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ORIGINAL ARTICLE

Coagulation function and mechanisms in various clinical phenotypes of patients with acquired factor V inhibitors

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Summary. *Background:* The clinical phenotype of individuals with acquired factor V (A-FV) inhibitors varies from asymptomatic (non-B group) to life-threatening bleeding (B group), but the mechanism(s) underlying this variation in hemorrhagic phenotype are poorly understood. *Objective:* To investigate coagulation mechanistically in a range of patients with A-FV antibodies. *Methods and Results:* Ten cases of A-FV inhibitors in the non-B ($n = 5$) and B groups ($n = 5$) were studied. Thrombin generation assays in these plasmas revealed little thrombin generation, despite similar FV activity levels in both groups. However, prothrombin time-based clot waveform analysis revealed that the clot times were significantly prolonged and the maximum velocity and acceleration of coagulation were lower in the B group than in the non-B group, suggesting that this technique might be useful for predicting and monitoring hemorrhagic symptoms. A-FV inhibitors from the non-B group recognized predominantly the FV heavy chain, whereas those from the B group recognized the light chain. Purified anti-FV autoantibodies (autoAbs) from the B group inhibited FV binding to phospholipid by 60–90%, whereas there was little effect on this reaction in the non-B group. In addition, anti-FV autoAbs from the non-B group impaired the activated protein C (APC) cofactor activity of FV in FVIIIa inactivation mechanisms, and delayed APC-catalyzed cleavage of FVa at Arg306, but not at Arg506, indicating the presence of APC resistance in the non-B group. *Conclusions:* The results suggest that the different hemorrhagic phenotypes in A-FV inhibitors depend on the specific epitope of anti-FV autoAbs, and appear to be associated with an imbalance of procoagulant and anticoagulant function.

Keywords: APC resistance; blood coagulation factor inhibitors; clinical laboratory techniques; factor V; hemostatic techniques.

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Introduction

Factor V is a single-chain molecule consisting of 2196 amino acids arranged in six domains, A1–A2–B–A3–C1–C2 [1,2]. FV governs the balance of coagulation by regulating opposing functional mechanisms. 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]. Thrombin proteolyses FV, generating the activated form (FVa), a heterodimer composed of a 105-kDa heavy chain (HCh), containing the A1 and A2 domains, and 71/74-kDa light chain (LCh), containing the A3, C1 and C2 domains. The development of a hypercoagulant state is controlled, however, by downregulation of cofactor activity by activated protein C (APC) with protein S (PS). FVa is rapidly inactivated by proteolytic cleavage at Arg506, and then at Arg306 and Arg679 [4]. Cleavage at Arg506 is essential for the exposure of other cleavage sites, but is unlikely to contribute significantly to the reduction in activity. Cleavage at Arg306 results in almost complete loss of FVa activity (FVa:C), but that at Arg679 has a more modest impact [5]. Irregularities in the mechanism of APC-mediated inactivation of FVa are therefore associated with thrombotic episodes in the presence of sustained prothrombin activation.

An alternative function of FV is an as anticoagulant cofactor of APC in the inactivation of FVIIIa [6]. FVIIIa is inactivated by cleavage at Arg336 by APC [7,8]. In the process of APC-mediated inactivation of FVIIIa, FV functions as an anticoagulant cofactor of APC, resulting in acceleration of FVIIIa inactivation [9]. This anticoagulant activity of FV is mediated by a product of proteoly-

sis by APC, prior to cleavage by thrombin. Cleavage at Arg506 of FV attached to the B domain is essential for the anticoagulant FV activity, whereas cleavage at Arg306 appears to be unlikely to contribute to this mechanism [9–12]. Any molecular defect of these cleavage reactions confers APC resistance (APCR). Clinically, individuals with the Arg506Gln mutation (FV Leiden) have a poor anticoagulant response to APC, which is associated with a significant increase in the risk of deep vein thrombosis (DVT) [13,14].

Acquired FV (A-FV) inhibitors occur rarely, but may develop spontaneously as autoantibodies (autoAbs) in previously normal individuals, after exposure to topical hemostatic agents containing bovine thrombin, antibiotic administration, cancer, and autoimmune disorders [15–18]. These anti-FV autoAbs are frequently associated with hemorrhagic symptoms, which are usually mild but are occasionally severe. Some patients remain asymptomatic, however, and hemorrhagic symptoms appear to be limited in ~ 20% of patients diagnosed with A-FV inhibitors [17,18]. It is of note that only four patients with A-FV inhibitors have presented with thrombotic manifestations [18]. Among these cases with DVT, Kalafatis *et al.* reported that the anti-FV autoAbs from one individual diminished both APC-mediated FVa inactivation and FV cofactor activity in APC-mediated FVIIIa inactivation, reflecting APCR [19]. Thrombotic mechanism(s) in other three cases appear not to have been explored, however. Moreover, the precise reasons for the variation in hemorrhagic phenotype in patients with A-FV inhibitors are poorly understood. We therefore investigated coagulation mechanisms in a range of patients with A-FV inhibitors, using a combination of established functional techniques.

Materials and methods

Reagents

Recombinant FVIII was a generous gift from Bayer (Osaka, Japan). Purified FV/FVa, FIXa, FX/FXa, prothrombin, α-thrombin, APC, PS, 5-dimethylamino-naphthalene-1-sulfonylarginine-*N*-(3-ethyl-1,5-pentanediyl)-amide (DAPA) and anti-FV HCh and LCh mAbs, AHV-5146 and AHV-5112, respectively (Hematologic Technologies, Essex Junction, VT, USA), hirudin (Calbiochem, San Diego, CA, USA) and chromogenic substrates S-2222 and S-2238 (Chromogenix, Milano, Italy) were commercially purchased. The activated partial thromboplastin time (APTT) and prothrombin time (PT) reagents, ellagic acid (Sysmex, Kobe, Japan), FV-deficient plasma (George King Biomedical, Overland Park, KS, USA), lipidated tissue factor (Innovin, Dade Behring, Marburg, Germany) and the thrombin substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) were purchased. PL vesicles (phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine; 10% : 60% : 30%) were prepared with *N*-octylglucoside [20]. HBS buffer (20 mM Hepes, pH 7.2, 0.1 M NaCl, 0.01% Tween-20) containing 2.5 mM $CaCl_2$ was used for dilution.

Blood samples

Whole blood was obtained by venepuncture from patients into tubes containing a 1 : 9 volume of 3.8% (w/v) trisodium citrate. Platelet-poor plasma was recovered after centrifugation of citrated whole blood for 10 min at 1500 × *g*. Normal pooled plasmas were prepared from 30 normal healthy individuals (25 : 5 male/female). All plasmas were stored at − 80 °C and thawed at 37 °C immediately prior to the assays. All samples were obtained after informed consent had been obtained, following local ethical guidelines.

FV activity (FV:C), FV antigen (FV:Ag) and FV inhibitor levels

FV:C and FV:Ag were measured with PT-based clotting assays with FV-deficient plasma and with ELISAs, respectively. A-FV inhibitor titers were determined with the Bethesda method described for FVIII antibodies [21].

Anti-FV inhibitor autoAbs

Anti-FV IgGs were purified from the plasma of patients with A-FV inhibitors. IgG preparations were fractionated by affinity chromatography on protein G–Sepharose. F $(ab')_2$ fragments were prepared by the use of immobilized pepsin–Sepharose (Pierce, Rockford, IL, USA). Specific regions of FV/FVa recognized by these antibodies were determined with SDS-PAGE and western blotting. The binding of FV/FVa fragments to anti-FV autoAbs was detected by the addition of anti-human peroxidase-linked antibody. The effects of FV inhibitors were expressed as a function of IgG concentration in this study, although the percentage of anti-FV in IgG preparations varied between patients.

Clot waveform analysis (CWA)

PT and APTT measurements were performed with the MDA-II Hemostasis System (Tcoag Ireland, Bray, Ireland). The clot waveforms obtained were computer-processed with the commercial kinetic algorithm [22]. The minimum value of the first derivative (min1) was calculated as an indicator of the maximum velocity of coagulation achieved. The minimum value of the second derivative (min2) was calculated as an indicator of the maximum acceleration of the reaction achieved. As the minima of min1 and min2 are derived from negative changes, the data were expressed as min1 and min2. The

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

Prothrombinase assay

The rate of conversion of prothrombin to thrombin was monitored in a purified system. FVa (2 nM) was incubated with various concentrations of anti-FV autoAbs at 37 °C for 30 min. The reactants were mixed with prothrombin (1.4 μM), PL (20 μM), and DAPA (30 μM), and this was followed by initiation of the addition of FXa (10 pM). Aliquots were removed at appropriate times to assess the initial rates of product formation, and were mixed with EDTA (final concentration of 50 mM) to quench the reactions. Rates of thrombin generation were determined at an absorbance of 405 nm after the addition of S-2238 (final concentration of 0.46 mM). Thrombin generation was quantified from a standard curve prepared with known amounts of thrombin.

FV–PL binding assay

Binding of FV to immobilized PL was examined in a solid-phase based ELISA [23]. α-Phosphatidyl-L-serine (5 μg mL^{-1}) in methanol was added to microtiter wells and air-dried at 4 °C overnight. After washing of the wells, the wells were blocked by the addition of gelatin solution (5 mg mL^{-1}) at 37 °C for 2 h. FV (1 nM) and anti-FV autoAbs were incubated for 2 h. After washing, the mixture reactants were added to the PL-coated well, and incubated for 2 h. Bound FV was quantified by the addition of the anti-FV mAb AHV-5146 (2.5 μg mL^{-1}), followed by a goat anti-mouse peroxidase-linked antibody (3 μg mL^{-1}) and *O*-phenylenediamine dihydrochloride substrate. Reactions were quenched by the addition of 2 M H_2SO_4, and absorbance at 492 nm was measured. The amount of non-specific IgG binding without FV was < 3% of the total signal. Specific binding was estimated by subtracting the amount of non-specific binding.

APCR assays

Chromogenic assay FXa generation in plasma samples was performed with the COATEST SP FVIII assay (Chromogenix). The test specifically quantifies FVIIIa activity in 16-fold-diluted plasma, by measuring intrinsic FXa generation mediated by excess exogenous FIXa and FX with PL and $CaCl_2$. The simultaneous addition of APC (40 nM) with the cofactors PS and FV contained in plasma inhibits intrinsic FXa generation by inactivating FVIIIa. The APC sensitivity ratio (APCsr) was expressed as the absorbance in the absence of APC divided by that in its presence. A low APCsr value indicates a defect in the inactivation of FVIIIa, reflecting the APCR.

APC cofactor activity of FV The APC cofactor activity of FV was measured with an FVIIIa degradation assay, as previously reported, with minor modifications [9]. FVIII (10 nM) and PL (20 μM) were activated by thrombin (5 nM) for 30 s, and the reaction was terminated by the addition of hirudin (2.5 U mL^{-1}). The generated FVIIIa was then incubated for 7 min with APC (0.5 nM), PS (5 nM), and the mixtures of FV (1 nM) and the indicated amounts of anti-FV autoAbs or normal IgG. The mixture was 10-fold-diluted prior to incubation with FIXa (2 nM) and FX (200 nM) for 1 min. After the addition of EDTA, generated FXa was evaluated by the use of S-2222 at 405 nm. Control experiments were performed without either APC or FV. FXa generation was quantified from a standard curve prepared with known amounts of FXa.

APC-catalyzed cleavage of FVa FV (8 nM) and anti-FV autoAbs (30 μg mL^{-1}) were incubated for 2 h at 37 °C. The mixtures were incubated with thrombin (30 nM) for 5 min at 37 °C, and the thrombin reaction was rapidly terminated by the addition of hirudin (10 U mL^{-1}). Samples containing the generated FVa (2 nM) were incubated with PL (20 μM), PS (30 nM), and APC (0.7 nM). Aliquots were removed from the mixtures at the indicated times, and reactions were immediately terminated by adding SDS and boiling for 3 min.

Western blot analysis

SDS-PAGE was performed with 8% gels, and this was followed by western blotting [24]. Protein bands were probed with the indicated anti-FV mAb, and this was followed by the addition of goat anti-mouse peroxidase-linked antibody. Signals were detected with enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA, USA). Densitometric scans were quantified with IMAGEJ 1.34 (NIH, Bethesda, MD, USA).

Results

Global coagulation function in plasmas from patients with A-FV inhibitors

The properties of five patients with A-FV inhibitors in the asymptomatic group (non-B group) and five patients with severe bleeding symptoms (B group) are summarized in Table 1. To define the bleeding symptoms, we used the Vicenza Bleeding Score [25] to evaluate hemorrhage. In the B group, the scores ranged from 3 to 7, whereas in the non-B group the scores were 0. In both groups, both the PT and the APTT were markedly prolonged, and FV:C ranged from undetectable to low levels (~ 10 IU dL^{-1}), showing that the routine clotting tests and FV:C levels did not well reflect the clinical phenotype. FV:Ag levels

Table 1 Properties of patients' plasmas with acquired factor V inhibitors

Case	Sex	PT (s)	APTT (s)	FV:C (IU dL^{-1})	FV:Ag (IU dL^{-1})	Inhibitor titer (BU mL^{-1})	Ig subtype	Bleeding score*	Underlying disease
Asymptomatic (non-B group)									
1	M	53.5	> 150	2.3	93.8	4.3	IgG	0	Chronic thyroiditis
2	M	47.4	133	11.5	86.3	5.4	IgG	0	Progressive supranuclear palsy
3	M	67.7	> 150	< 1.0	137	11.8	IgG	0	Intraductal papillary mucinous tumor of pancreas
4	M	83.6	> 150	< 1.0	180	1.7	IgG	0	Atrial fibrillation
5	F	34.1	66.8	1.7	55.6	8.7	IgG	0	None
Severe bleeding (B group)									
6	M	94.5	> 150	8.0	81.3	118	IgG	4	Aspiration pneumonia, asthma
7	F	56.0	> 150	< 1.0	75.0	16	IgG	4	Surgery for valve replacement (TR, AS, MR)
8	M	> 100	> 150	< 1.0	57.8	64	IgG	3	Chronic renal failure
9	M	> 100	> 150	< 1.0	2.1	9.9	IgG	5	None
10	M	81.1	92.3	< 1.0	36.2	8.2	IgG	7	None
Control	–	12.1	30.3	–	–	–	–	–	–

APTT, activated partial thromboplastin time; AS, aortic stenosis; F, female; FV:Ag, FV antigen; FV:C, FV activity; M, male; MR, mitral regurgitation; PT, prothrombin time; TR, tricuspid regurgitation. *Numbers represent the bleeding score calculated according to Rodeghiero *et al.* [25].

(except for case 9) were not significantly different between groups, supporting the contention that low FV:C in patients was attributable to functional inhibition by A-FV inhibitors. FV inhibitor titers were 6.4 ± 3.9 and 43 ± 48 BU mL^{-1} in the non-B group and B group, respectively. The immunoglobulin class was IgG in all patients, and none had been exposed to antibiotic therapy or bovine thrombin products. In all cases, platelet counts, other coagulation proteins, anticoagulant proteins (protein C [PC]/PS, antithrombin) and fibrinolytic proteins showed normal plasma levels. No autoAbs, except for those against FV, were detectable (data not shown). These findings typified the difficulties in identifying the different clinical phenotypes on the basis of basic coagulation tests.

We compared the different inhibitor groups by using the following global coagulation assays: the thrombin generation test (TGT) and CWA. There was little evident thrombin generation in patient plasmas, even after 60 min of reaction time (data not shown), suggesting that evaluation of the different A-FV inhibitors with this technique was not informative. Unlike the TGT, CWA reflects the process of fibrin formation. Representative curves of the PT-based CWA are shown in Fig. 1 (upper). Because of the small sample volume from case 7, this case failed to show the clot waveform. The clot time observed in the non-B group was markedly shorter that that in the B group (56.5 ± 19.6 vs. 104 ± 20 s, $P = 0.0044$) (Fig. 1A), and the parameters min1 and min2 were significantly greater in the non-B group than in the B group (min1, 3.09 ± 1.00 vs. 0.87 ± 0.32, $P = 0.0022$; min2, 0.90 ± 0.48 vs. 0.15 ± 0.06, $P = 0.009$) (Fig. 1B,C). These findings appeared to be in keeping with the contrasting hemorrhagic symptoms observed in the different patients. The data suggested that CWA might be a useful method for predicting and monitoring the bleeding tendency in patients with A-FV inhibitors.

Binding epitope(s) of A-FV inhibitors

To clarify the precise mechanism(s) involved in the distinct phenotypes in patients with A-FV inhibitors, we initially attempted to determine the FV epitopes of the antibodies by immunoblotting. Figure 2 shows that, in the non-B group, three inhibitors reacted with the HCh alone. In contrast, in the B group, two inhibitors reacted with the LCh alone, and two inhibitors reacted with both chains. The results suggested the possible presence of distinct epitopes of anti-FV autoAbs between both groups. The reason for the presence of some bands in FV lanes (cases 1 and 10) was unclear, but partially proteolysed FV may be a contaminant of the single-chain FV preparation. Binding to FV(a) fragments was not observed in two cases from the non-B group and in one case from the B group. This may be attributable to weak binding reactivity of individual inhibitors and/or to low sensitivity of this assay.

Effects of purified anti-FV autoAbs on FV–PL binding

The specific properties of A-FV inhibitors were further examined with immune-purified IgGs from the patients' plasmas. With mixtures of normal plasma and purified anti-FV IgG, all CWA parameters were similar to those obtained with native patients' plasmas (data not shown), confirming that the defective coagulation function in both groups was attributable to the presence of antibody. The LCh of FV, and in particular the C2 domain, contains PL-binding site(s) [26]. The effects of purified anti-FV autoAbs on FV binding to immobilized PL were therefore examined with a solid-phase-based ELISA. All IgGs from

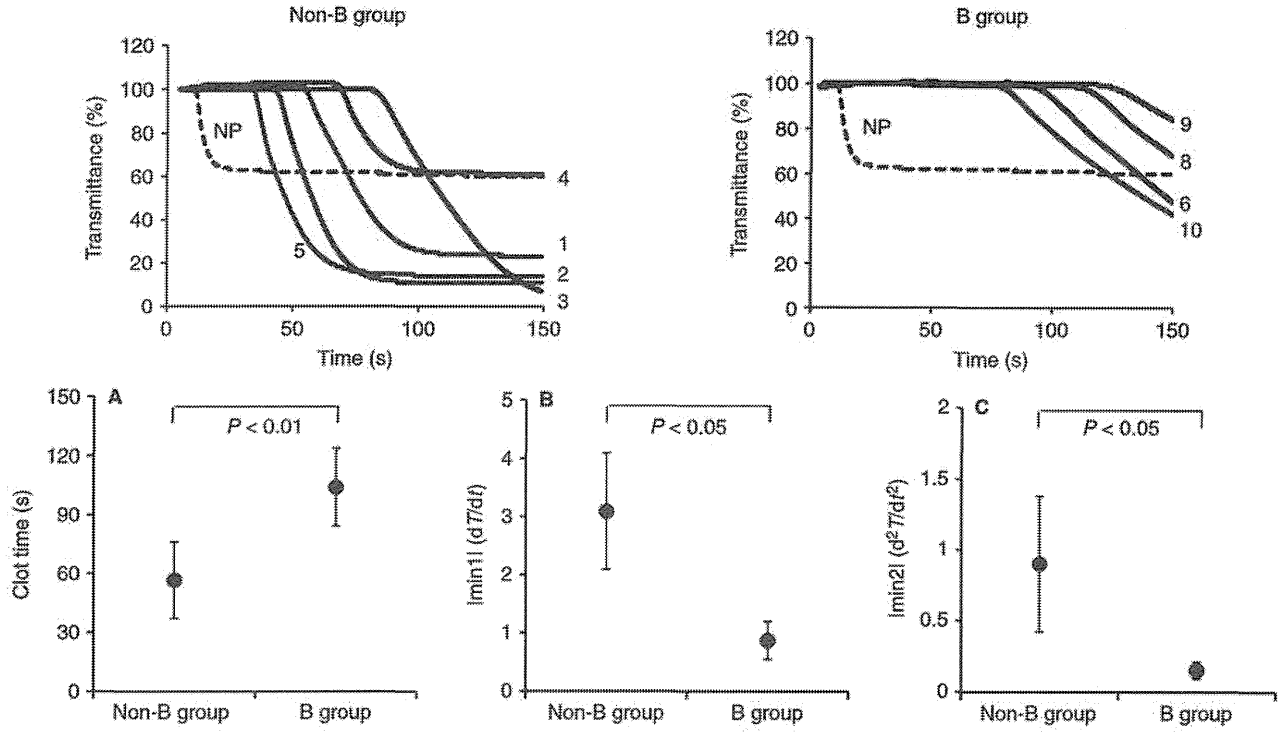

Fig. 1. Clot waveform analysis in plasmas from patients with acquired factor V inhibitors. The prothrombin times in patients' plasmas in both groups were measured with the MDA-II system. The parameters (lower panels) clot time (A), min1 (B) and min2 (C) were calculated from the clot waveform (upper panels) 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

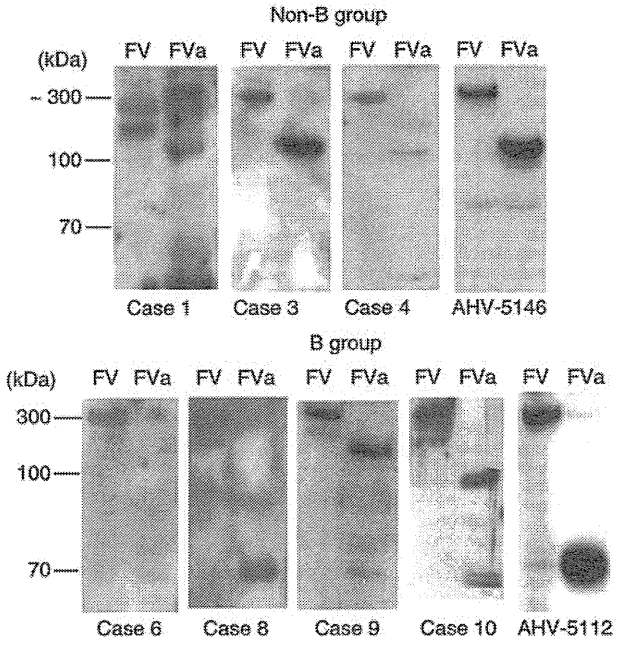

Fig. 2. Identification of epitope(s) of acquired factor V inhibitors. Samples of FV and thrombin-cleaved FVa (100 nM) were analyzed by 8% gel SDS-PAGE, and this was followed by transfer to poly (vinylidene difluoride) membranes. The membranes were incubated with patients' plasmas, and binding to FV(a) 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.

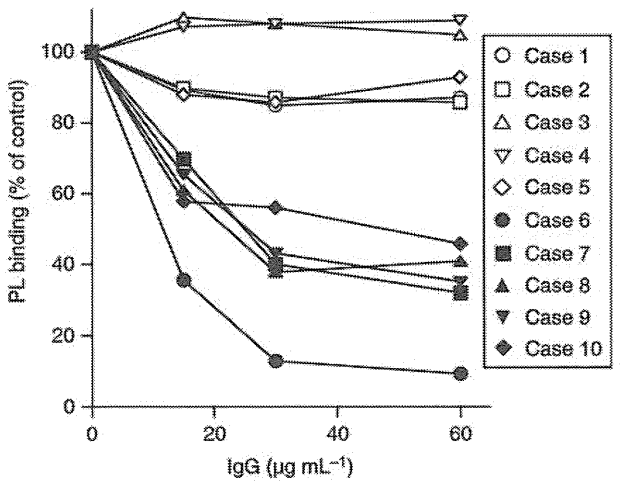

Fig. 3. Effects of anti-FV autoantibodies (autoAbs) on FV binding to phospholipid (PL) in solid-phase-based ELISA. α-Phosphatidyl-L-serine (5 μg mL^{-1}) in methanol was added to microtiter wells, and air-dried at 4 °C overnight. After blocking with gelatin solution, the reactant mixtures with FV (1 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.

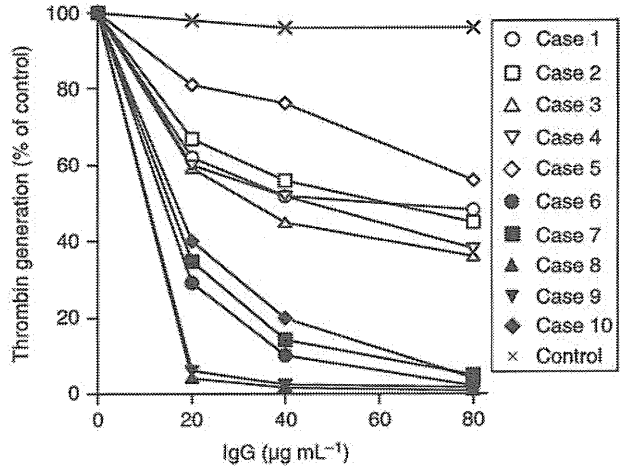

Fig. 4. Prothrombinase activity in the presence of anti-FV autoantibodies (autoAbs). Mixtures of FVa (2 nM) and anti-FV autoAbs were added to prothrombin (1.4 μM), phospholipid vesicles (20 μM), and 5-dimethylamino-naphthalene-1-sulfonylarginine-*N*-(3-ethyl-1,5-pentanediyl)-amide (30 μM). Thrombin generation 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

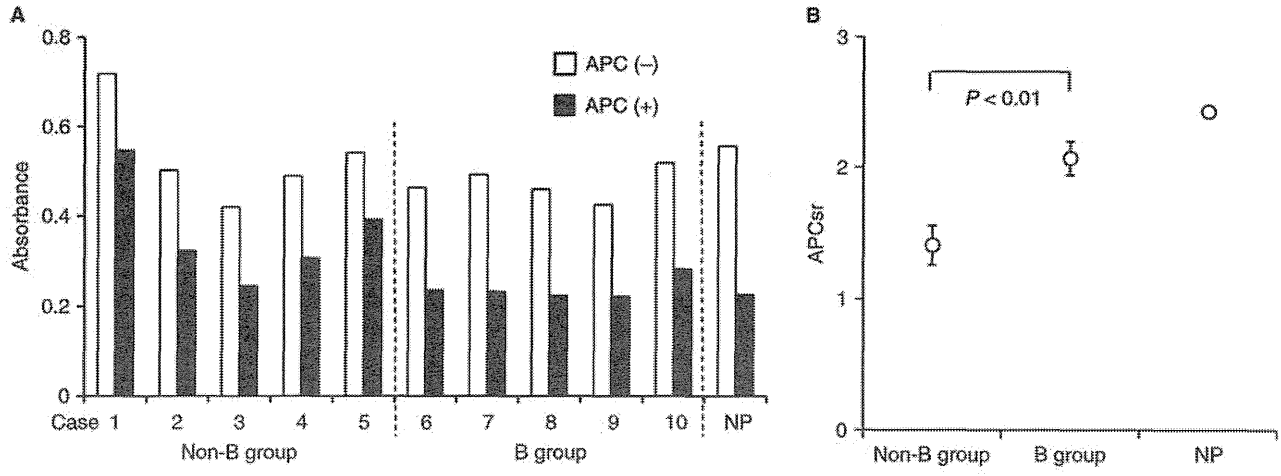

Fig. 5. Activated protein C (APC) sensitivity ratio (APCsr) in the non-B group or B group with acquired factor V inhibitors. FXa generation assays in the presence of APC with patients' plasmas or normal plasma (NP) were performed with COATEST SP FVIII as described in Materials and methods. (A) The absorbance 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.

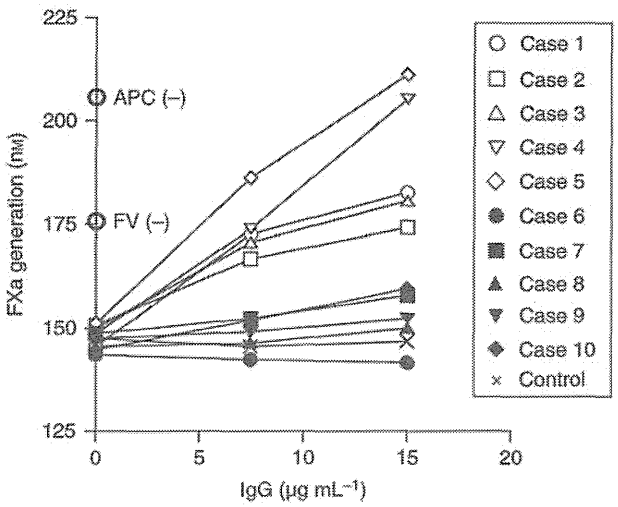

Fig. 6. Effects of anti-FV autoantibodies (autoAbs) on the activated protein C (APC) cofactor activity of FV assessed by FVIIIa degradation. FVIII (10 nM) and phospholipid (20 μM) were activated by thrombin (5 nM), and this was followed by the addition of hirudin. Generated FVIIIa was incubated with 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

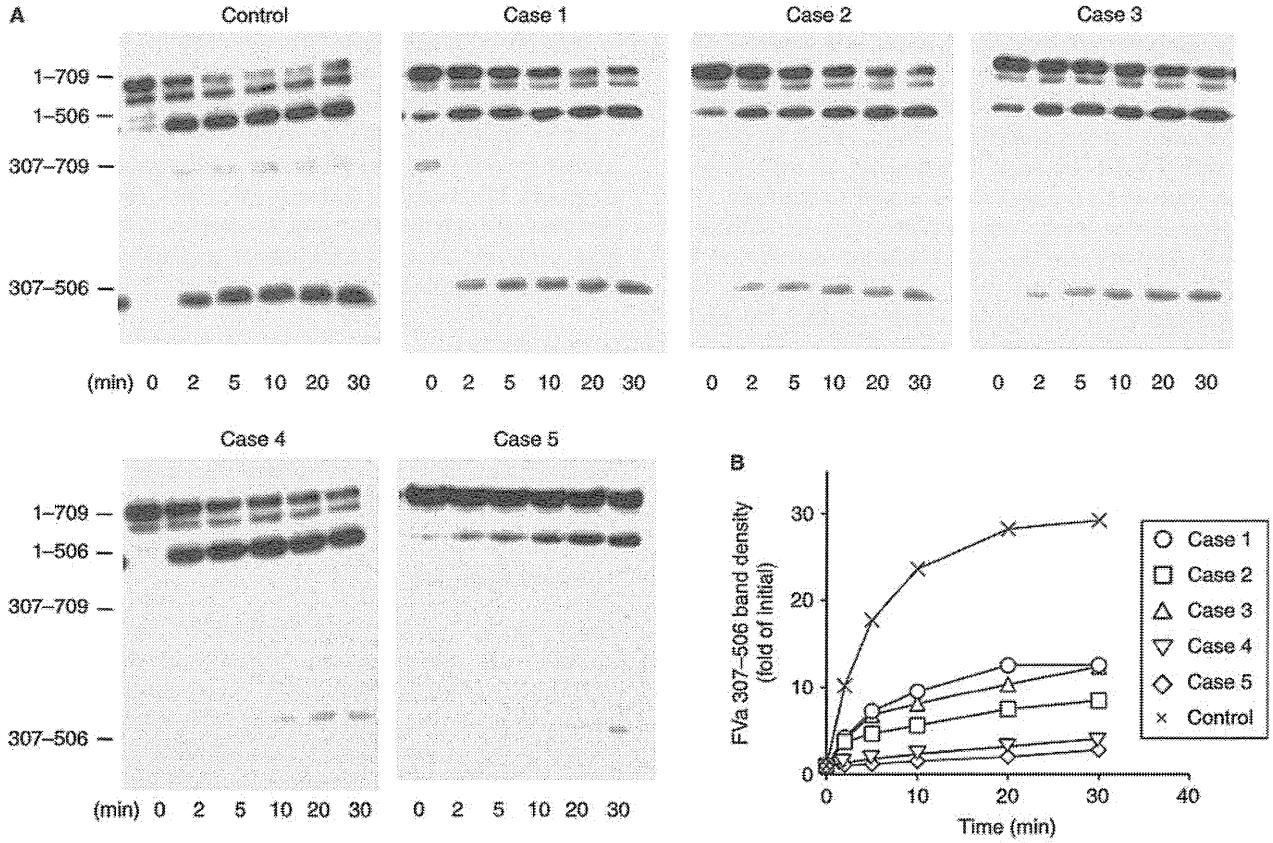

Fig. 7. Activated protein C (APC)-catalyzed proteolytic cleavage of the FVa heavy chain in the presence of anti-FV autoantibodies (autoAbs) from the non-B group. Mixtures of FV (8 nM) and anti-FV autoAbs (30 μg mL^{-1}) from the non-B group were incubated with thrombin (30 nM) for 5 min, and the reaction 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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Contribution of ADAMTS13 to the better cell engraftment efficacy in mouse model of bone marrow transplantation

The adhesive protein von Willebrand factor (VWF) plays an essential role in physiological hemostasis, mediating platelet adhesion and aggregation under high shear stress conditions.[1,2] The VWF-cleaving protease ADAMTS13 precisely down-regulates VWF activity to avoid pathological intravascular thrombosis in the microvasculature, including arterial capillaries, where blood flow typically creates high shear stress.[1-3] Indeed, the functional deficiency of ADAMTS13 is known to cause thrombotic thrombocytopenic purpura, a typical thrombotic occlusion of the microvasculature.[2,4,5] Thus, the proper equilibrium between VWF and ADAMTS13 is necessary for robust microcirculation *in vivo*. In this context, we hypothesized that ADAMTS13 might contribute to better donor cell homing and engraftment in various cell therapy approaches, in which fluent blood flow in the microcirculation system could be critical. To test this hypothesis, we investigated the role of ADAMTS13 on donor cell engraftment using a bone marrow transplantation (BMT) model in ADAMTS13 gene-deleted (*Adamts13* -/-) mice.

Adamts13 -/- (KO) mice were back-crossed for more than 15 generations to the C57BL/6 background, as described.[6] Wild-type (WT) mice (C57BL/6-background) were purchased from Japan SLC (Shizuoka, Japan). All mice used in this study were 8-12 weeks old with body weights of 25-30 grams. Mouse experiments were performed in accordance with protocols approved by the Ethics Review Committee for Animal Experimentation of Nara Medical University. In the BMT experiment, recipient WT or KO mice were conditioned for cellular transplantation with lethal total body irradiation (TBI: 5.5 × 2 = total 11 Gray) using a cesium irradiator (MBR-1520, Hitachi, Tokyo,

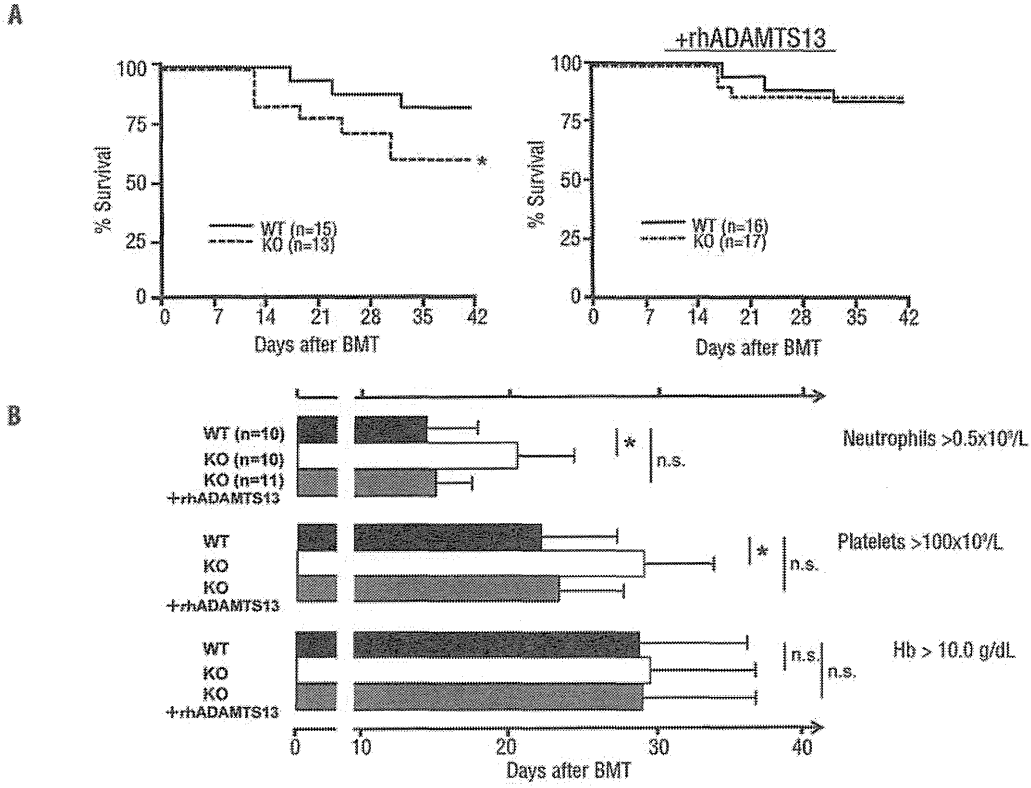

Figure 1. Survival rates and bone marrow suppression of wild-type (WT) or *Adamts13* $^{-/-}$ (KO) mice receiving TBI and subsequent BMT. (A) Kaplan-Meier analysis of survival rates of WT or KO mice receiving TBI and BMT. GFP-positive donor bone marrow cells (5 × 10⁶/mouse) were transplanted to sex-matched WT (n=15) or KO (n=13) mice within 6 hours after TBI (total dose of 11 Gray/mouse) via tail vein. In some indicated experiments (right panel), recombinant human ADAMTS13 (rhADAMTS13; 5 μg/mouse, equivalent to 200 U/kg) was added to the donor bone marrow cell suspension prior to cellular transplantation. Significance of survival studies was quantified using Kaplan-Meier analysis and log rank tests. Note that the survival rate of KO mice began declining significantly (*P<0.05) at Day 14 of BMT, as compared to the WT mice (left panel; WT: 100% *vs.* KO: 76.9%). Following bolus administration of rhADAMTS13, this impaired survival rate in KO mice (WT: 81.0% *vs.* KO: 61.5% at Day 35) improved and became nearly indistinguishable from WT mice (see right panel). (B) Sequential peripheral blood analysis of WT or KO mice after TBI and BMT. Recipient mice were anesthetized using isoflurane inhalation, and 70 μL of blood was collected from the saphenous vein. Complete blood counts of recipient WT (n=10) or KO (n=10) were determined with an automatic blood cell counter (pocH®-100i: Sysmex, Kobe, Japan) every three days following BMT. Each bar represents the mean ± standard deviation (SD) duration that neutrophils counts were > 0.5x10⁹/L, platelet tcounts were > 100x10⁹/L, or hemoglobin values (Hb) were > 10.0 g/dL. Differences between groups were evaluated by Student's t-test. Note that nadir periods of KO mice are significantly (*P<0.05) longer than those of WT mice with regard to neutrophil and platelet counts (WT: 14.4 ± 3.3 and 22.4 ± 3.5 days *vs.* KO: 20.2±3.8 and 28.5±4.8 days, respectively), while no differences between these 2 groups are seen in Hb (WT: 28.2 ± 7.8 days *vs.* KO: 29.7 ± 7.5 days). These nadir period prolongations were improved by rhADAMTS13 (n=11) to an extent comparable to those of WT (n.s.: not significant).

Japan). Bone marrow cells to be transplanted were collected from femurs and tibias of donor green fluorescence protein (GFP) mice[7] (purchased from Japan SLC: C57BL/6-background), as described.[8] After removing the red blood cells by lysing with Tris-buffered ammonium chloride, suspended donor bone marrow mononuclear cells were transplanted into irradiated sex-matched recipient mice via tail vein. In some indicated experiments, recombinant human ADAMTS13 (rhADAMTS13), which was prepared as previously described,[9] was added to the donor bone marrow cell suspension prior to cellular transplantation. The VWF-cleaving activity of rhADAMTS13 was determined by *in vitro* FRETS-VWF73 assay.[10]

Kaplan-Meier analysis showed that the mean survival rate of KO mice receiving TBI and subsequent BMT was significantly lower than that of WT mice starting at Day 14 after BMT, and recombinant ADAMTS13 restored the survival rate of KO mice to that of WT mice (Figure 1A). Since all WT and KO mice that underwent TBI without BMT died within 21 days (*results not shown*), the mortality rates under our experimental conditions most likely depended upon the cell engraftment efficacy during BMT and indicate an important contribution of ADAMTS13 in this regard. Indeed, peripheral blood analysis following BMT revealed the longer nadir period in KO mice with regard to neutrophils and platelets (Figure 1B), which was shortened significantly by recombinant ADAMTS13, with the resulting nadir periods comparable to those of WT mice (Figure 1B).

In addition to the above long-term observation experiment, some recipient mice were sacrificed at Days 1, 7, and 14 after BMT to check the extent of donor cell engraftment to the bone marrow and to assess the pathohistological conditions of major organs. After removing the red blood cells, the recipients' bone marrow was collected from the femurs and tibias and used to assess donor cell engraftment efficacy based on the percentage of GFP-positive cells rela-

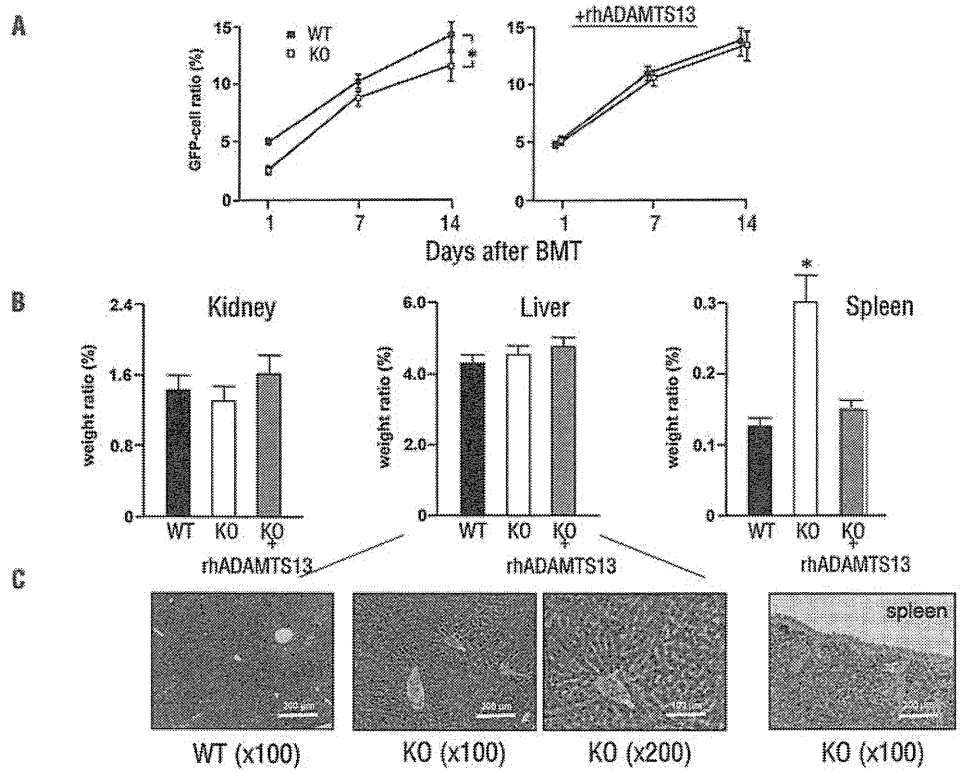

Figure 2. Bone marrow analysis and pathohistological studies in WT or KO mice that received TBI and subsequent BMT. These series of experiments, in which the recipient mice were sacrificed at Days 1, 7, and 14 after BMT (n=5 each), were performed independently of the long-term observation experiments in Figure 1. (A) Flow cytometric analysis of bone marrow cells from WT or KO mice that received TBI and BMT. Each data point represents the average ± SD of "GFP-cell ratio," the percentage of GFP-positive cells relative to total mononuclear cells in bone marrow. Note that a significant ($*P < 0.05$) reduction of donor GFP-positive cells in KO mice is already seen at Day 1 and continues throughout the observation period. In terms of cell propagation in KO mouse marrow, GFP-cells gradually increased in a time-dependent manner similar to that of WT (left panel). This GFP-cell reduction in KO mice was eliminated by rhADAMTS13 (see right panel). (B) Macroscopic findings of major organs in mice sacrificed at Day 7. Each bar represents the average ± SD of "weight ratio," the percentage of each organ weight (kidney, liver, or spleen) relative to total mouse body weight. In terms of macroscopic appearance, no particular differences were seen between WT and KO mice, except for a larger spleen in KO mice (*results not shown*). In fact, the calculated weight ratio confirmed the significant ($*P < 0.05$) splenomegaly in KO mice, which was eliminated by rhADAMTS13 administration. Mild splenomegaly, the extent of which was improved, remained in the corresponding Day 14 samples of KO mice (*results not shown*). (C) Microscopic findings of liver or spleen in mice sacrificed at Day 7. Images displayed are representative of 5 independent mouse samples. The liver samples with hematoxylin-eosin staining (×100 or ×200; original magnification) demonstrate slight dilation of the portal and central veins as well as mild sinusoidal congestion in both WT and KO mice, albeit less pronounced in WT mice. KO mouse livers do not exhibit either typical thrombotic legions in micro-vessels or SOS-lesions. As consistent with macroscopic splenomegaly, mild congestion and external capsule hypertrophy are observed in spleen of KO mice. These microscopic findings are basically similar to the corresponding Day 14 samples (*results not shown*).

tive to total mononuclear cells using flow cytometer (BD LSR-II: Nippon Becton Dickinson Company Ltd., Tokyo, Japan). Consistent with the findings in the peripheral blood, flow cytometric analysis of recipient bone marrow revealed the reduction of donor GFP-positive cells in KO mice that was already significant at Day 1 after BMT (Figure 2A). The population of GFP-positive cells in the bone marrow of KO mice expands gradually in a time-dependent manner similar to that of WT mice (Figure 2A), suggesting that ADAMTS13 is likely to play a role in the initial donor cell homing rather than cell propagation in the bone marrow cell engraftment. Thus, our results could verify the initial hypothesis that ADAMTS13 may contribute to better donor cell homing to the target recipient marrow, a process that requires fluent blood flow in the microvasculature including arterial capillaries.

Thrombotic microangiopathy (TMA) is a well-recognized serious complication of BMT, especially in the liver in the form of sinusoidal obstruction syndrome (SOS), and is known to be associated with functional ADAMTS13 deficiency.[11] Our histological studies, however, have only confirmed mild congestion and sinusoidal dilatation in the liver as well as significant splenic enlargement and congestion in KO mice, without typical thrombotic or SOS lesions of microvessels (Figure 2B and C). These histological findings may be consistent with possible portal hypertension, perhaps reflecting transient occlusion of the microvasculature by enhanced leukocyte plugging or platelet micro-aggregate formation that may occur in systemic microcirculation. Thus, the reduced local microcirculation could result in the poor donor cell homing to bone marrow that was observed in KO mice. Indeed, some clinical symptoms of TMA with functional deficiency of ADAMTS13 are known to be labile and variable,[5] suggesting the existence of transient microvasculature occlusion that cannot be reproducibly demonstrated in final tissue sample sections.

Recent mouse model studies by us and others demonstrated that proper functional regulation of VWF by ADAMTS13 significantly ameliorates the severity of fatal arterial thrombosis in conditions such as cerebrovascular accident or myocardial infarction.[12-15] ADAMTS13 reduces VWF-dependent platelet microaggregate formation as well as inflammatory responses such as leukocyte accumulation at ischemic sites, both of which may result in local microvasculature occlusion.[5] Thus, this property of ADAMTS13 can protect against impaired microcirculation *in vivo*, and may also contribute to better donor cell homing and engraftment in various cell therapy approaches that require fluent blood flow in the microvasculature.

In conclusion, our results illustrate that the regulation of VWF-mediated thrombotic or inflammatory responses by ADAMTS13 may contribute to the improved systemic microcirculation critical for efficient donor cell homing and engraftment in BMT, suggesting a clinical therapeutic potential of ADAMTS13 in cell therapy approaches.

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Key words: bone marrow transplantation, cell engraftment, ADAMTS13, efficacy, mouse model.

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Letters to the Editor

Late vitamin K deficiency bleeding in an infant born at a maternity hospital

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Kaga *et al.* described an additional case of late vitamin K deficiency bleeding (VKDB) in an infant with inadequate prophylactic vitamin K.[1]

In the fifth Japanese nationwide survey of late VKDB in infancy performed between January 1999 and December 2004, 71 cases of late VKDB were reported.[2] At that time, a three-dose oral regimen with 2 mg of vitamin K was recommended in Japan.[3] The consensus on prevention of late VKDB by vitamin K prophylaxis is well known, but late VKDB is still occurring, with insufficient vitamin K treatment noted even in healthy infants: 42/71 infants (59%) had inadequate vitamin K (of the three standard doses required, it was given only once in 27 cases, and only twice in 15 cases) in the fifth nationwide survey in Japan.[2]

Recently, the current status of vitamin K treatment for infants has been modified as follows: oral vitamin K prophylaxis at birth with 2 mg phytomenadione, followed by weekly oral vitamin K prophylaxis; 2 mg should be given by the parents until 3 months of age (1 month of age in formula-fed infants).[4] Although weekly oral vitamin K prophylaxis for infants effectively reduced the incidence of late VKDB,[5,6] a repeated oral dose regimen may not be practical because of poor patient compliance.[7] Many parents lack basic knowledge about the importance and safety of vitamin K.[8]

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The regimen of weekly 1 mg vitamin K treatment for 3 months in Denmark, where compliance was high, resulted in 100% protection against VKDB.[6] In order to ensure successful vitamin K treatment for infants, there is an urgent need to inform parents about the risk and benefit of safe vitamin K treatment, as well as to provide them with perinatal facilities.

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D 血小板と止血・血栓の異常

3 血栓症と血栓性素因

a. 遺伝性血栓症(栓友病)

▶定義と概念

血栓症とは，止血機構が過剰に作動することにより病的血栓が血管内に形成され，組織・臓器の血液循環が障害される多元病である．血栓塞栓を起こしやすい体質に，さまざまな誘因が加わって発症する．血栓症を唯一の表現型とする単一遺伝子病が“栓友病(thrombophilia)”であるが，未発症者は“血栓性素因”とよぶのが適切である．

▶病因・病態

血管壁，血流の変化および凝血能が相互に作用して，血栓が形成される．動脈血栓には血管壁の変化が，静脈血栓には凝血能が深く関与する．血栓性素因のあるおもな遺伝病を**表**に示す．

3大自然抗凝固因子であるプロテインC(protein C：PC)，プロテインS(protein S：PS)およびアンチトロンビン(antithrombin：AT)異常は，最も血栓発症リスクが高い．PCは肝臓で合成されるビタミンK依存性抗凝固因子で，トロンボモジュリン(thrombomodulin：TM)とトロンビンの作用により活性化PCとなる．活性化PCはPSを補酵素に，凝固第VaおよびVIIIa因子を選択的に不活化し，またプラスミノーゲンアクチベーターインヒビター1(plasminogen activator inhibitor-1：PAI-1)を不活化して抗凝固作用をもたらす．活性化PCには抗炎症作用や細胞保護機能がある．PSもビタミンK依存性抗凝固因子で，肝臓と血管内皮で産生され，血小板顆粒にも存在する．活性化PCの補酵素として，また単独でも第VaとXa因子を不活化し，抗凝固因子として働く．約60％は補体C4結合蛋白と結合し，約40％が遊離型で補酵素活性をもつ．ATはトロンビン，第IXa・第Xa・第XIa・第XIIa因子などのセリンプロテアーゼに対する阻害因子で，肝にて合成される．PC欠損症は抗原量・活性ともに低下するtypeIと，抗原量は正常で活性のみ低下するtypeIIに分類される．PS欠損症は全抗原量，遊離型抗原量，活性ともに低下するtypeIと，活性のみ低下するtypeII，遊離抗原量のみ低下するtypeIIIに分類される．AT欠損症は抗原量・活性ともに低下するTypeI，AT活性のみ低下するtypeII(RS：活性部位の異常，HBS：ヘパリン結合部位の異常，PE：両方の異常)に分類される．欧米人に頻度の高いFV Leidenやプロトロンビン変異G20210Aは日本人には見つからないが，活性化PC抵抗性を示すFV Nara[1]やAT抵抗性を示すFII Yukuhashi[2]が近年同定された．PC，PSおよびAT欠損症は，常染色体優性遺伝病で，各蛋白をコードする遺伝子は*PROC*(2q13-q14に局在)，*PROS1*(3q11.2に局在)および*SERPINC1*(1q23-25に局在)である．両アレル変異による新生児電撃性紫斑病が有名である．活性低下を示すヘテロ変異保有者は，思春期頃から深部静脈血栓症や肺血栓塞栓症などの静脈血栓塞栓症を発症する．プラスミノーゲン異常症，FXII欠損症およびTM異常症の血栓性素因は確立していない．

代謝異常として，高ホモシステイン血症は活性酸素による内皮傷害が動脈血栓を誘導する．メチレンテトラヒドロ葉酸還元酵素遺伝子多型(methylenetetrahydrofolate reductase C677T：MTHFR C677T)はホモシステイン血症をきたし，脳梗塞を起こす．新生児自己炎症性疾患にも血栓症が報告されている．小

表 血栓塞栓症の遺伝性素因*

凝固因子とその制御因子の異常
プロテインC欠損症，プロテインS欠損症，アンチトロンビン欠損症，FVLeiden(G1691A, Nara)，プロトロンビン変異(G20210A, Yukuhashi)，ADAMTS13異常症，FXII欠損症，フィブリノゲン異常症など
高FVIII血症，高FIX血症，高FXI血症，高TAFI血症，低TFPI血症
代謝疾患など
高ホモシステイン血症(cystathionineβ合成酵素欠乏症，MTHFR欠乏症，MTHFR C677Tなどの葉酸代謝異常)，Marfan症候群，Fabry病，IL-1受容体欠損症など

＊：全身性に血栓症を起こしやすい素因であり，脳血栓，冠動脈血栓など狭義(局所)の血栓症は含めない

TAFI：thrombin-activatable fibrinolysis inhibitor, TFPI：tissue factor pathway inhibitor

児の発作性夜間血色素尿症はきわめてまれで，血栓症も通常みられない．

▶疫学

全国調査から，20歳未満での遺伝性血栓症の発症は年間10例以上と推定される．その70％は3大因子欠損症(PC異常45％，PS異常15％，AT異常10％)で，残り30％がADAMTS13欠損症などである[3]．成人静脈血栓塞栓症の内訳(PS異常40％，PC異常20％，AT異常10％)とは異なり，3歳未満，特に新生児のPC異常が多い．思春期発症にPS異常とAT異常が多くなる．日本人における先天性PS欠損症の頻度は1.12％で，欧米0.03～0.13％より高く，PS-Tokushima多型などがあるためと考えられる．新生児から乳児早期にPC欠損症が多い理由は，明らかではない．先天性PC欠損症の発症は人口約10万人に1人とされるが，PC遺伝子ヘテロ変異を有する日本人は620人に1人と推定されている．ATヘテロ変異保有者の頻度は0.1～0.3％と3大因子欠損症のうち最も低い．

▶臨床徴候

小児の血栓症は新生児期に最も多く，次いで思春期である．新生児から乳児期に多い重症PC欠損症は，生後1か月以内に頭蓋内出血および梗塞か，電撃性紫斑病を発症する．その2/3は両者を合併し，頭蓋内病変が先行する[3]．胎児期の脳室拡大から先天性水頭症と診断される例，たまたま眼病変で見つかる例などがある．脳静脈洞血栓症，硝子体出血，腎不全もPC欠損症を疑う症候である．子宮内発育遅延はまれで，多くは正期産児である．思春期発症例はPS欠損症が多く，成人型の深部静脈血栓症や肺血栓塞栓症などの発症様式をとる．小児血栓症全体に占める3大遺伝性欠損症の割合は，5～10％と想定される．中心静脈カテーテルは新生児血栓症の重要な誘因であるが，抗凝固因子欠損症はまれである．小児悪性腫瘍治療中の血栓リスクに及ぼす遺伝的素因の寄与も低い[4]

▶診断・検査

止血機構の成熟にしたがい，PC，PS，AT濃度は年齢とともに上昇する．AT濃度は学童期まで，PCは成人までに上昇する．PSはC4BPと結合しないfree PSが活性を示す．新生児期はC4BP値が低くfree PS濃度が相対的に高い．三大抗凝固因子のうち最も成人域への到達が遅れるのがPCで，個人差も大きい．PC濃度にはASE(age-related stability element)や内皮PC受容体(endothelial protein C receptor：EPCR)多型が関与している．測定法(amidolytic法・clotting法など)，ビタミンK欠乏などの検討が必要である．3大因子を同時測定し，各因子活性の乖離から欠損症を推測する．成人ではPS/AT活性比が遺伝性PS欠損症の，小児ではPC/PS活性比が遺伝性PC欠損症のスクリーニングに用いられる．3か月未満ではPC活性による診断はむずかしい．

▶治療と予後

血栓症の治療は，抗凝固療法と血栓溶解療法からなる．初回の場合は，年齢に応じて再発予防を行う．抗凝固因子欠損症に対する濃厚血小板と新鮮凍結血漿による補充療法の必要性は，特に新生児・乳児で高い．生理的止血効果を期待するには凝固因子活性20～30％は必要である．国内ではAT製剤と活性化PC製剤が使用可能であるが，PSの濃縮因子製剤はない．

小児血栓症のうち，特発性の再発例および遺伝性栓友病患者にはワルファリンカリウム(ワーファリン®)の長期投与が必要となる．PC欠乏がある場合，奇異性血栓症に注意して少量から開始しプロトロンビン時間国際標準比(PT-INR)をモニターする．ワルファリンカリウム(ワーファリン®)は骨と成長に対する影響にも注意する．ワルファリン過剰のカウンターに用いられる乾燥人血液凝固第Ⅸ因子複合体(PPSB-HT®)に含まれるPC濃度はきわめて高いが，PC欠損症に対する保険適用はない．PC欠損症は肝移植が根治療法となるが，最近，国内で小児のドミノ移植が成功した[5]．

▶ピットフォール・対策

新生児から乳児期早期にはPC欠損症を，それ以降はPS欠損症とAT欠損症を念頭におき，活性値から推測して遺伝子診断を進める．ワーファリン®投与後はPCおよびPS活性値から診断できないので注意する．小児血栓症では補充療法を十分に行う．

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Case Report

Fetal hydrocephalus and neonatal stroke as the first presentation of protein C deficiency

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Abstract

Severe protein C-deficiency is a rare heritable thrombophilia of the newborn. Infants with biallelic *PROC* mutations present purpura fulminans and intracranial thromboembolism, while the prenatal onset of mutated heterozygotes remains unclear. We herewith present the first case of fetal ventriculomegaly and neonatal stroke associated with heterozygous *PROC* mutation. The infant was born to a healthy mother at 38 gestational weeks. The fetal growth had been normal, but the routine ultrasound screening had indicated mild hydrocephalus at 28 weeks of gestation. He developed convulsions two days after birth. Computed tomography of the brain revealed multiple hemorrhagic infarctions and ventriculomegaly. Dissociated levels of the plasma activity between protein C (21%) and protein S (42%) reached to determine the heterozygote of *PROC* c.574_576delAAG, a common thrombophilic predisposition in Asian ancestries. PC-mutant heterozygotes may have a limited high risk of cerebral thromboembolism during the perinatal course.

Keywords: Ventriculomegaly; Heritable thrombophilia; Perinatal stroke

1. Introduction

Hydrocephalus, or ventriculomegaly, occurs in association with anomaly, infection, tumor, and hemorrhagic complication. It begins at any age with an incidence of 0.48–0.81 per 1000 live births [1]. Fetal ventriculomegaly arises from loss of cerebral tissue, excessive production of cerebrospinal fluid (CSF) or obstruction to CSF pathways. Thromboembolic and hemorrhagic events result in hydrocephalus involving both cerebral atrophy and decreased CSF reabsorption.

Protein C (PC), protein S (PS) and antithrombin (AT) deficiencies are the most potent thrombophilias

Abbreviations: AT, antithrombin; CSF, cerebrospinal fluid; CT, computed tomography; MRI, magnetic resonance imaging; PC, protein C; PS, protein S

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beyond the ethnicity. Heterozygotes with the gene mutation of PC (*PROC*), PS (*PROS1*) or AT (*SERPINC1*) are at the established high risk of venous thromboembolism in adolescents and young adults [2]. Pediatric thrombosis occurs at the highest incidence in the newborns. The neonatal onset of the natural anticoagulant factor defects is exclusively PC-deficiency [3]. Purpura fulminans and intracranial thromboembolism are the first presentation of severe PC-deficiency. Congenital hydrocephalus was reported in patients with homozygous or compound heterozygous *PROC* mutations of parental origin [3,4]. However, there is no information about the prenatal onset of heterozygous carriers.

We report the first case of fetal hydrocephalus secondary to cerebral thromboembolism associated with a heterozygous *PROC* mutation registered in adult patients with thrombosis [5]. The perinatal course indicated a critical role of PC-deficient hypercoagulability.

2. Case report

A 2-day-old infant was transferred to our hospital because of seizures. He was born at 38 gestational weeks, weighing 2750 g, by vaginal delivery to a healthy mother. Fetal growth was normal. Ultrasound and magnetic resonance imagings (MRI) suggested slight enlargement of the left lateral ventricle with ischemic atrophy at 28 and 34 weeks of gestation, respectively. Family history was unremarkable. He had normal umbilical cord and placenta with full Apgar scores at birth.

On admission, the infant was intubated for generalized convulsions. He was afebrile showing no anomaly, anemia, skin eruption or abnormal jaundice. There was no bulging fontanelle. Electro-/echo-cardiographies revealed no cardiovascular diseases. Computed tomography (CT) scanning of the brain (Fig. 1a) showed obsolete hemorrhagic lesions in bilateral ventricles and subarachnoid cavities and low intensity area in the right frontal lobe. Peripheral blood counts showed leukocytes 17.2×10^9/L (reference range [rr]: $3.30–8.60 \times 10^9$), hemoglobin 16.7 g/dL (rr: 13.7–16.8) and a platelet count of 259×10^9/L (rr: $158–348 \times 10^9$). Coagulation studies revealed normal prothrombin time 16.2 s (rr: 10.0–13.5) and prolonged activated thromboplastin time 144.1 s (rr: 26.0–41.0) because of vitamin K deficient state. Fibrinogen concentration was 88 mg/dL (rr: 200–400) with increased levels of fibrinogen degradation products 8.4 μg/mL and D-dimer 4.7 μg/mL (rr: <1.0). AT activity (43%) was normal for age. Blood chemistry was unremarkable. C-reactive protein was 0.10 mg/dL (rr: <0.14). On day 1 after admission, he was extubated

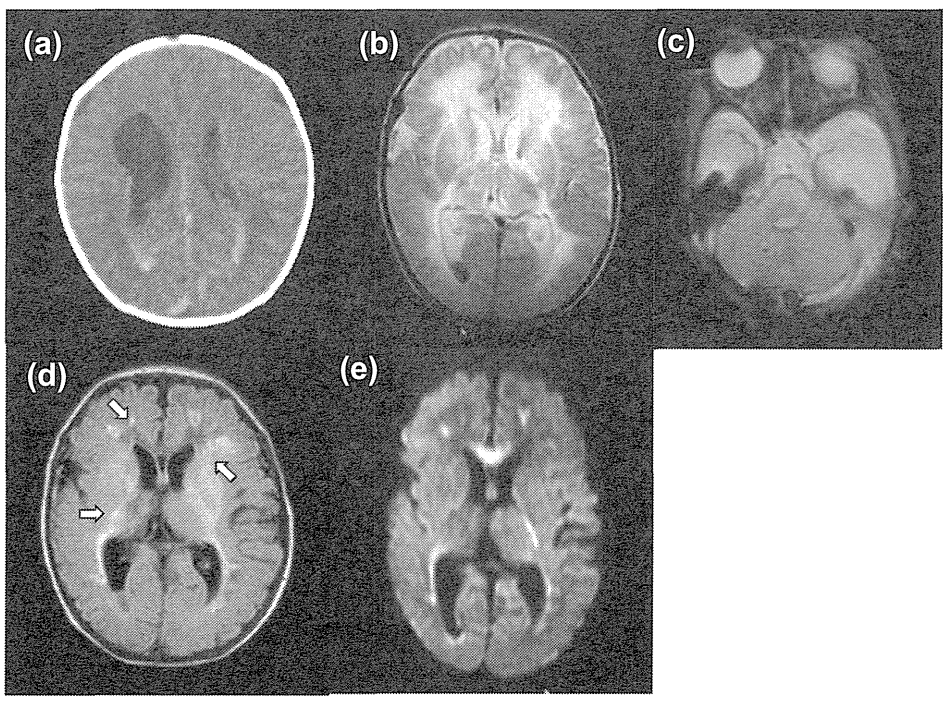

Fig. 1. Computed tomography (CT) of the brain on admission (a) indicates obsolete hemorrhagic lesions in bilateral ventricles and subarachnoid cavities and low intensity area in the right frontal lobe. Magnetic resonance imaging (MRI) of the brain on day 8 after birth (b–e) indicates multiple infarctions including obsolete (d, *arrows*) and acute lesions, along with bilateral ventriculomegaly. T2-weighted image (b) shows multiple low intensity areas in the left frontal lobe, the left frontal horn of lateral ventricle and bilateral cerebellar hemisphere. T2 star-weighted image (c) shows low intensity area in cerebellar hemisphere. Fluid attenuation inversion recovery (d) and diffusion weighted images (e) show multiple high intensity areas in different distribution.

Table 1
Reported patients of heritable protein C deficiency who first presented fetal ventriculomegaly.

Pt	Gestational weeks at the detection of enlarged ventricles	Protein C activity (%)	*PROC* mutation		Postnatal condition			Refs.
			Zygosity	Genotype	Disease	Specific treatment	outcome	
1	33	2	Homo. or comp. hetero.	n.d.	Hydrocephalus, ocular bleeding	Plasma	n.d.	[9]
2	34	2	Homo.	c.1042C>T, exon 9	Hydrocephalus, ocular bleeding	n.d.	n.d.	[10]
3	33	<10	Homo.	c.1235G>A, exon 9	hydrocephalus, purpura fulminans	PC-product, AT-product	Death	[3]
4	34	<10	Comp. hetero.	n.d.	Hydrocephalus, vitreous bleeding, purpura fulminans	PC-product, warfarin	PMR	[3]
5	28	21	Hetero. c.574_6delAAG, exon 7		Hydrocephalus, convulsion	None	PMR	Our case

AT-/PC-product: plasma-derived concentrates of antithrombin or activated protein C, n.d.: not described, PMR: psychomotor retardation. Clinical records of patients 3 and 4 were obtained from the information of meeting reports according to the reference.

after the control of seizures on phenobarbital therapy. MRI showed multiple low intensity areas in the frontal lobe, the left frontal horn of lateral ventricle, and bilateral cerebellar hemisphere in T2-weighted images (Fig. 1b) and T2 star-weighted images (Fig. 1c). High intensity lesions in the white matters suggested obsolete (Fig. 1d, *arrows*) and acute infarctions (Fig. 1e). No vasculopathy or extracranial thrombosis was found by MR angiography, lung scintigraphy, renography and funduscopic examinations.

Reassessed coagulation profiles 8 days after birth were as follows; plasma PC-activity 21%, PS-activity 42%, and AT-activity 83% along with factor VII activity 74%. These indicated non-severe isolated PC-deficiency. Genetic analysis was performed using peripheral blood-derived DNA after the informed consent was obtained. Direct sequencing of the coding and promoter regions of *PROC* determined a heterozygous mutation in exon 7 of *PROC* c.574_576delAAG, p.Lys193del in both the infant and his healthy father. The 3-base deletion raises the thromboembolic risk in Japanese [5] and Chinese populations [6]. The infant had no explainable causes of ventriculomegaly and infarction other than the deletion, although PC-activity of his father was 100% (one time study). He was discharged from the hospital 29 days after birth. Thereafter, plasma PC-activity subnormally increased to 56% until age 15 months. He had normal growth but slightly delayed development.

3. Discussion

Cerebral infarction and purpura fulminans are the hallmark of severe PC-deficiency (<0.01 IU/mL or <1% of plasma PC-activity). However, biallelic *PROC* mutations are found only in half of the neonatal cases [3]. This is the first report of fetal ventriculomegaly associated with heterozygous *PROC* mutation.

Perinatal hypercoagulability with PC-deficiency is a concern. Fetal hydrocephalus occurs in congenital anomaly, intracranial bleeding, infection or tumors. Yamazaki et al. [7] reported that 9 of 156 fetuses with hydrocephalus (5.8%) suffered from prior hemorrhages. Symptomatic stroke in full-term neonates occurs with an overall incidence of 1.35 per 100,000 live births. In 92 term newborns with ischemic strokes [8], 62 patients (68%) had at least one prothrombotic risk factor compared with 44 controls (24%), and PC-type I deficiency was found in 6 neonates. Table 1 shows the summary of heritable PC-deficiency associated with fetal ventriculomegaly [3,5,6]. Four reported patients were considered to carry the biallelic mutation. All showed bilateral ventriculomegaly at the late gestational stage. Fetal growth was arrested in patient 3. Regular monitoring might lead to the earliest detection in our patient. Intracranial hemorrhages and infarctions precede purpura fulminans in neonatal PC-deficiency [3]. Cerebral blood flow is the target of thrombosis during the dynamics of fetal to neonatal circulation. Absolutely low PC concentration causes hypercoagulable state, irrespective of the consumption or defective production. The plasma PC-activity of preterm infants is at the lowest levels throughout the life. The onset of PC-deficiency may thus converge to the period from the later gestation to the first several days of life.

The full-term infant presented multiple hemorrhagic infarctions after birth. Regular ultrasound screening showed ventriculomegaly at 28 weeks of gestation. Fetal MRI determined slight enlargement of the left lateral ventricle and low signal intensity in T2-weighted image. There was no evidence of cerebrovascular accidents related to vasculopathy, heart disease, metabolic disorders, tumors and infections. Imaging analyses of the fetus implied ischemic stroke (Otera et al, submitted). The PC-activity (21%) at the first evaluation could