TFPI down-regulates the initiation of blood coagulation by inhibiting both FVIIa/TF and FXa [19]. The full-length molecule comprising free TFPI represents the most functionally active-form, whilst the majority of TFPI in plasma is truncated, is associated with lipoprotein and is inactive [20]. The concentration of free TFPI in plasma varies between 6 and 50 % of total TFPI [10, 21]. Free TFPI binds sequentially to FXa and FVIIa/TF, and hence free TFPI appears to function preferentially in the presence of FXa [19], suggesting the likelihood that free TFPI has a broader effect on the early coagulation phases than on the propagation phases. It is notable that in both representative cases in the present study, new bleeding episodes occurred, distinct from the presenting hemorrhage, despite consecutive aPCC infusions. In each case, the first hemorrhage was resolved and it seemed likely that the new bleeding resulted from an impairment of the initiation phase of blood coagulation. In these circumstances it would seem reasonable to change from aPCC to rFVIIa for clinical effectiveness. In this context, rFVIIa might neutralize and/or overcome TFPI function, and restore the initiation phase of blood coagulation. Alternatively, it is also believed that large

Table 2 TGT parameters and TFPI assays in patients classified as aPCC-poor, aPCC-good, and rFVIIa-good

Patients	Period of last infusion	TGT parameters (pre/post-infusion)				Free TFPI (pre/post-infusion) (ng/ml)
		Lagtime (min)	Peak Th (nM)	Peak Th with anti-TFPI (nM)	Peak ratio[a]	
aPCC-poor						
Case 1 (day 6)[b]	12 h	8.5/8.1	296/273	–/–	–/–	41/51
Case 1 (day 250)	12 h	–/6.8	–/114	–/459	–/4.0	–/59
Case 2 (day 6)	12 h	5.5/4.5	77/72	402/437	5.2/6.1	48/60
Case 2 (day 34)	12 h	6.0/6.3	92/64	721/748	7.8/11.7	32/45
Case 3	12 h	5.8/6.8	156/93	771/1,127	4.9/12.1	47/58
Case 4	12 h	5.0/5.3	225/198	691/999	3.1/5.0	31/52
aPCC-good						
Case 1 (day 18)[b]	12 day	5.4/5.1	257/382	–/–	–/–	30/44
Case 1 (day 37)	>2 week	5.9/5.0	63/195	126/377	2.0/1.9	16/27
Case 2 (day 32)	7 day	3.0/3.5	48/86	187/287	3.9/3.3	26/37
Case 5	>4 week	5.0/4.3	44/86	267/467	6.1/5.4	36/38
Case 6	none	4.0/3.5	34/105	239/429	7.0/4.1	30/38
FVIIa-good						
Case 1 (day 7)[b]	>4 week	5.7/3.3	49/58	133/138	2.7/2.4	30/26
Case 2 (day 154)	3 day	3.0/2.4	82/166	231/446	2.8/2.7	19/19
Case 4	2 day	3.8/2.3	43/73	166/182	3.9/2.5	19/14
Case 5	>4 week	5.8/2.3	28/85	126/217	4.5/2.6	18/17
Case 6	none	5.3/2.8	53/128	287/321	5.4/2.5	25/26
Control		5.3	201	367	1.8	28

[a] Peak ratio shows ratio of peak thrombin level after adding anti-TFPI compared to its absence. Control; normal plasma

TGT was initiated using TF (1 pM) in all cases, except for [b] using TF (5 pM)

amounts of rFVIIa activate FX on the surface of activated platelets without TF [22].

Although aPCC contains several pro- and anticoagulant factors including prothrombin, FVII(a), FIX, FX(a) and protein C [14], the presence of TFPI in aPCC has not been reported. We confirmed that the concentration of total TFPI in several different batches of aPCC was ~12 ng/unit, and most of this was free TFPI. Plasma concentrations of aPCC in treated HA patients could be expected to reach ~2 unit/ml after a dose 80 unit/kg. On this basis, therefore, free TFPI levels in plasma would increase by ~24 ng/ml, corresponding to a level ~85 % above that in normal plasma (28 ng/ml, Table 2). Our findings showed that free TFPI levels in HA patients treated with aPCC increased by 10–15 ng/ml, in keeping with the concentrations of free TFPI in aPCC itself. It seems possible that enhanced procoagulant activity mediated by aPCC might accelerate the release of endogenous TFPI from vascular endothelium, but rFVIIa infusions had little effect on TFPI in plasma despite inducing increased thrombin generation.

The presence of excess of free (full-length) TFPI in FVIII-deficient plasma in vitro resulted in a prolonged lagtime and decreased peak thrombin levels in the TGT experiments, similar to those observed with aPCC-unresponsive plasmas. In particular, the addition of ~20 ng/ml free TFPI, corresponding to that in aPCC at 2 unit/ml, induced similar changes in the TGT parameters to those seen in HA plasma with corresponding levels of TFPI (~50 ng/ml). Free TFPI levels post-infusion and pre-infusion in the aPCC-poor patients were significantly greater than those in those classified as aPCC-good. In addition, TFPI activities post-infusion in the aPCC-poor group were significantly increased compared to those in the aPCC-good individuals. These data strongly support a possible relationship between the poor response to aPCC and TFPI accumulated after consecutive infusions. Our data indicate that measurements of free TFPI before and/or during the consecutive aPCC infusions could provide a useful aid for predicting the effectiveness of treatment.

FEIBA® (aPCC) appears to have been a safe therapeutic product for many years, although there have been occasional reports of adverse clinical side effects including thrombosis caused by hyper-coagulant activity. It may be, therefore, that TFPI contributes to the safety of aPCC products of this nature by suppressing excess procoagulant activity and/or maintaining functional ability. In contrast, in some circumstances removal of TFPI from aPCC could be beneficial and prevents the resistance to therapy described in this report. In Japan, insurance limitations have previously prevented the use of aPCC for more than 4-consecutive days, and most patients treated with aPCC would have remained responsive by not accumulating undesirable levels of TFPI. In some instances of life-threatening hemorrhage, aPCC has been used for greater

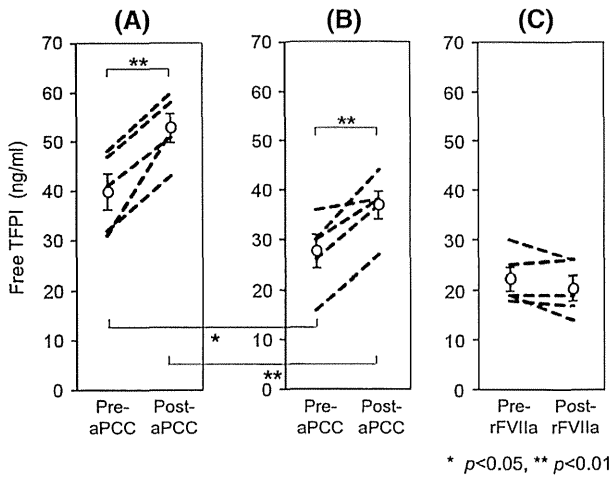

Fig. 5 Free TFPI antigen levels and the different response to aPCC or rFVIIa—Free TFPI antigen levels were measured in fifteen pairs of plasma samples (pre/post-infusion of aPCC or rFVIIa) from 6 patients (cases 1–6) classed as aPCC-poor (**a**), aPCC-good (**b**), and FVIIa-good (**c**). Average ± SEM values are shown as *open circles* and *bars*, respectively. Significant differences are shown as $* p < 0.05$, $** p < 0.01$

Table 3 Free and total TFPI content in aPCC and rFVIIa

Batch Number	Total TFPI (ng/unit)	Free TFPI (ng/unit)	Free/total
FEIBA®			
VNF 1J006A	12.2	13.3	1.09
VNF 1J008A	8.4	9.0	1.07
VNF 1H028	12.7	13.9	1.10
VNF 1H044	18.5	18.8	1.02
VNF 2J036BA	7.3	7.0	0.96
Average	11.8 ± 2.0	12.4 ± 2.1	1.05 ± 0.03
NovoSeven®			
X1002	n.d.	n.d.	–

n.d. not determined

than 4 days, and some poor responses have been recorded [6, 7]. Resistance to aPCC has not been raised as a major difficulty, however, and our findings show that treatment might be re-instated successfully in some previously unresponsive cases.

It remains to be determined how free TFPI increases in these circumstances despite the very short half-life of free TFPI ($T_{1/2}$ ~1 h). The heterogeneity of normal TFPI levels, clearance of TFPI, and TFPI-related factors (protein S, FV, and FXa) amongst individuals and different ethnic populations might contribute to variable responses. Nevertheless, our findings show that levels of free TFPI antigen and activity in the plasma of most patients treated with aPCC would increase, and our results have demonstrated that the reduced effectiveness of aPCC therapy in some patients with FVIII inhibitors after consecutive infusions could be related to the accumulation of TFPI. In future, because of a low number of cases in this study, much more cases such as a multicenter study would be required to consolidate our conclusion.

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Conflict of interest M.S. is a board member of the Feiba and Advate Safety Board in Japan organized by Baxer and a board member of the Benefix Post Marketing Surveillance Study Board in Japan organized by Pfizer has received payment for consultancy meetings with Baxter, Pfizer, Biogen Idec, Bayer, CSL Behring, Kaketsuken, Chugai Therapeutic Company and Novo Nordisk, has received unrestricted grants supporting research from Baxter, Pfizer, Bayer, Kaketsuken, Novo Nordisk, Chugai Pharmaceutical Company and CSL Behring. The other authors declare that they have no conflict to declare.

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Regular Article

THROMBOSIS AND HEMOSTASIS

Novel FV mutation (W1920R, FV_{Nara}) associated with serious deep vein thrombosis and more potent APC resistance relative to FV_{Leiden}

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Key Points

- FV_{Nara} (W1920R), associated with serious deep vein thrombosis, is more resistant to APC relative to FV_{Leiden} (R506Q).
- This mechanism results from significant decreases in FVa susceptibility to APC and FV cofactor activity for APC.

Factor V (FV) appears to be pivotal in both procoagulant and anticoagulant mechanisms. A novel homozygote (FV_{Nara}), a novel mechanism of thrombosis associated with Trp1920→Arg (W1920R), was found in a Japanese boy and was associated with serious deep vein thrombosis despite a low level of plasma FV activity (10 IU/dL). Activated partial thromboplastin time–based clotting assays and thrombin generation assays showed that FV_{Nara} was resistant to activated protein C (APC). Reduced susceptibility of FVa_{Nara} to APC-catalyzed inactivation and impaired APC cofactor activity of FV_{Nara} on APC-catalyzed FVIIIa inactivation contributed to the APC resistance (APCR). Mixtures of FV-deficient plasma and recombinant FV-W1920R confirmed that the mutation governed the APCR of FV_{Nara}. APC-catalyzed inactivation of FVa-W1920R was significantly weakened, by ~11- and ~4.5-fold, compared with that of FV–wild-type (WT) and FV_{Leiden} (R506Q), respectively, through markedly delayed cleavage at Arg506 and little cleavage at Arg306, consistent with the significantly impaired APC-catalyzed inactivation. The rate of APC-catalyzed FVIIIa inactivation with FV-W1920R was similar to that without FV, suggesting a loss of APC cofactor activity. FV-W1920R bound to phospholipids, similar to FV-WT. In conclusion, relative to FV_{Leiden}, the more potent APCR of FV_{Nara} resulted from significant loss of FVa susceptibility to APC and APC cofactor activity, mediated by possible failure of interaction with APC and/or protein S. (*Blood.* 2014;123(15):2420-2428)

Introduction

Factor V (FV) contributes to opposing mechanisms in the regulation of coagulation.[1,2] The procoagulant action of FV is associated with cofactor activity for FXa in the prothrombinase complex, which catalyzes the conversion of prothrombin to thrombin on a phospholipid (PL) surface.[3-5] FV is converted to FVa by proteolytic cleavage by thrombin. Development of a hypercoagulant state is controlled by downregulation by activated protein C (APC) with protein S (PS). Hence, FVa is rapidly inactivated by proteolytic cleavage of the heavy chain (HCh) at Arg306, Arg506, and Arg679.[6,7] Cleavage at Arg506 is essential for the exposure of other cleavage sites but is not directly required for the decrease in activity. Cleavage at Arg306 results in near-complete loss of FVa activity. Nevertheless, any defect of 1 or more cleavage reactions significantly affects the processes of APC-induced inactivation.[8,9] The alternative function of FV is as an anticoagulant cofactor of APC in FVIIIa inactivation.[1] FVIIIa functions as a cofactor in the tenase complex and is responsible for PL-dependent FXa generation by FIXa.[10-12] In the process of APC-induced FVIIIa inactivation, FV acts as an anticoagulant cofactor of APC with PS, resulting in acceleration of FVIIIa inactivation through cleavage at Arg336.[13,14] This anticoagulant activity of FV is mediated by a product of proteolysis by APC before cleavage by thrombin. Cleavage at Arg506 of FV attached to the B domain is essential to the anticoagulant activity of FV, whereas cleavage at Arg306 appears to contribute less to this mechanism.[15,16] Any molecular defect of these cleavage reactions confers APC resistance (APCR).[1]

A point mutation of the *F5* gene, Arg506→Gln (R506Q; FV_{Leiden}), is the major cause of APCR[2] and is detected in ~20% of Caucasians with deep venous thrombosis (DVT).[17,18] The loss of the APC cleavage site at Arg506 in FV_{Leiden} results in a loss of APC-induced FVa inactivation and impairment of FV cofactor activity of APC in FVIIIa inactivation. Rare FV point mutations Arg306→Thr (R306T; $FV_{Cambridge}$)[19,20] and Arg306→Gly (R306G; $FV_{Hong\ Kong}$)[20,21] affect the APC cleavage site at Arg306 and are associated with mild APCR.[22] No FV mutations linked to APCR have been identified in Japanese populations, however. We describe the findings in a Japanese boy with severe DVT in the paradoxical presence of FV deficiency with FV activity (FV:C) 10 IU/dL. We have identified a novel mechanism of thrombosis associated with a Trp1920→Arg (W1920R) mutation in the *F5* gene (FV_{Nara}). The defect resulted in APCR more potent than that seen with FV_{Leiden}.

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Materials and methods

Blood samples were obtained after informed consent following local ethical guidelines. DNA direct sequencing and the expression of recombinant protein were approved by the Medical Research Ethics Committee of Tokyo Medical University. This study was conducted in accordance with the Declaration of Helsinki.

Reagents

The pMT2/FV mammalian expression plasmid containing the full-length *F5* cDNA was provided by Dr Kaufman (University of Michigan, Ann Arbor, MI). The EZ1 DNA Blood Kit, QIAquick Gel Extraction Kit, and QIAfilter Plasmid Kit (Qiagen, Dusseldorf, Germany) and the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) were purchased. Recombinant FVIII was a generous gift from Bayer Corporation, Japan. A monoclonal antibody (mAb)C5,[23] recognizing the C-terminus of the FVIII A1 domain, was provided by Dr Carol Fulcher (Scripps Research Institute, La Jolla, CA). FV, FIXa, FX, FXa, α-thrombin, APC, PS, mAbAHV-5146 against the FV HCh (Hematologic Technologies, Essex Junction, VT), lipidated TF (Innovin; Dade Behring, Marburg, Germany), and fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) were purchased commercially. FV-deficient plasmas (George King. Overland Park, KS), PT, and activated partial thromboplastin time (aPTT) reagent (Instrumentation Laboratory, Bedford, MA; Sysmex, Kobe, Japan) were purchased. PL vesicles containing phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine, 10%/60%/30%, were prepared using *N*-octylglucoside.[24]

DNA direct sequencing

Genomic DNA was extracted from leukocytes, using the BioRobot EZ1 workstation. PCR assays were performed with *Taq* DNA polymerase (TaKaRa-Bio, Otsu, Japan). PCR products were electrophoresed on agarose gels and purified by gel extraction. Purified PCR products from genomic DNA and *F5* plasmids were confirmed by sequencing using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and analyzed with a 3730 DNA analyzer. All sequences were compared with wild-type (WT) *F5* sequences (GenBank number Z99572).

Expression of recombinant FV

The mutations were introduced independently into a pMT2/FV plasmid by site-directed mutagenesis.[25] The WT and mutant plasmids used in transfection experiments were purified. Vectors expressing recombinant proteins were transfected into HEK293 cells, using the lipofection method. After 60 hours, the culture media and cells were harvested. Conditioned media (CM) were collected, centrifuged to remove cell debris, and stored at −80°C. FV antigen (FV:Ag) levels in CM and cell lysates were measured by enzyme-linked immunosorbent assay (ELISA; Affinity Biologicals). FV:C in CM was measured in PT-based clotting assays, using the ACL9000 coagulation analyzer (Instrumentation Laboratory). The specific activity of FV was calculated as the ratio of FV:C to the concentration of FV:Ag, both of which were measured in CM. The proteins were harvested in serum-free medium and concentrated by filtration (cutoff ~100 kDa).

APCR assay

aPTT-based assays. The APC-resistance kit, which is not approved and not commercially available in Japan, was provided by Instrumentation Laboratory for research use. This assay was performed using ACL9000 with predilution of sample plasmas in FV-deficient plasma. The APC sensitivity ratios (APCsrs) were expressed as ratios of aPTT clotting times in the presence of APC divided by clotting times in its absence. This assay reflects the effect of APC on inactivation of both FVa and FVIIIa; hence, a low level of APCsr indicates a defect in the inactivation of FVa and/or FVIIIa and, consequently, reflects APCR.

Thrombin generation-based assays. Calibrated automated thrombin generation assay (Thrombinoscope) was performed as previously reported.[26] Platelet-poor plasma (PPP) or platelet-rich plasma (PRP) was preincubated for 10 minutes with TF (5 pM), APC (8 or 40 nM), and PL (0 or 10 μM), respectively. PRP was adjusted to 15×10^4 platelets/μL. Measurements were commenced after the addition of $CaCl_2$ and fluorogenic substrate (final concentration [f.c.] 16.7 mM and 417 μM, respectively). Fluorescent signals were monitored continuously in a Fluoroskan microplate reader (Thermo Fisher Scientific, Franklin, MA). For data analyses, the parameters (lag time, peak thrombin, time to peak, and endogenous thrombin potential [ETP]) were derived. The APCsrs were expressed as ratios of the parameter in the absence (or presence) of APC, divided by the ratio in its presence (or absence).

FXa generation-based assays. Normal or patient's plasma was mixed with FV-deficient plasma in various proportions and assayed using the FXa generation assay (COATEST SP-FVIII, Chromogenix, Milan, Italy), with minor modifications.[27] The test specifically quantifies FVIIIa:C in 16-fold diluted plasma by measuring intrinsic FXa generation mediated by excess exogenous FIXa and FX with PL. The simultaneous addition of APC (40 nM) with cofactors PS and FV in plasma inhibits intrinsic FXa generation by inactivating FVIIIa. The APCsrs were expressed as ratios of the amount of generated FXa in the absence of APC divided by that in its presence. A low level of APCsr indicates a defect in FVIIIa inactivation and, consequently, reflects APCR.

Prothrombinase assay

FV (2 nM) was activated by thrombin (20 nM) for 1 minute, followed by the addition of hirudin. The reactants were mixed with prothrombin (1.4 μM), PL, and 5-dimethylamino-naphthalene-1-sulfonylarginine-*N*-(3-ethyl-1,5-pentanediyl)-amide (30 μM), followed by initiation by the addition of FXa (10 pM). Aliquots were removed to assess the initial rates of product formation, and the reactions were quenched with EDTA (f.c. 50 mM). Rates of thrombin generation were determined at absorbance 405 nm (Abs_{405}) after the addition of S-2238 (f.c. 0.46 mM). Thrombin generation was quantified from a standard curve prepared using known amounts of thrombin.

FV–PL binding

Binding of FV to immobilized PL was examined in ELISAs.[28] α-phosphatidyl-*L*-serine (5 μg/mL) in methanol was added to microtiter wells and air-dried. The wells were blocked by the addition of gelatin solution (5 mg/mL), and serial dilutions of FV were added and incubated at 37°C for 2 hours. Bound FV was quantified by the addition of anti-FV mAbAHV-5146 (2.5 μg/mL) and goat anti-mouse peroxidase-linked antibody, followed by measuring at Abs_{492}. The amount of nonspecific immunoglobulin G (IgG) binding without FV was <3% of the total signal. Specific binding was estimated by subtracting the amount of nonspecific binding.

APC-catalyzed inactivation of FVa

FV (8 nM) was incubated with thrombin (100 nM) for 5 minutes at 37°C, and reaction was terminated by the addition of hirudin (25 U/mL). Samples containing the generated FVa (2 nM) were incubated with APC (25 pM), and PS (30 nM) with PL (20 μM), for the indicated times. Aliquots were obtained from the mixtures and diluted ~30-fold. Residual FV:C was measured in aPTT-based clotting assays. The presence of thrombin and hirudin in the diluted samples had little effect in these assays.

APC cofactor activity of FV

The APC cofactor activity of FV variants was measured in a FVIIIa degradation assay,[29] with minor modifications. FVIII (10 nM) and PL (20 μM) were activated by thrombin (5 nM) for 30 seconds, and the reaction was terminated by the addition of hirudin (2.5 U/mL). The generated FVIIIa was then incubated with APC (0.5 nM) and PS (5 nM) with various concentrations of FV variants for 20 minutes. The reactants were diluted 9-fold before incubation with FIXa (2 nM) and FX (200 nM) for 1 minute. Generated FXa was measured in a chromogenic assay with S-2222 at Abs_{405}. Relative FVIII:C was calculated from the amounts of generated FXa.

Table 1. FV levels and APCR in plasmas of the patient and family members

Case	FV:C (IU/dL)	FV:Ag (IU/dL)	*F5* mutation	aPTT-based APCR assay		
				aPTT, seconds		
				Minus APC	Plus APC	APCsr (plus/minus APC)
FV_{Nara} family members						
Patient	10.0	40.0	W1920R homozygote	79.7	131	1.64
Father	74.1	74.0	W1920R heterozygote	43.3	98.0	2.26
Mother	87.6	75.0	W1920R heterozygote	39.0	104	2.67
Brother	109	77.0	WT	32.2	98.4	3.06
Sister-1	113	101	WT	32.6	107	3.28
Sister-2	132	115	WT	32.2	99.8	3.10
FV_{Leiden} patient						
Patient 1	56.5	96.0	R506Q heterozygote	35.2	57.2	1.63
Patient 2	65.2	61.0	R506Q heterozygote	36.8	61.2	1.66
FV-deficient patient						
Patient 1	50.0	44.0		39.2	124	3.16
Patient 2	54.0	53.0		38.4	118	3.07
Patient 3	55.0	47.0		43.4	124	2.85
Healthy controls						
Male (n = 17)				32.6 ± 0.70	108.4 ± 4.50	3.32 ± 0.11
Female (n = 15)				33.5 ± 1.47	108.6 ± 3.64	3.24 ± 0.08

All data were measured at least 3 times, and the average values are shown. For the levels of healthy controls, the average value ± standard deviation is shown.

Western blotting

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed using 8% gels, followed by western blotting.[30] Protein bands were probed using the indicated mAbs, followed by the addition of goat anti-mouse peroxidase-linked antibody.[30] Signals were detected using enhanced chemiluminescence, and densitometric scans were quantified using Image J 1.34.

Results

Patient's profile

A 13-year-old boy was admitted for massive DVT in association with swelling of the lower extremities. There was no personal or family history of thrombosis. Laboratory findings demonstrated prolonged PT and aPTT (18.9/67.6 seconds; control, 12.2/30.2 seconds). FV:C and FV:Ag were 10 and 40 IU/dL, respectively, indicating a cross-reactive material–reduced reaction. Anti-FV inhibitor was not detected. Other procoagulant and anticoagulant factors, including fibrinolytic factors and antiphospholipid syndrome-associated factors, were within normal range. Free tissue factor pathway inhibitor was 20.2 ng/mL (normal, 15-35 ng/mL).[31] His parents and 3 siblings had normal levels of FV:C and FV:Ag (Table 1). He was treated with warfarin to maintain prothrombin time-international normalized ratio 2.5 to 3.0. Nevertheless, a fresh thrombus developed in his left external iliac-vein, and the right inferior vena cava was completely occluded. After heparinization and urokinase therapy, the patient was treated with higher doses of warfarin to maintain prothrombin time-international normalized ratio 4.0 to 5.0, and he has since been free of recurrent DVT.

Gene analysis

Direct sequencing identified a W1920R homozygous mutation of exon 20 of *F5* in the patient (Figure 1). His parents heterozygously carried this mutation, but it was undetected in his siblings. Neither the FV_{Leiden} mutation (R506Q) nor FV-HR2 haplotype (H1299R and D2194G) were found in the patient or his family members. The W1920R mutation was not detected in 100 alleles from Japanese control subjects using direct sequencing, and the novel *F5* missense mutation that we identified was designated FV_{Nara}.

APCR in FV_{Nara} plasma

We investigated whether the FV_{Nara} was resistant to APC. aPTT-based APCR assays, reflecting APC inactivation of FVa and FVIIIa, were performed. The APCsr obtained in patient's plasma was similar to those of FV_{Leiden} patients and significantly lower than those in healthy control patients (Table 1). APCsrs in the parents were intermediate, and those in the siblings were equal to the levels of healthy controls. APCsrs of 3 mild FV-deficient patients with FV:C ~50 IU/dL were similar to those of control. APCsrs of inherited FV-deficient patients with FV:C ~10 IU/dL were not measurable, however, because the clotting times after the addition of APC were markedly prolonged (data not shown). These results demonstrated that the FV_{Nara} mutation conferred APCR.

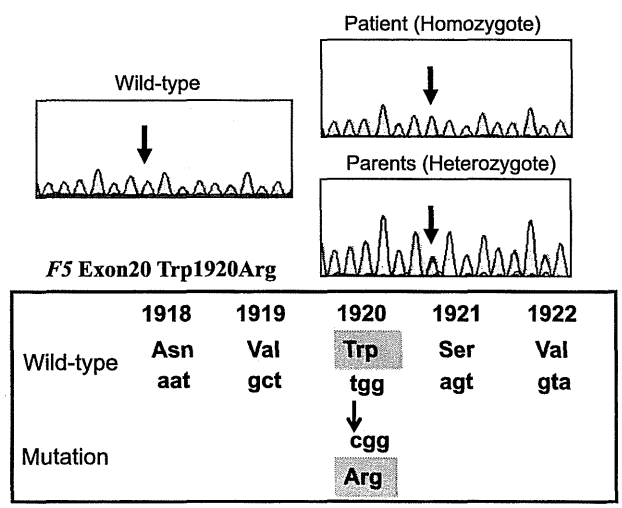

Figure 1. DNA direct sequencing of exon 20 of *F5* gene from the patient, his parents, normal WT. The mutation (T–C) is present at codon 1920, resulting in a Trp1920→Arg substitution in the FV protein (FV_{Nara}).