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ORIGINAL ARTICLE *Clinical haemophilia*

A Phase II clinical trial of a mixture of plasma-derived factor VIIa and factor X (MC710) in haemophilia patients with inhibitors: haemostatic efficacy, safety and pharmacokinetics/pharmacodynamics

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Summary. MC710, a mixture of plasma-derived activated factor VII and factor X at a protein weight ratio of 1:10, is a novel bypassing agent for haemostasis in haemophilia patients with inhibitors. In a Phase II trial, we evaluated the haemostatic efficacy and safety of single doses of MC710, and investigated pharmacokinetic and pharmacodynamic parameters in nine joint bleeding episodes in six male haemophilia patients with inhibitors. This trial was a multi-centre, open-label, non-randomized study of two doses (60 and 120 μg kg^{-1} as FVIIa dose), allowing the re-administration of different MC710 dosages to the same subjects. Haemostatic efficacy was assessed by evaluating reduction in pain and swelling, as well as increase in range of motion in a bleeding joint. The results of the study showed that in nine bleeding episodes, seven treatments were rated as 'excellent' or 'effective' according to investigator's rating system of efficacy at 8 h after administration. No serious or severe adverse events were observed after administration; furthermore, measurement of several diagnostic markers revealed no signs or symptoms of disseminated intravascular coagulation (DIC). The haemostatic potential of MC710 was confirmed at doses of 60 and 120 μg kg^{-1} in this trial. MC710 is thus expected to be a safe and efficacious novel bypassing agent for controlling bleeding in haemophilia patients with inhibitors.

Keywords: bypassing agents, factor VIIa, factor X, haemophiliacs with inhibitors, haemostatic efficacy, joint bleeding

Introduction

To control bleeding in haemophilia patients with inhibitors, recombinant activated factor VII (rFVIIa; NovoSeven) and activated prothrombin complex concentrates (APCC; FEIBA) are commonly used as bypassing agents. However, a considerable number of patients experience treatment failure or insufficient

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efficacy. Because refractory bleeding cannot be controlled by monotherapy, several regimens of combination therapy using rFVIIa and APCC are currently in development [1–4]. On the other hand, rFVIIa analogues in which several amino acids are mutated have been developed to strengthen the haemostatic effect of rFVIIa [5,6], and an rFVIIa albumin fusion protein with a prolonged half-life is under development for prophylaxis [7].

MC710 is a new bypassing agent that is currently being developed by *KAKETSUKEN* (Kumamoto, Japan). It was designed as a lyophilized product prepared by mixing plasma-derived activated factor VII (FVIIa) and factor X (FX) at a protein weight ratio of 1:10 under acidic conditions to suppress the generation of activated FX (FXa). In the manufacturing of MC710, there are three viral elimination and inactivation processes (solvent/detergent treatment, virus removal membrane with 15-nm pore size, and dry-heat process at 65°C for 96 h) [8].

We have already reported the results of a Phase I trial wherein MC710 was intravenously administered at a single dose to 11 haemophilia patients with inhibitors in non-bleeding state and the dose dependency of pharmacokinetic (PK) and pharmacodynamic (PD) parameters from 20 to 120 μg kg^{-1} were confirmed [9]. The thrombin generation profiles of subjects' plasma triggered by tissue factor (TF)/phospholipids (PL) after administration at any dose of MC710 were different from those of both rFVIIa and APCC at clinical doses [9,10]. The thrombin generation potential of MC710 120 μg kg^{-1} has also been reported to be maintained at normal human plasma (NHP) levels for 24 h [10]. No serious or severe adverse events were observed after administration of MC710 in the Phase I trial, and incidences of adverse events were not dependent on MC710 dose. Furthermore, no signs or symptoms of disseminated intravascular coagulation (DIC) were observed, and MC710 was tolerated up to 120 μg kg^{-1} [9].

In this Phase II trial, we evaluated the haemostatic efficacy and safety of MC710 in the treatment of joint bleeding, and investigated PK and PD parameters during the bleeding state on administration of two doses (60 and 120 μg kg^{-1}) in haemophilia patients with inhibitors.

Materials and methods

Subjects and trial design

This trial was a multi-centre, open-label, non-randomized study of male Japanese haemophilia patients with inhibitors. The institutional review board of each participating institute approved this trial and the informed consent procedures. All subjects provided written informed consent. The key inclusion and exclusion criteria for the subjects are shown in Table 1. Bleeding severity was categorized in three grades, 'severe', 'moderate' and 'mild', judged by the investigator's observation. In this study, 'severe' bleeding was excluded for MC710 administrations due to safety concerns. MC710 was intravenously administered at a single dose to the patients within 3.5 h after the onset of joint bleeding (shoulder, elbow, knee or ankle) to determine haemostatic efficacy, safety and PK and PD parameters in bleeding state. MC710 was investigated at two doses: 60 μg kg^{-1}, which was predicted to have a stronger haemostatic potential than the clinically available bypassing agent, NovoSeven at 120 μg kg^{-1} [9]; and 120 μg kg^{-1}, which was the maximum tolerable dose identified in the Phase I trial [9]. The minimum sample size for each MC710 dosage was set at $n = 4$, which is the minimum number required for statistical evaluation. Blood samples were taken prior to MC710 treatment, at 10 min, and 2, 8 and 24 h after administration. MC710 120 μg kg^{-1} was given after completing more than four treatments with 60 μg kg^{-1}, allowing administrations of both doses in the same subjects. After completing dosing steps at 60 and 120 μg kg^{-1}, a safety and efficacy committee (SEC) which was organized by the coordinating investigators of the trial groups, was convened to evaluate safety and efficacy for each dose or to decide if a dose-up or dose-down was necessary.

Table 1. Key inclusion and exclusion criteria.

(i) Key Inclusion Criteria
patients: male congenital haemophilia A or B patients with inhibitors
age: ≥16 years and ≤65 years
inhibitor titre: ≥0.5 BU mL^{-1}
(ii) Key Exclusion Criteria
Patients with the following symptoms
• hypercoagulability
• history of DIC
• development of AIDS
• hypersensitivity to NovoSeven, FEIBA, and other plasma products
• decompensated liver cirrhosis
• heart failure, angina or pathologic arrhythmia such as atrial fibrillation
• anaemia (<9.5 g dL^{-1} of haemoglobin)

Haemostatic efficacy

Haemostatic efficacy was determined for each treatment by investigator's evaluation, using changes in visual analogue scale (VAS) for pain reduction, girth of the knee joint (only if applicable) for decrease in swelling and range of motion (ROM) of the bleeding joint for improvement of joint mobility. VAS was indicated by the subjects using a 10 cm line in which pains were ranged from 'no pain' (0 cm) to 'the worst pain experienced in the past' (10 cm), prior to, and at 1, 2, 4, 8 and 24 h after administration of MC710. Girth of joint (only applicable for a bleeding knee

joint) and ROM were measured with a tape measure and goniometer, respectively, prior to, and at 4, 8 and 24 h after administration of MC710.

Using these data, haemostatic efficacy for the treatment of each bleeding episode was assessed at 4 and 8 h after administration of MC710 according to a modified version of an investigator's rating system of efficacy described in the previous report [11].

1. Excellent – abrupt pain relief and unequivocal improvement in signs (swelling and/or mobility) of joint bleeding.
2. Effective –
   i. Abrupt pain relief, and no changes in signs of joint bleeding,
   ii. Definite pain relief and ≥slight improvement in signs of joint bleeding
   iii. Slight pain relief, and improvement in signs of joint bleeding.
3. Partially effective – slight pain relief and slight improvement in signs of joint bleeding.
4. Ineffective – no improvement or worsening of symptoms.The primary endpoint for this trial was the haemostatic efficacy at 8 h after administration of MC710.

Safety assessment

For the assessment of safety, subjective symptoms and objective findings were observed, including recording of vital signs and laboratory findings before and after MC710 administration. For the evaluation of DIC, platelet counts, fibrinogen and D-dimer were measured prior to, and at 10 min, and 2, 8 and 24 h after administration of MC710. Further, thrombin–antithrombin complex (TAT) and prothrombin fragment F_{1+2} (F1+2) were measured on the same time course to evaluate amount of generated thrombin in subject plasma. The observation period for adverse events was 1 week after the administration. Twelve weeks after the administration of MC710, virologic and serologic tests were conducted to detect the production of new viral antigens (hepatitis B surface) or antibodies against viruses (hepatitis B surface, hepatitis B core and hepatitis C virus), and anti-FVII, anti-FX or anti-mouse IgG antibodies.

PK/PD assessments

PK parameters of the active ingredients of MC710 were determined by measuring FVII clotting activity (FVII:C), FX clotting activity (FX:C), FVII antigen (FVII:Ag) and FX antigen (FX:Ag) using the same methods described previously [9], and data were analyzed using a non-compartmental model with WinNonlin® (ver 5.1; Pharsight, Mountain View, CA, USA) software using baseline adjusted values (differences from values before administration). Recovery was calculated as the percentage of the each active ingredient concentration in the plasma at 10 min after administration of MC710. In the PD parameter analysis, APTT and PT were measured, and APTT clot waveform analysis (CWA) and thrombin generation test (TGT) were performed using previously described methods [10].

Statistical analysis

The differences in MC710 dose on PK and PD parameters were analyzed using a mixed-effects model using subject as a random effect and dose (including observation time in the PD parameter analysis) as a fixed effect. For these statistical analyses, SAS Release 9.1 (SAS Institute Inc., Cary, NC, USA) was used. All reported *P* values are two-tailed and not adjusted for multiple testing. *P* values less than 0.05 were considered statistically significant.

Results

Subjects and administration

In this trial, 19 subjects [nine haemophilia A (HA) patients with inhibitors and 10 haemophilia B (HB) patients with inhibitors] were enrolled. MC710 was given to six subjects including 3 HA and 3 HB patients with inhibitors (Table 2). The other 13 subjects were not given the drug due to lack of bleeding or >3.5 h from the onset of bleeding. MC710 was given at 60 μg kg^{-1} to five subjects (Nos.1, 2, 3, 5 and 6). The SEC determined that the dose should be raised to 120 μg kg^{-1} after considering the efficacy and safety data obtained with the 60 μg kg^{-1} treatment. MC710 was then given at 120 μg kg^{-1} to four subjects (Nos.1, 4, 5 and 6). In nine bleeding episodes,

Table 2. Subject characteristics

Subject No.	Haemophilia type	Age (years)	Body weight (kg)	Inhibitor titer (BU mL^{-1})		MC710 dosing (μg kg^{-1})
				FVIII inhibitor	FIX inhibitor	
1	HA	39	58.0	8.0	–	60, 120
2	HB	16	61.0	–	40.6	60
3	HA	24	83.6	60.5	–	60
4	HA	43	50.5	44.2	–	120
5	HB	20	80.7	–	2.6	60, 120
6	HB	19	48.0	–	3.2	60, 120

three of six subjects received both the 60 and 120 μg kg^{-1} doses (Table 2).

Haemostatic Efficacy

The bleeding joints that were assessed during the treatment with MC710 included knee (n = 3) and ankle (n = 2) for 60 μg kg^{-1}, and knee (n = 2), elbow, and ankle for 120 μg kg^{-1} (Table 3). Bleeding severity was 'moderate' for the patient with a bleeding elbow that received 120 μg kg^{-1} and all other bleedings were 'mild'. Seven of nine episodes were evaluated as 'excellent' or 'effective', and two treatments with 120 μg kg^{-1} were determined 'partially effective'. The rate of VAS change in four (Nos.2, 3, 5 and 6) of five subjects administered 60 μg kg^{-1} decreased to a range of 0–59% of preadministration levels at 8 h, and that in subject No.5 returned to the preadministration level at 24 h (Fig. 1a). All VAS measured in subject No.1 were 0 cm, as the subject did not complain of pain in the bleeding joint (data not plotted in Fig. 1a). At 60 μg kg^{-1}, the girth of the knee joints in three subjects (Nos.1, 2 and 5) at 8 h decreased 0.70 ± 0.44 cm compared to preadministration, and the ROM of the knee joints in three subjects (Nos.1, 2 and 5) at 8 h increased 11.7° ± 5.8° compared to preadministration and that of the ankle joint in subject No.3 at 8 h did not change, but that in subject No.6 increased 30° compared to preadministration. The rate of VAS change in three (Nos.4, 5 and 6) of four subjects administered 120 μg kg^{-1} decreased to a range of 6–71% of preadministration levels at 8 h. All VAS measured in subject No.1 were 0 cm for the same reason described for 60 μg kg^{-1} (data not plotted in Fig. 1b). At 120 μg kg^{-1}, the girth of the knee joints in two subjects (Nos.1 and 6) at 8 h decreased 1.0 and 0.3 cm compared to preadministration, respectively, and the ROM of the knee joints in two subjects (Nos.1 and 6) and that of the elbow joint in subject No.4 at 8 h did not change compared to preadministration and that of the ankle joint in subject No.5 at 8 h increased 20° compared to preadministration.

Safety

Two mild adverse reactions, fever and increased blood pressure, were observed in the same subject (No.3) 2–8 h after administration of MC710 at 60 μg kg^{-1}, however, these adverse reactions spontaneously disappeared within 24 h after administration without treatment. On the other hand, no adverse reactions were observed after administration of MC710 at 120 μg kg^{-1}. No serious or severe adverse events were observed within 1 week after administration of MC710, and no subject discontinued the trial due to

Table 3. Administered dose, bleeding conditions, and efficacy.

	60 μg kg^{-1} (n = 5)				120 μg kg^{-1} (n = 4)			
Subj. No.	Bleeding joint	Severity	Time from bleeding to dosing (h)	Haemostatic efficacy	Bleeding joint	Severity	Time from bleeding to dosing (h)	Haemostatic efficacy
1	left knee	mild	3.12	effective	left knee	mild	2.50	excellent
2	left knee	mild	1.95	excellent	–	–	–	–
3	right ankle	mild	2.50	effective	–	–	–	–
4	–	–	–	–	right elbow	moderate	2.20	partially effective
5	left knee	mild	3.40	effective	left ankle	mild	1.58	effective
6	right ankle	mild	2.45	effective	left knee	mild	2.90	partially effective

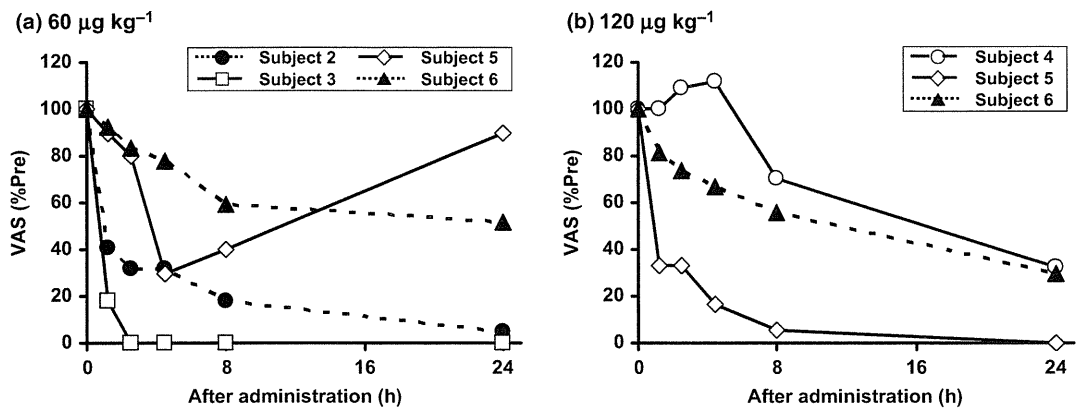

Fig. 1. Changes in visual analogue scale (VAS). VAS were measured at 1, 2, 4, 8, and 24 h after administration of MC710. Time-dependent changes after administration of 60 μg kg^{-1} (a) or 120 μg kg^{-1} (b) for each subject, No.2 (●), 3 (□), 4 (○), 5 (◇), 6 (▲) are shown as percentage of post- to pre-administration.

an adverse event. No clinical symptoms or changes in laboratory tests (platelet count, fibrinogen or D-dimer) indicating a hypercoagulable state, such as DIC, were observed after administration of MC710 (data not shown). In addition, the results of virologic and serologic tests confirmed that no subject developed a new viral antigen or produced a new antibody after administration of MC710.

Pharmacokinetics (FVII:C, FVII:Ag, FX:C, and FX:Ag)

Plasma FVII:C, FVII:Ag, FX:C and FX:Ag levels rapidly increased after administration of either MC710 60 or 120 μg kg^{-1}. FVII:C and FVII:Ag levels returned to preadministration values at 24 h (Figs. 2a and b) and increased levels of FX:C and FX:Ag persisted in the blood until 24 h at both doses of MC710 (Figs. 2c and d). In the PK analysis, area under the curve (AUC) and maximum plasma concentration (C_{max}) of FVII:C, FVII:Ag, FX:C and FX:Ag at MC710 120 μg kg^{-1} were 1.5 to 2.5 times of those at 60 μg kg^{-1} ($P < 0.05$). Other parameters; half-life ($t_{1/2}$), mean residence time (MRT), clearance (CL), volume of distribution at steady state (Vd_{ss}) and recovery at MC710 120 μg kg^{-1} were almost similar to those at 60 μg kg^{-1} (Table 4).

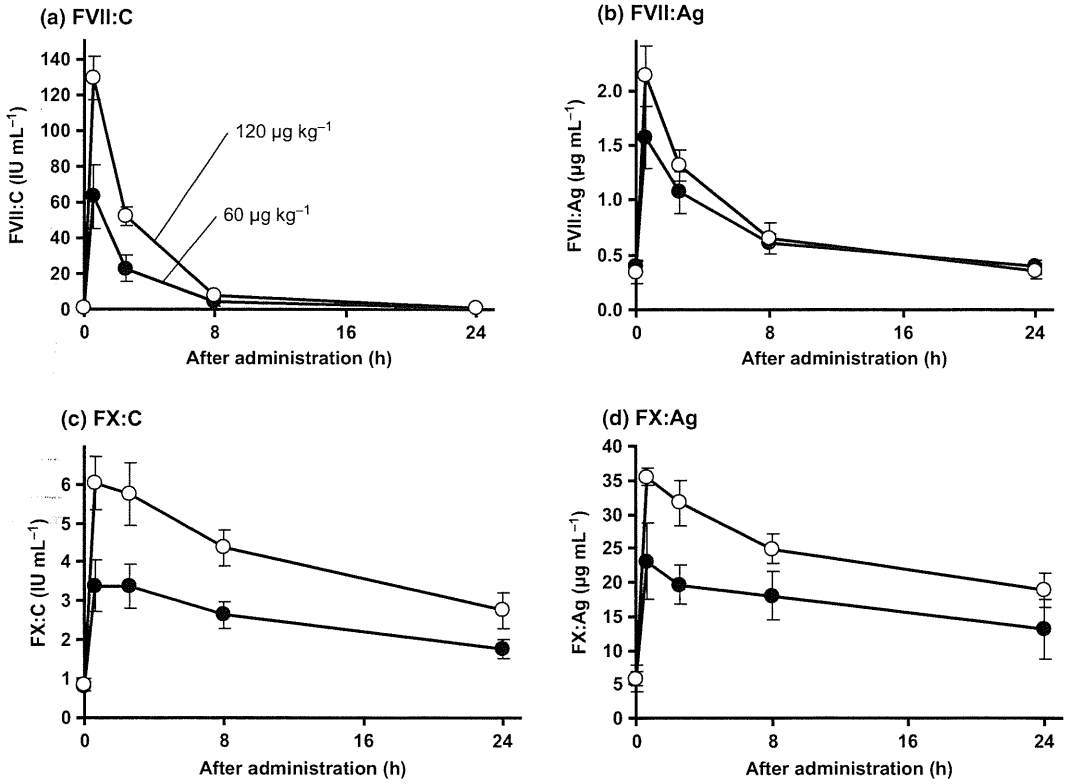

Fig. 2. Pharmacokinetics of MC710 active ingredients. Time-dependent changes in the pharmacokinetics of FVII clotting activity (FVII:C) (a), FVII antigen (FVII:Ag) (b), FX clotting activity (FX:C) (c), and FX antigen (FX:Ag) (d) are shown. The mark represents the mean ± SD. MC710 doses are denoted with the following symbols; 60 μg kg^{-1} (n = 5, •), 120 μg kg^{-1} (n = 4, ○).

Table 4. PK parameters of MC710 active ingredients.

MC710 active ingredients	MC710 dose	n	$AUC_{0\rightarrow24}$ (IU h mL^{-1}) (μg h mL^{-1})	C_{max} (IU mL^{-1}) (μg mL^{-1})	$t_{1/2}$ (h)	MRT (h)	CL (mL h^{-1} kg^{-1})	Vd_{ss} (mL kg^{-1})	Recovery (%)
FVII:C	60 μg kg^{-1}	5	157.8 ± 63.7	61.7 ± 17.7	1.8 ± 0.5	1.7 ± 0.5	20.8 ± 7.5	37.0 ± 9.3	97.2 ± 25.5
	120 μg kg^{-1}	4	394.4 ± 57.1	128.1 ± 12.0	2.6 ± 0.1	2.3 ± 0.1	15.4 ± 2.1	36.3 ± 4.5	99.2 ± 4.3
FVII:Ag	60 μg kg^{-1}	5	4.6 ± 2.6	1.2 ± 0.3	3.1 ± 1.9	2.5 ± 1.7	13.3 ± 5.1	44.5 ± 11.4	92.9 ± 20.5
	120 μg kg^{-1}	4	8.2 ± 0.7	1.8 ± 0.2	3.6 ± 1.0	3.5 ± 1.0	13.8 ± 0.8	59.2 ± 10.8	69.7 ± 4.6
FX:C	60 μg kg^{-1}	5	38.9 ± 7.9	2.6 ± 0.6	15.8 ± 2.5	8.6 ± 0.5	1.7 ± 0.5	36.1 ± 7.4	127.4 ± 30.8
	120 μg kg^{-1}	4	75.2 ± 9.1	5.2 ± 0.7	16.0 ± 1.4	8.7 ± 0.2	1.7 ± 0.3	36.6 ± 4.2	125.4 ± 11.2
FX:Ag	60 μg kg^{-1}	5	255.6 ± 72.5	17.3 ± 5.1	26.5 ± 17.2	9.2 ± 1.1	1.4 ± 0.8	38.5 ± 6.3	137.3 ± 43.4
	120 μg kg^{-1}	4	427.6 ± 53.6	29.7 ± 1.3	22.5 ± 3.3	9.4 ± 0.4	1.5 ± 0.4	46.0 ± 6.0	114.8 ± 1.9

$AUC_{0\rightarrow24}$ and C_{max} were denoted in two mode; 'IU h mL^{-1}' and 'IU mL^{-1}' for procoagulant activity and 'μg h mL^{-1}' and 'μg mL^{-1}' for antigen level, respectively. Data are shown as the mean ±SD.

Pharmacodynamics (APTT, PT, CWA and TGT)

APTT, prolonged for 120 s or more before administration, was improved and become close to the normal range for healthy individuals after administration of MC710. The APTT improving effect at 120 μg kg^{-1} was superior to that at 60 μg kg^{-1} ($P < 0.05$) (Fig. 3a). PT reached approximately 6 s (the determination limit) after administration of either dose and remained at this level for up to 2 h. PT reduction persisted until 8 h without returning to the preadministration level after treatment with either dose (Fig. 3b). CWA profiles at both doses changed drastically after administration (Fig. 4a i) and the transmittance changes at 120 μg kg^{-1} were closer to

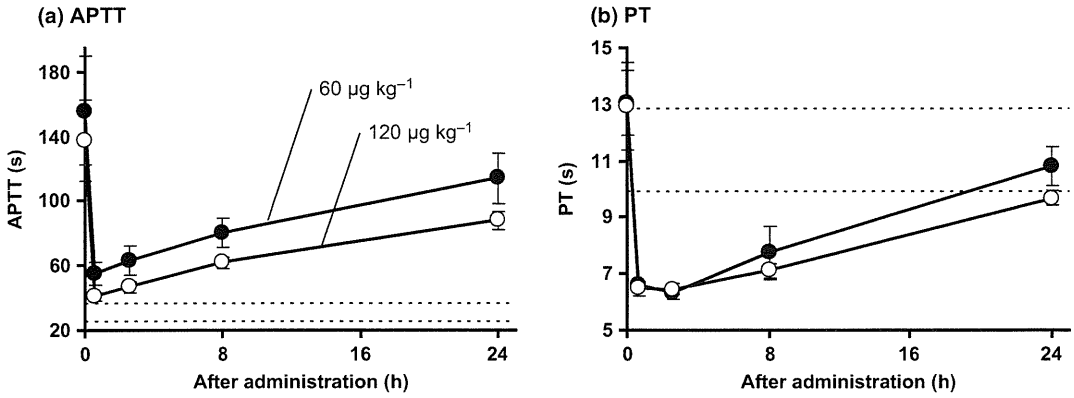

Fig. 3. Changes in APTT and PT. Time-dependent changes in APTT (a) and PT (b) are shown. The normal ranges for healthy individuals (---) for APTT were defined as 36.0 (upper limit) and 25.0 (lower limit) s and for PT as 12.8 (upper limit) and 9.9 (lower limit) s. The mark represents the mean ± SD. MC710 doses are denoted with the following symbols; 60 μg kg^{-1} (n = 5, •), 120 μg kg^{-1} (n = 4, ○).

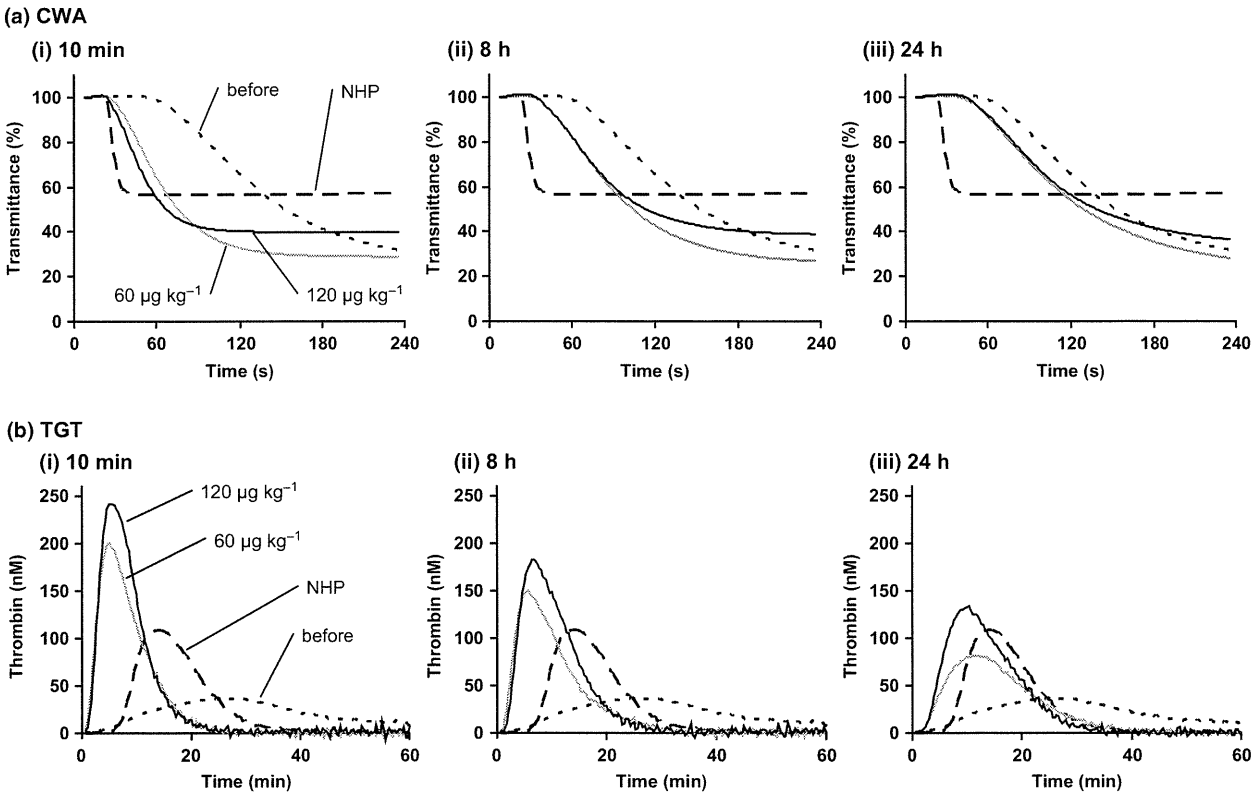

Fig. 4. Changes in clot waveform analysis (CWA) and thrombin generation test (TGT). Time-dependent changes in CWA profiles (a) and TGT profiles (b) are shown. The assay was conducted using the plasma collected before and after administration of MC710; (i) 10 min, (ii) 8 h, and (iii) 24 h for 60 μg kg^{-1} and 120 μg kg^{-1}. In each panel at the indicated time course, CWA and TGT average profiles before administration (n = 9, dotted line ----), 60 μg kg^{-1} (n = 5, gray-solid line ·····), 120 μg kg^{-1} (n = 4, black-solid line —), and normal human plasma (NHP) (n = 18, broken line — —) are shown.

normal plasma than at 60 μg kg^{-1}. Changes in CWA profiles were observed even at 24 h after administration (Fig. 4a iii). TGT at both doses at 8 h after administration indicated a shorter 'lag time' and 'time-to-peak' and a larger peak thrombin amount than in normal plasma (Fig. 4b ii). The thrombin generation potential was maintained at the level of normal plasma for 24 h after treatment with either dose (Fig. 4b iii).

Discussion

The haemostatic effects of MC710 were evaluated using an investigator's rating system of efficacy regarding changes in pain (VAS), swelling (girth of the knee joint, only if applicable) and mobility (ROM) of a bleeding joint after administration of MC710. At 8 h after administration of MC710, seven of nine treatments were considered 'excellent' or 'effective', and two of nine treatments (120 μg kg^{-1}) were 'partially effective' (Table 3). However, SEC concluded that the two episodes determined 'partially effective' were haemostatically effective considering the following findings at 24 h after the treatment: (i) VAS was reduced from 3.4 to 1.1 cm and ROM was increased from 25° to 60° (+35°) in subject No.4, and (ii) VAS was reduced from 2.7 to 0.8 cm, swelling did not worsen, and heat on the bleeding joint disappeared in subject No.6.

Regarding pharmacokinetics, as shown in Table 4, the mean recoveries of FVII:C and FVII:Ag at 60 and 120 μg kg^{-1} ranged from 69.7% to 99.2%, and those of FX:C and FX:Ag at both doses were ≥114.8%, indicating values which exceeded our predictions based on the data obtained in subjects that were not bleeding [9]. Recoveries of FX:C and FX:Ag from subject No.5 at 60 μg kg^{-1} were 168.7% and 212.6%, and others ranged from 93.7% to 146.9% and 110.5% to 136.1% respectively. These higher recoveries of FX might be due to the small sample size, or to biased individual assay results.

We evaluated APTT, CWA, PT and TGT as PD parameters. The results of APTT and CWA measurements showed that just after the administration of MC710 at 60 and 120 μg kg^{-1}, values in both indicators were close to NHP, and that this improvement persisted for 24 h (Fig. 3a and Figs. 4a i–iii). PT was observed to be shortened for 24 h (Fig. 3b), and TGT showed that the thrombin generation potential just after the administration at both doses was greater than NHP (Fig. 4b i), and that this effect was maintained from 8 to 24 h after administration (Fig. 4b ii, iii). TAT increased from 2.73 ± 0.59 ng mL^{-1} to 18.20 ± 12.84 ng mL^{-1} and F1+2 increased from 101.0 ± 28.8 pmol L^{-1} to 484.5 ± 212.4 pmol L^{-1} at 2 h after administration of MC710 at 120 μg kg^{-1}. This thrombin generation potential of MC710 might estimate that there are some risks of hypercoagulability through the extrinsic pathway, however; any signs of DIC were not observed in the subjects of this trial. According to the changes in PD parameters, TAT and F1+2, it might be suggested that MC710 has a strong- and long-acting effect on thrombin generation potential with no hypercoagulability up to 24 h after administration in the presence or absence of TF at the bleeding site.

MC710 was designed as an agent to allow co-administration of FVIIa and FX. Administration of MC710 120 μg kg^{-1} increased the plasma FVIIa concentration to 129 IU mL^{-1} (FVII:C) and 2.1 μg mL^{-1} (FVII:Ag) (Figs. 2a and b), and FX concentration to 6.0 IU mL^{-1} (FX:C) and 35 μg mL^{-1} (FX:Ag) (Figs. 2c and d) respectively. It has been suggested that the surface of activated platelets might be the site where FVIIa activates FX in the haemostasis of bleeding of haemophilia patients by rFVIIa therapy [12–14]. Enzymologic analysis has revealed that the *Km* for FVIIa-catalyzed FX activation (0.16 to 0.25 μM) is higher than the FX concentration in plasma (approximately 8 μg mL^{-1}, 0.14 μM) in the presence or absence of TF, therefore, increased FX concentration in plasma would generate more FXa by FVIIa more quickly [15,16]. Furthermore, the long half-life of FX might contribute to its prolonged haemostatic potential in plasma.

In this trial, the haemostatic efficacy of MC710 was determined 'excellent' or 'effective' in seven of nine joint bleeding episodes at 8 h after single dosing 60 or 120 μg kg^{-1}, and both doses was judged by SEC to be effective for controlling bleeding in haemophilia patients with inhibitors. Furthermore, no serious or severe adverse events, or signs or symptoms of DIC were observed in both doses. Therefore, we judged that it was appropriate to set up a clinical dose of MC710 within the range of 60 to 120 μg kg^{-1}.

MC710 is anticipated to be a safe and novel bypassing agent for controlling bleeding in haemophilia patients with inhibitors. An expanded study (Phase III trial) in haemophilia patients with inhibitors is planned to investigate the efficacy and safety of repeated administration of MC710 in a dose range of 60–120 μg kg^{-1}.

Acknowledgements

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Author contribution

Akira Shirahata developed the clinical trial protocol, evaluated efficacy and safety data, and discussed clinical events in the role of medical expert. Hidehiko Saito, Katsuyuki Fukutake, Junki Takamatsu and Midori Shima acted as the coordinating investigators, ensuring that investigators at the different institutions had a common understanding of the protocol and the implementation of the trial, as well as giving general advice on the conduct of the trial. The investigators had responsibility for all medical judgments associated with this trial in their institution and conducted the trial in accordance with the protocol including the selection of subjects, obtaining

of informed consent, provision of data and information, reporting of adverse events, documentation of case reports and storage of essential documents. Yasuo Ohashi, acting as the statistical advisor, gave advice and instruction on statistical analysis methods for the trial. *KAKETSUKEN* (The Chemo-Sero-Therapeutic Research Institute) managed the trial overall, including development and amendment of the protocol, data management, statistical analysis, quality control and assurance and data preservation. Mitsubishi Chemical Medience Corporation collected the samples taken at the trial sites, conducted the tests, maintained measurement results and guaranteed the reliability of the results of the analyses. Shin Nippon Biomedical Laboratories, Ltd. (Kagoshima, Japan) was responsible for the estimation of PK parameters and conducted the statistical analysis.

Disclosures

Akira Shirahata and Yasuo Ohashi received a fee from *KAKETSUKEN* for the implementation of the trial. The other authors have no conflict of interest to declare.

MC710 Phase II Trial group

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References

1 Schneiderman J, Rubin E, Nugent DJ, Young G. Sequential therapy with activated prothrombin complex concentrates and recombinant FVIIa in patients with severe haemophilia and inhibitors: update of our previous experience. *Haemophilia* 2007; **13**: 244–8.

2 Economou M, Teli A, Tzantzaroudi A, Tsatra I, Zavitsanakis A, Athanassiou-Metaxa M. Sequential therapy with activated prothrombin complex concentrate (FEIBA) and recombinant factor VIIa in a patient with severe haemophilia A, inhibitor presence and refractory bleeding. *Haemophilia* 2008; **14**: 390–1.

3 Livnat T, Martinowitz U, Zivelin A, Seligsohn U. Effects of factor VIII inhibitor bypassing activity (FEIBA), recombinant factor VIIa or both on thrombin generation in normal and haemophilia A plasma. *Haemophilia* 2008; **14**: 782–6.

4 Gringeri A, Fischer K, Karafoulidou A, Klamroth R, López-Fernández MF, Mancuso E; European Haemophilia Treatment Standardisation Board (EHTSB). Sequential combined bypassing therapy is safe and effective in the treatment of unresponsive bleeding in adults and children with hemophilia and inhibitors. *Haemophilia* 2011; **17**: 630–5.

5 Møss J, Scharling B, Ezban M, Møller Sørensen T. Evaluation of the safety and pharmacokinetics of a fast-acting recombinant FVIIa analogue, NN1731, in healthy male subjects. *J Thromb Haemost* 2009; **7**: 299–305.

6 Mahlangu JN, Coetzee MJ, Laffan M *et al.* Phase I, randomized, double-blind, placebo-controlled, single-dose escalation study of the recombinant factor VIIa variant BAY 86-6150 in hemophilia. *J Thromb Haemost* 2012; **10**: 773–80.

7 Weimer T, Wormsbächer W, Kronthaler U, Lang W, Liebing U, Schulte S. Prolonged *in-vivo* half-life of factor VIIa by fusion to albumin. *Thromb Haemost* 2008; **99**: 659–67.

8 Nakatomi Y, Nakashima T, Gokudan S *et al.* Combining FVIIa and FX into a mixture which imparts a unique thrombin generation potential to hemophilic plasma: an in vitro assessment of FVIIa/FX mixture as an alternative bypassing agent. *Thromb Res* 2010; **125**: 457–63.

9 Shirahata A, Fukutake K, Mimaya J *et al.* Clinical pharmacological study of a plasma-derived factor VIIa and factor X mixture (MC710) in haemophilia patients with inhibitors—Phase I trial. *Haemophilia* 2012; **18**: 94–101.

10 Shirahata A, Fukutake K, Takamatsu J *et al.* Results of clot waveform analysis and thrombin generation test for a plasma-derived factor VIIa and X mixture (MC710) in haemophilia patients with Inhibitors—Phase I trial: 2nd report. *Haemophilia* 2013; **19**: 330–7.

11 Tarantino MD, Collins PW, Hay CR *et al.* Clinical evaluation of an advanced category antihaemophilic factor prepared using a plasma/albumin-free method: pharmacokinetics, efficacy, and safety in previously treated patients with haemophilia A. *Haemophilia* 2004; **10**: 428–37.

12 Monroe DM, Hoffman M, Oliver JA, Roberts HR. Platelet activity of high-dose factor VIIa is independent of tissue factor. *Br J Haematol* 1997; **99**: 542–7.

13 Monroe DM, Hoffman M, Oliver JA, Roberts HR. A possible mechanism of action of activated factor VII independent of tissue factor. *Blood Coagul Fibrinolysis* 1998; **9** (Suppl 1): S15–20.

14 Hoffman M, Monroe DM. The action of high-dose factor VIIa (FVIIa) in a cell-based model of hemostasis. *Semin Hematol* 2001; **38**(Suppl 12): 6–9.

15 Tomokiyo K, Nakatomi Y, Araki T *et al.* A novel therapeutic approach combining human plasma-derived factors VIIa and X for haemophilia patients with inhibitors: evidence of a higher thrombin generation rate in vitro and more sustained haemostatic activity in vivo than obtained with factor VIIa alone. *Vox Sang* 2003; **85**: 290–9.

16 Tomokiyo K, Nakatomi Y, Hamamoto T, Nakagaki T. Prospective efficacy and safety of a novel bypassing agent, FVIIa/FX mixture (MC710) for hemophilia patients with inhibitors. In: Batorova A eds. Hemophilia. Chapter 6: Rijeka, Croatia: InTech, 2012: 79–96.

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—【総　　説】— Review —

凝固障害に対する補充療法—標準的治療法の整理

松下　　正

キーワード：新鮮凍結血漿，フィブリノゲン，希釈性凝固障害，第 VII 因子，アンチトロンビン

凝固因子補充療法の基本戦略

生理的条件下のヒトの凝固システムではクロット(凝血塊）形成を促進する方向と抑制する方向（抗凝固システム）が絶妙なバランスで成り立っている（図 1）．先天性凝固障害においてはあくまでも生理的条件下での単一の凝固因子欠乏症であるため，先天性障害に対する凝固因子補充療法では，欠乏している凝固因子の濃度を十分上昇させることが必要である．表 1 に出血傾向を来たし，補充療法の必要な先天性凝固障害と対応する濃縮因子製剤について掲げた．

「血液製剤の使用指針」(厚生労働省医薬食品局血液対策課，平成 17 年 9 月(平成 24 年 3 月一部改正)(以下，指針))は「生理的な止血効果を期待するための凝固因子の最少の血中活性値は，正常値の 20～30% 程度である」としているが，この考え方は教科書の一章[1]をオリジナルとするものであり，もともとは(先天性選択的)凝固因子欠乏症の最低限止血レベルについて述べたものである．すなわちここでいう最小の活性値とは生理的環境（図 1A）における出血の予防に最低限必要な活性値のことであり，出血の重症度によっては濃縮凝固因子製剤により 20～30% を上回る凝固因子活性を得る必要がある（表 2，次章参照）．

一方，後天的な希釈性凝固障害は複合型凝固因子欠乏症であり，血漿中凝固因子は全体的に最低必要量近くまで低下しているが，線溶因子・抗凝固因子も同様に低下している．しかしこのバランスは所詮不安定であり容易に崩れやすい（図 1B）．

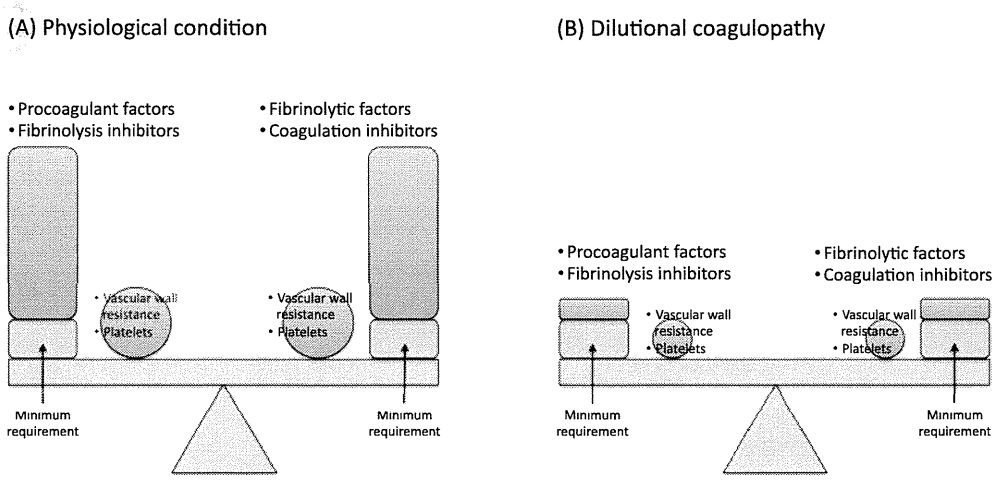

図 1　止血の均衡に関与する因子

(A）生理的環境，(B）希釈性凝固障害における止血の均衡性．血漿中凝固・線溶因子（Procoagulant factor，Fibrinolysis inhibitor）は最低必要量（Minimum requirement）近くまで低下しているが，線溶因子（Fibrinolytic factors)・凝固因子インヒビター（Coagulation inhibitors）も同様に低下している場合はバランスが取れ症状も発現は少ない．ただし，双方とも，さらには血管抵抗性（Vascular wall resistance）や血小板（platelets）数などその他の因子も減少しているため容易にバランスが崩れやすい．

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表1 出血傾向を来す先天性凝固障害（インヒビターのないもの）と対応濃縮凝固因子製剤

疾患	使用製剤	我が国における発売品®
無フィブリノゲン症	フィブリノゲン濃縮製剤	フィブリノゲン HT
プロトロンビン欠乏症	プロトロンビン複合体（PCC）	PPSB*
第 V 因子欠乏症	新鮮凍結血漿（FFP）	
第 X 因子欠乏症	プロトロンビン複合体（PCC）	PPSB*
第 VII 因子欠乏症	活性化第 VII 因子（rFVIIa）， プロトロンビン複合体（PPSB）	ノボセブン HI，PPSB*
血友病 A	第 VIII 因子濃縮製剤 （血漿由来または遺伝子組み換え）	文献[2] 参照
血友病 B	第 IX 因子濃縮製剤 （血漿由来または遺伝子組み換え）	
第 XI 因子欠乏症	新鮮凍結血漿（FFP）	
第 XIII 因子欠乏症	第 XIII 因子濃縮製剤	フィブロガミン
フォン・ヴィレブランド病	DDAVP またはフォン・ヴィレブランド 因子を含む第 VIII 因子濃縮製剤	デスモプレシンまたは コンファクト F

*保険適応外である

表2 血友病における急性出血に対する補充療法[2]

出血部位	症状	目標因子レベル	追加投与（症状に応じて）
関節内出血 筋肉内出血[注1]	前兆または初期	20-40%	なし
	重症出血[注2]	40-80%	目標レベル 20-80% 12-24 時間毎に出血症状消失まで
口腔内出血	局所処置にて止血しないとき	20-40%	12-24 時間毎に 1-2 日間
	舌・舌小体・口唇小体・口唇裂傷の出血	40-60%	12-24 時間毎に 3-7 日間
消化管出血		80-100%	12-24 時間毎に 3-7 日間以上
気道出血	閉塞のおそれのある場合		
皮下出血	大きな血腫や頚部・顔面の血腫	20-40%	12-24 時間毎に 1-3 日間
鼻出血	局所処置とトラネキサム酸の投与を 行っても止血しない場合	20-40%	12-24 時間毎に 1-3 日間
肉眼的血尿[注3]	安静臥床と水分摂取で軽快しない場合	20-40%	12-24 時間毎に 1-3 日間
	疼痛，遷延する場合	40-60%	
骨折 外傷		トラフ 80-100% ピーク 100%	12 時間毎に 1 週間程度
頭蓋内出血		トラフ 100% とした持続輸注	最低 5-7 日以上継続
		持続輸注困難な場合は ピーク最低 100%	12 時間毎に最低 5-7 日以上
乳幼児の頭部打撲		速やかに 50-100%[注4]	

注1 腸腰筋出血は原則として入院加療とし，安静を図りつつ，12 時間毎のボーラス投与，または関節手術に準じて持続投与を行う．

注2 初期の出血の自覚症状に気づかず，何らかの理学的所見が出現してから気づいた場合，もしくは何らかの理由で速やかな補充療法が行われなかった場合や頻繁に出血を繰り返す target joint に出血が連続して起こった場合

注3 トラネキサム酸の投与は禁忌である．

注4 必要に応じその後 CT スキャンなどの検査を行ない，頭蓋内出血が否定された場合でも一両日中は経過観察を十分行なう．

先天性凝固障害に対する凝固因子補充療法

1. 血友病に対する凝固因子補充療法

血友病の出血症状に対する達成目標因子レベルは 20〜30% ですむこともあるが，むしろそれ以上に必要なことが多い（表2）．適切な目標因子レベルを 100% よりどこまで減量して良いかの根拠となるエビデンスを科学的に明らかにした文献は厳密な意味では存在しないが，本邦初のガイドライン[2]では，少数の患者を診療する非専門施設においても最低限対応できることを目的としている．表2では血友病患者における様々な出血症状に対し，適切な第 VIII 因子または第 IX 因子レベルを詳説した．

2. インヒビター保有血友病患者に対する凝固因子補充療法

一般に，インヒビター保有患者の急性出血に対するバイパス止血療法に用いる製剤としては活性化プロトロンビン複合体(aPCC)と遺伝子組換え型活性型第VII因子（rFVIIa）製剤がある．両製剤の止血効果の優劣に関する十分なエビデンスはなく，両者の特性を十分に考慮した上でいずれかを選択する必要がある[3]．

後天性希釈性凝固障害に対する凝固因子補充療法

1. 希釈性凝固障害の病態生理

外科的止血が達成されるまで循環血液量の1～2倍の輸液を行った場合は，血管内の血小板や凝固因子，線溶系因子が希釈され，相対的な凝固因子欠乏症に陥る．一方大量出血による循環破綻が改善されないと低酸素に陥った組織では嫌気性代謝により乳酸が産生され，代謝性アシドーシスがもたらされ，生理的な凝固因子活性に影響を与える．このような状態を一般的に希釈性凝固障害と呼び，さらに外傷早期は血管内に流入した組織因子（TF）などにより凝固亢進状態となるので二次線溶が活性化され線溶亢進状態となることから出血傾向が助長される．

2. 大量出血時の凝固検査モニタリング

欧州のガイドライン[4]はじめ，術中管理に関する各種ガイドラインではPT-INR，APTT，フィブリノゲン，血小板数の測定を推奨している．これらに加えて，thrombelastometry（TEG）またはTEGを改良発展させたROTEM®が推奨される[5]．

Chowdaryらは少数例での検討ではあるが，FFP投与を行った大量出血患者の後ろ向きの解析において，フィブリノゲンからXII因子までの（IV，VIを除く）各凝固因子レベルをすべて測定した．各凝固因子活性が「指針」でも取り上げられている「30%」を下回った症例と，実際にFFP投与を行った症例はほとんど一致せず，少なくともこれらのスクリーニング検査は補充療法の必要性を予見できないとした[6]．ただしフィブリノゲンについては検討されておらず，希釈性凝固障害における最適なモニタリング法には未だベストプラクティスが得られているとは言いがたい．

3. 凝固因子補充療法のエビデンス

凝固因子補充療法に用いられる製剤としてはFFP，FFPを氷温静置して得られる沈殿物を遠心回収したクリオプレチピテート（フィブリノゲン，フィブロネクチン，von Willebrand因子など高分子血漿タンパクが多く含まれる）もしくは各種の濃縮凝固因子製剤がある．凝固因子濃縮製剤については，我が国の健康保険ではrFVIIa製剤はインヒビター陽性血友病等の先天性凝固障害，フィブリノゲン製剤は先天性フィブリノゲン欠乏症，プロトロンビン複合体製剤（Prothrombin complex concentrates：PCC）は血友病B，アンチトロンビン（AT）製剤についてはDICと先天性アンチトロンビン欠乏症に対してのみそれぞれ使用することができる．

1）FFP

FFPの使用開始基準として「指針」が推奨するPT，APTTの基準と欧米の基準には大差がなく，欧米では一般にPTまたはAPTTが正常の1.5倍を超えると必要とされる[4]．フィブリノゲンについては多くの場合100 mg/dl以上が必要とされ[7]，FFP 450ml投与で約30 mg/dl上昇する（体重60kgでロスがない場合）．

FFPは非常に汎用される凝固因子製剤でありながら，希釈性凝固障害に対する有効性に関するエビデンスはきわめて少ない[7][8]．しかしながら，ガイドラインでは推奨される傾向にある．FFPについてはvolume overload，輸血感染症，輸注副作用（非溶血性・アレルギー性），Transfusion-related acute lung injury（TRALI）など副作用も無視できない．

近年，米陸軍における後ろ向きの検討をきっかけとして，赤血球ばかりでなくFFP/RBC unit比を高く保って出血対策を立てることが推奨されるようになってきた．ただし，こうした考え方に関しては反論もあり，一般施設の806例の外傷患者の後ろ向き観察結果ではPRBC：FFP比は入院期間，死亡率に影響を与えず，RBC/FFP比を1：1で投与した患者の予後は影響されなかった[9]との報告もある．

こうした混乱については，FFPは融解して輸注するまで時間がかかるため，赤血球投与が先行した後FFP投与に至る前に早期死亡した症例は，低FFP/RBC比群としてカウントされるためバイアスがかかっている可能性がある．また軍隊の検討では患者は若い男性が多く，ほとんど鋭的外傷であることから必ずしも一般施設とは同率に論じられない．

FFPの投与開始量は多くの場合10～15ml/kg（成人で約5～10単位）が推奨されることが多いが，この投与量では凝固因子の上昇程度はわずかである．実際にはこの倍の用量が必要であることが少数例の検討ではあるが示されており[6]，現場の診療感覚とも近い．しかし30ml/kg（成人で10～20単位）のFFPを短期間で投与することはvolume overload等，様々な問題がある．

2）フィブリノゲンとクリオプレチピテート

フィブリン形成は血液凝固において最も重要なステップであり，フィブリノゲンがなければこのステップは起こりえない[7]．Hiippalaらは，60例とやや少数ではあるが大量出血後，各凝固パラメーターの，活動性出血における止血最低レベルといわれる値に低下するまでの出血量を計算した．血小板数が5万/μlに低下するの

に総血液量(輸液・輸血量も含む)の230(169～294)%の出血を要したが，わずか142%(117～169)%の失血でフィブリノゲンは100mg/dl以下に達し，早期のフィブリノゲンの補充の重要性が示された[10]．ちなみにプロトロンビン，第V因子，第VII因子が20～25%以下に達するまではそれぞれ201%(160%～244%)，229%(167%～300%)，236%(198%～277%)の失血を要した．

出産後(大量)出血(Post-partum hemorrhage：PPH)では短時間に致命的な出血が起こるためフィブリノゲン低下のスピードが速く，フィブリノゲンやクリオプレチピテート輸注が救命に寄与したとする報告が多い．Charbitらは PPH においてフィブリノゲン濃度200mg/dl以下が重症出血と有意に関連していた[11]と報告したが，検査とその結果の入手までのタイミングを考えると妥当なレベルかもしれない．また人工心肺：cardiopulmonary bypass(CPB)を使用する冠動脈バイパス術[12]，様々な術後の大量出血[13]においてもフィブリノゲン濃度は出血量，輸血必要量と関連していたとされる．ただし前向き Randomized Controlled Trial(ランダム化比較試験，以下 RCT)は今のところ少ない．Fenger-Eriksenらのプラセボ対照試験では大量出血を伴う広汎子宮全摘において希釈性凝固障害に陥った患者で，フィブリノゲン製剤45mg/kg(成人で2～3g)の投与により，有意に出血量・輸血必要量に好影響を与えている．ただし各アーム10例と少数例での検討であった[14]．

多くのガイドライン，指針ではフィブリノゲン値150～200mg/dl以下，あるいはTEGでの低フィブリノゲン血症の兆候(Fibrinogen Thromboelastometry；FIBTEM)などをもとにフィブリノゲン濃縮製剤の投与を勧めている[4]．開始用量は成人で3～4gのフィブリノゲン濃縮製剤か50mg/kgのクリオプレチピテートである．フィブリノゲンの補充が血栓塞栓症のリスクになるかどうかは確定した情報はない．フィブリノゲンは急性期反応物質であり，出血がなければ術後には自然に700mg/dl以上になる可能性もあることから注意を要する．

3) rFVIIa

TFは活性型第VII因子(FVIIa)と強く結合し，FVIIaの酵素活性を大幅に上昇させる．一方でrFVIIaにはTF非依存性の作用メカニズムもある．血友病に対するrFVIIaの治療量(90μg/kg)を投与して得られるFVIIa血中濃度は25nM(正常の250倍)となり，この濃度ではTF非依存性に活性化した血小板上で第IX因子，第X因子を直接活性化させることができる．TEGにおいてrFVIIaなどの作用をモニターする場合はこのTF非依存性を主にみていることになる．

近年，インヒビター陽性血友病に対する強力な止血作用が，非血友病患者における希釈性凝固障害に伴う出血に応用されるようになり，外科的止血が追いつかない場合の有効性が検討されるようになってきた．26件の非常に多数のRCTが実施されている[15][16]が，rFVIIaはその作用機序から血栓塞栓症という重要な副作用を発症する危惧があり，また非常に高価な製剤であることから，その効果をエビデンスに基づいて詳細に検証することが必要である．

①外傷患者に対するエビデンス

大量出血事例へのRCTとしては，Boffardらが4時間以内に赤血球輸血を6単位以上要した16歳から65歳の鈍的外傷患者143例を対象に，rFVIIaを200μg/kg，1時間後に100μg/kg，3時間後に100μg/kgの3回投与する群と投与しない群で比較検討したところ，rFVIIa投与群では赤血球輸血量が平均2.6単位有意に減少した．しかしながら30日後の死亡率は有意差を認めず，大量出血症例に受傷早期から第VII因子製剤を投与しても輸血量を減少させることはできても明らかな救命率を向上させることはできていない[17]．我が国におけるRCTはないが，清水らが止血術後の致死的出血傾向・凝固障害を第VII因子製剤投与の適応とする治療プロトコールにより，少数例ではあるが良好な成績を報告している[18]．

②心臓血管系手術におけるエビデンス

CPBを併用する心臓血管手術においては回路内の凝血を防ぐためやむを得ず全身をヘパリン化するため，またCPB中は血小板の機械的破壊が起こりやすいこともあり，外科的止血の追いつかない大量出血が起きやすい．CPB患者の80%が輸血を必要とすると言うデータもある[19]．

DiproseらのRCTではrFVIIa(90μg/kg)の単回投与をCPBからの離脱後に行い，赤血球輸血の量は減少したが，rFVIIaがあまりにも高額なため症例数が不足し，有意な差は得られていない[19]．Gillらの RCTは30施設の共同研究で，プラセボを対照としてrFVIIa(40μg/kgと80μg/kg)がCPB離脱後投与された．80μg/kgを投与された群で有意に再手術率が低くまたどのrFVIIa群でも赤血球輸血量の減少が有意に見られた．しかしながら有意差はないものの，rFVIIa群では脳梗塞などの副作用が多数出現し，死亡率の改善は得られなかった[20]．Ekertらは1歳以下の76例の先天性心疾患手術において40μg/kgのrFVIIa投与群とプラセボ群に分け，CPB離脱後の投与20分後に追加投与が必要になるほどの出血が見られるかどうか比較した．結果はむしろ逆となり，閉胸までの時間は投与群の方が長くなり，赤血球，FFP必要量にも差がなかった[21]．

③重症肝疾患におけるエビデンス

Child-Pugh分類BまたはCの重症肝硬変患者で上部

表3　非血友病患者に対してrFVIIaを使用したRCTのまとめ

(A) 有効性の検討

	予防投与（14件）			治療的投与（12件）		
	効果	95% CI	検討研究数	効果	95% CI	検討研究数
死亡率減少（相対危険度）	0.82	0.36～1.83	13	0.90	0.76～1.06	12
出血量減少量（ml，体重補正あり）	−276	−411～−141	8			
出血コントロール（相対危険度）				1.05	0.97～1.14	4
赤血球輸血量（ml，体重補正あり）	−281	−433～−129	10	+21	−108～150	4

文献[15]より

(B) 血栓性有害事象のリスク

	rFVIIa投与例（2583名）中の血栓塞栓症発症数（%）	プラセボ投与例（1536名）中の血栓塞栓症発症数（%）	オッズ比（95% CI），p値
全出血栓塞栓症	264（10.2）	134（8.7）	1.17（0.94～1.47），$p=0.16$
動脈系	141（5.5）	4（3.2）	1.68（1.20～2.36），$p=0.003$
冠動脈血栓症	76（2.9）	17（1.1）	2.39（1.39～4.09），$p=0.002$
脳血管系血栓症	45（1.7）	20（1.3）	1.27（0.74～2.17），$p=0.39$
静脈系	137（5.3）	88（5.7）	0.93（0.70～1.23），$p=0.61$

文献[16]より

CI：Confidence interval

消化管出血を呈する患者に対し，内視鏡的止血後rFVIIaを投与したが，赤血球輸血量の減少には至っていない[22]．肝移植においては数カ所でRCTが組まれ，rFVIIaの予防投与が行われているが．いずれの研究でも死亡率の改善は得られていない[23]．

④産科大量出血におけるエビデンス

産科における大量出血例では，動脈塞栓術・内腸骨動脈結紮術・子宮摘出術などの外科的処置を講じてもなお止血困難な症例に対して，近年rFVIIaの有効性に関する報告が相次いだ．日本産婦人科・新生児血液学会が行ったrFVIIaの後ろ向き使用調査では2007年から2010年まで24例が解析された．15例で止血，8例で出血の減少，1例で不変であった[24]が，3例に血栓塞栓症がみられ，合併症が皆無ではなかったことは注目すべきである．

オーストラリアとニュージーランドでは2002～2008年の間に90例が登録され，出血量の減少は64%に見られたが，2例(1.9%)に非致死的な血栓塞栓症がみられている[25]．北ヨーロッパの登録（92例）においては83%に有効性を報告している[26]．イタリアからの報告では，2005～2007年までのPPH 35症例について，使用前後でINRの低下，フィブリノゲン濃度の上昇を観察し，また赤血球輸血，血小板輸血，FFPの使用量の減少をみている[27]．他の領域に比べて産科出血の領域ではRCTはまだ報告がなく，今後より高いエビデンスがのぞまれる．

⑤効果と副作用のメタアナリシス

Cochrane Libraryのデータを用いたメタアナリシス[15]（2010年時点）によれば，rFVIIaの血友病以外の疾患の使用に関しては26件のRCTがあり，14件は予防的な投与（1,137例），12件は治療的な投与（2,538例）であった（表3）．治療群とプラセボ群の間には死亡率において有意な差は見られなかったが，出血量と輸血量においてはrFVIIaの有用性が観察された．しかし治療的な投与を行った研究をまとめると，死亡率の改善は得られず止血効果もはっきりしなかった．

同年，Leviらは上記の26件に加えて9件の正常人を対象とした研究を加えて35件のRCTについて血栓塞栓症のリスクに関するメタアナリシスを行った．rFVIIa群ではプラセボ群に比べ，動脈血栓塞栓(5.5%対3.2%，75歳以上では10.8%対4.1%）および冠動脈血栓塞栓（2.9%対1.1%）の発生率が有意に高かった（表3）[16]．

現在得られている効果と副作用のエビデンスの解析によれば，rFVIIaの適応外投与を臨床試験以外の設定でこれ以上続ける根拠はないと言うことになる．しかしながら臨床の現場においては「これまで経験したことのない明らかな止血効果」を経験したという声は消えない[18][24]．外科的止血の追いつかない凝固障害において少なくとも著効を示す病態は確実に存在すると考えられ，この病態の解明が今後の課題となるだろう．

4）PCC

PCCは血漿のバリウム吸着分画から中等度に精製され，第IX因子を多量に含むため以前は血友病Bの治療に頻用された(現在も日本の保険適用は血友病Bのみ)混合製剤である．バリウム吸着分画は2価イオンに高親和性に結合するペプチド，すなわちビタミンKによるカルボキシル化を受けたGlaドメインを保有するビタミンK（VK）依存性血漿タンパク：プロトロンビン，第VII因子，第IX因子，第X因子，protein C，protein Sを豊富に含む．

一般にビタミンK（VK）の使用は，血栓症リスクの増加や，Vitamin K antagonist（VKA：ワルファリン）耐性を引き起こすことなく，急速にINRを低下させることができるとされている．VKAの過剰作用によりINRが4.5から10.0となり，出血もしくは出血のリスクのある患者に対してはまず経口VKが使用される．しかしこれを早急に是正する場合にはPCC 500〜1,000UもしくはFFPを投与することが日本循環器学会のガイドラインでも保険適応外であるが紹介されている[28]．

このようにPCCがVKAの効果の抑制（VKA reversal）に非常に有用であることは様々な研究により実証されている．RCTは3件のみであるが，VK依存性凝固因子欠乏症を適切な濃縮製剤で補うという観点からは，VKA reversalの方法としては医学的蓋然性の高い治療であるとも言える．その意味では後年の後ろ向き試験の方がむしろ緻密である[29]．

VKA reversalを伴わない大量出血に対するPCCの使用にはまだ一定の見解はない．PCCには血栓塞栓症のリスクがあるため，回復後は血栓予防に留意する必要があるが，APCC，rFVIIa等に比べて血栓塞栓症の頻度は低いという報告もある[30]．

5）アンチトロンビン

アンチトロンビン（AT）は主として基礎疾患として重症敗血症を有するDIC患者に対する単独投与の有効性が臨床的に示されている．重症敗血症に伴うDICで認められる血中AT活性低下は，DICによるものに加えて炎症（血管外漏出）もしくは肝の蛋白合成能低下によるものもあると考えられ，著しい低AT血症を来すことが多い．

AT活性が70%以下のとき，「指針」では投与することが可能とされているが，出血症状を有する患者にはたとえ異常低値を示しても必ずしも直ちに補充することは推奨されない．近年行われた重症敗血症に対する多施設RCT（KyberSept trial）におけるDIC合併例のサブ解析では，ヘパリンを併用した群で出血有害事象による有意な死亡率上昇がみられた[31]．今後新たなエビデンスが登場するまでは，ATは大量出血を呈する外傷患者には出血を助長する可能性が高く，敗血症・DICの病態改善以外の目的で積極的に投与すべきではない．

終わりに

先天性凝固障害の血友病の出血症状は単一凝固因子欠乏であり，目指すべき凝固因子レベルが設定されている．これに対して大量出血に伴う希釈性凝固障害ではフィブリノゲン濃縮製剤やクリオプレチピテートの有効性が多く報告されているが，前向きRCTは今のところ少なく，今後の検討が待たれる．一方でrFVIIaやPCCの有効性に関しては多くのRCTによっても総合的な有用性は高いとは言えず，今後はこれらの有効性が得られる病態を探索する努力が必要となるだろう．

文　献

1) Edmunds L., Salzman E.: Hemostatic problems, transfusion therapy, and cardiopulmonary bypass in surgical patients, In: Colman R, Hirsh J, Marder V, et al, eds, Hemostasis and thrombosis: basic principles and clinical practice, JB Lippincott, Philadelphia, 1994, 956—968.
2) 松下　正，天野景裕，瀧　正志，他：インヒビターのない血友病患者の急性出血，処置・手術における凝固因子補充療法のガイドライン．日本血栓止血学会誌，19：510—519, 2008.
3) 田中一郎，天野景裕，瀧　正志，他：インヒビター保有先天性血友病患者に対する止血治療ガイドライン．日本血栓止血学会誌，19：520—539, 2008.
4) Rossaint R., Bouillon B., Cerny V., et al: Management of bleeding following major trauma: an updated European guideline. Crit Care, 14: R52, 2010.
5) Rugeri L., Levrat A., David J.S., et al: Diagnosis of early coagulation abnormalities in trauma patients by rotation thrombelastography. J Thromb Haemost, 5: 289—295, 2007.
6) Chowdary P., Saayman A.G., Paulus U., et al: Efficacy of standard dose and 30 ml/kg fresh frozen plasma in correcting laboratory parameters of haemostasis in critically ill patients. Br J Haematol, 125: 69—73, 2004.
7) 山本晃士，西脇公俊，加藤千秋，他：術中大量出血を防ぐための新たな輸血治療　クリオプレシピテートおよびフィブリノゲン濃縮製剤投与効果の検討．日本輸血細胞治療学会誌，56：36—42, 2010.
8) Stanworth S.J., Brunskill S.J., Hyde C.J., et al: Is fresh frozen plasma clinically effective? A systematic review of randomized controlled trials. Br J Haematol, 126: 139—152, 2004.
9) Scalea T.M., Bochicchio K.M., Lumpkins K., et al: Early aggressive use of fresh frozen plasma does not improve outcome in critically injured trauma patients. Ann Surg, 248: 578—584, 2008.
10) Hiippala S.T., Myllyla G.J., Vahtera E.M.: Hemostatic factors and replacement of major blood loss with plasma-poor red cell concentrates. Anesth Analg, 81: 360—365, 1995.
11) Charbit B., Mandelbrot L., Samain E., et al: The decrease of fibrinogen is an early predictor of the severity of postpartum hemorrhage. J Thromb Haemost, 5: 266—273, 2007.

12) Karlsson M., Ternstrom L., Hyllner M., et al: Plasma fibrinogen level, bleeding, and transfusion after on-pump coronary artery bypass grafting surgery: a prospective observational study. Transfusion, 48: 2152—2158, 2008.
13) Fenger-Eriksen C., Lindberg-Larsen M., Christensen A. Q., et al: Fibrinogen concentrate substitution therapy in patients with massive haemorrhage and low plasma fibrinogen concentrations. Br J Anaesth, 101: 769—773, 2008.
14) Fenger-Eriksen C., Jensen T.M., Kristensen B.S., et al: Fibrinogen substitution improves whole blood clot firmness after dilution with hydroxyethyl starch in bleeding patients undergoing radical cystectomy: a randomized, placebo-controlled clinical trial. J Thromb Haemost, 7: 795—802, 2009.
15) Lin Y., Stanworth S., Birchall J., et al: Use of recombinant factor VIIa for the prevention and treatment of bleeding in patients without hemophilia: a systematic review and meta-analysis. CMAJ, 183: E9—19, 2011.
16) Levi M., Levy J.H., Andersen H.F., et al: Safety of recombinant activated factor VII in randomized clinical trials. N Engl J Med, 363: 1791—1800, 2010.
17) Boffard K.D., Riou B., Warren B., et al: Recombinant factor VIIa as adjunctive therapy for bleeding control in severely injured trauma patients: two parallel randomized, placebo-controlled, double-blind clinical trials. J Trauma, 59: 8—15, 2005.
18) 清水健太郎，小倉裕司，吉矢和久，他：Lethal triad に陥った外傷出血症例に対する遺伝子組換え　活性型血液凝固第 VII 因子製剤（ノボセブン）投与の有効性．日本救急医学会雑誌，20：133—141, 2009.
19) Diprose P., Herbertson M.J., O'Shaughnessy D., et al: Activated recombinant factor VII after cardiopulmonary bypass reduces allogeneic transfusion in complex non-coronary cardiac surgery: randomized double-blind placebo-controlled pilot study. Br J Anaesth, 95: 596—602, 2005.
20) Gill R., Herbertson M., Vuylsteke A., et al: Safety and efficacy of recombinant activated factor VII: a randomized placebo-controlled trial in the setting of bleeding after cardiac surgery. Circulation, 120: 21—27, 2009.
21) Ekert H., Brizard C., Eyers R., et al: Elective administration in infants of low-dose recombinant activated factor VII (rFVIIa) in cardiopulmonary bypass surgery for congenital heart disease does not shorten time to chest closure or reduce blood loss and need for transfusions: a randomized, double-blind, parallel group, placebo-controlled study of rFVIIa and standard haemostatic replacement therapy versus standard haemostatic replacement therapy. Blood Coagul Fibrinolysis, 17: 389—395, 2006.
22) Bosch J., Thabut D., Albillos A., et al: Recombinant factor VIIa for variceal bleeding in patients with advanced cirrhosis: A randomized, controlled trial. Hepatology, 47: 1604—1614, 2008.
23) Pugliese F., Ruberto F., Summonti D., et al: Activated recombinant factor VII in orthotopic liver transplantation. Transplant Proc, 39: 1883—1885, 2007.
24) Kobayashi T., Nakabayashi M., Yoshioka A., et al: Recombinant activated factor VII (rFVIIa/NovoSeven®) in the management of severe postpartum haemorrhage: initial report of a multicentre case series in Japan. Int J Hematol, 95: 57—63, 2012.
25) Phillips L.E., McLintock C., Pollock W., et al: Recombinant Activated Factor VII in Obstetric Hemorrhage: Experiences from the Australian and New Zealand Haemostasis Registry. Anesthesia & Analgesia, 109: 1908—1915, 2009.
26) Alfirevic Z., Elbourne D., Pavord S., et al: Use of recombinant activated factor VII in primary postpartum hemorrhage: the Northern European registry 2000-2004. Obstet Gynecol, 110: 1270—1278, 2007.
27) Barillari G., Frigo M.G., Casarotto M., et al: Use of recombinant activated factor VII in severe post-partum haemorrhage: data from the Italian Registry: a multicentric observational retrospective study. Thromb Res, 124: e41—47, 2009.
28) 2008 年度合同研究班報告：循環器疾患における抗凝固・抗血小板療法に関するガイドライン，日本循環器学会.
29) Leissinger C.A., Blatt P.M., Hoots W.K., et al: Role of prothrombin complex concentrates in reversing warfarin anticoagulation: a review of the literature. Am J Hematol, 83: 137—143, 2008.
30) Majeed A., Eelde A., Agren A., et al: Thromboembolic safety and efficacy of prothrombin complex concentrates in the emergency reversal of warfarin coagulopathy. Thromb Res, 129: 146—151, 2012.

31) Kienast J., Juers M., Wiedermann CJ., et al: Treatment effects of high-dose antithrombon without concomitant heparin in patients with severe sepsis with or without disseminated intravascular coagulation. J Thromb Haemost, 4: 90—97, 2006.

REPLACEMENT THERAPY OF COAGULATION FACTORS: CLINICAL EVIDENCE AND STANDARD MANAGEMENT

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ADAMTS13 unbound to larger von Willebrand factor multimers in cryosupernatant: implications for selection of plasma preparations for thrombotic thrombocytopenic purpura treatment

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BACKGROUND: Thrombotic thrombocytopenic purpura (TTP) is characterized by deficient ADAMTS13 activity. Treatment involves plasma exchange (PE). Both fresh-frozen plasma (FFP) and cryosupernatant (CSP) are used, but it remains to be determined which is more effective.

STUDY DESIGN AND METHODS: To analyze the interaction between von Willebrand factor (VWF) and ADAMTS13, we used large-pore isoelectric focusing (IEF) analysis followed by detection with anti-ADAMTS13 monoclonal antibody. FFP, CSP, cryoprecipitate (CP), and purified ADAMTS13 were analyzed for their effects on high shear stress–induced platelet aggregation (H-SIPA).

RESULTS: IEF analysis of normal plasma revealed three groups of ADAMTS13 bands with pI of 4.9 to 5.6, 5.8 to 6.7, and 7.0 or 7.5. Two band groups (pI 4.9-5.6 and 5.8-6.7) were found in plasma of a patient with Type 3 von Willebrand disease, in which VWF is absent, whereas no bands were found in plasma of a patient with congenital ADAMTS13 deficiency. Mixing these plasmas generated the bands at pI 7.0 or 7.5, representing the VWF-ADAMTS13 complex; these bands were absent in CSP. FFP and purified ADAMTS13 down regulated H-SIPA in a dose-dependent manner. However, CP did not inhibit H-SIPA in the initial phase, and the degree of inhibition at the endpoint was almost indistinguishable from those of the other two plasma products.

CONCLUSION: Both plasma products (FFP and CSP) are effective for PE in TTP patients. However, CSP may be more favorable, because it has lower levels of VWF and almost normal ADAMTS13 activity, but lower levels of ADAMTS13 in complex with larger VWF multimers.

Von Willebrand factor (VWF), a multimeric hemostatic glycoprotein, is secreted from vascular endothelial cells into circulation as unusually large VWF multimers (UL-VWFMs).[1] The UL-VWFM is the most biologically active form with regard to platelet (PLT) adhesion properties.[2] Under conditions of high shear stress, UL-VWFMs cause enhanced PLT aggregation and give rise to VWF-rich thrombi in the microvasculature. ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type-1 motifs 13) down regulates the function of UL-VWFMs by cleaving the VWF A2 domain at the Tyr1605–Met1606 bond, yielding

ABBREVIATIONS: ADAMTS13 = a disintegrin-like and metalloprotease with thrombospondin type-1 motifs 13; CP = cryoprecipitate; CSP = cryosupernatant; dp = depleted; H-SIPA = high shear stress–induced platelet aggregation; IAA = iodoacetamide; IEF = isoelectric focusing; pd = plasma derived; PE = plasma exchange; T3-VWD = Type 3 von Willebrand disease; TBS = Tris-buffered saline; TTP = thrombotic thrombocytopenic purpura; UL-VWFM(s) = unusually large VWF multimer(s); USS = Upshaw-Schulman syndrome.

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a series of smaller molecular forms.[3-5] The proteolytic activity of ADAMTS13 is located in its amino-terminal metalloprotease domain, but optimal enzyme activity requires cooperative interactions with other domains of the ADAMTS13 molecule.[6]

Deficiency of ADAMTS13 activity causes a life-threatening generalized disease, thrombotic thrombocytopenic purpura (TTP), which can be caused either by mutation of the ADAMTS13 gene (Upshaw-Schulman syndrome [USS]) or by acquisition of autoantibodies against the ADAMTS13 enzyme.[7] USS is often treated by prophylactic infusions of fresh-frozen plasma (FFP) as a source of ADAMTS13, but cryoprecipitate (CP) has also been effective.[8,9] On the other hand, for patients with acquired TTP, plasma exchange (PE) is the first-line treatment.[10] For PE treatment, cryosupernatant (CSP) is preferentially used in Canada, but FFP is used in many other countries, including Japan. However, it has not been firmly established which material is more favorable for PE.[11] In this context, immunoprecipitation using anti-VWF revealed that approximately 3% of ADAMTS13 in plasma is bound to VWF, with a stoichiometry of one ADAMTS13 molecule to 250 VWF monomeric subunits.[12] However, the characteristics of the VWF-ADAMTS13 complex in the plasma milieu remain unclear, as does the physiologic relevance of functional differences, if any, between the bound and unbound forms of ADAMTS13.

To address these issues and analyze the VWF-ADAMTS13 complex in the plasma milieu, we employed isoelectric focusing (IEF) analysis using a large-pore agarose-acrylamide composite gel. Using this method, we were able to visualize the VWF-ADAMTS13 complex. We found that in the plasma milieu, ADAMTS13 forms a complex with larger VWFMs, but is less likely to do so with smaller VWFMs (dimers and tetramers); the complex can be separated from its unbound counterpart by cryoprecipitation. Based on these observations, we hypothesize that the bound and unbound forms of ADAMTS13 possess functional differences with respect to the microvascular condition of the patient. Furthermore, we evaluated the functional differences between ADAMTS13 in CSP and CP, by testing their inhibitory effects on high shear stress–induced PLT aggregation (H-SIPA).

MATERIALS AND METHODS

Plasma samples

Anticoagulated blood containing 1/10 volume of 3.8% sodium citrate was collected from normal individuals and from patient with either Type 3 von Willebrand disease (T3-VWD) or USS (Patient USS-EE4). The citrated plasmas were then separated by centrifugation and stored at –80°C until use. T3-VWD plasma had less than 3% of the normal control levels of both VWF antigen and ristocetin cofactor. The USS-EE4 patient had plasma levels of both ADAMTS13 activity and antigen less than 0.5 and 0.1% of the normal control, respectively; the ADAMTS13 gene mutation was identified as 2259delA/2259delA.[8] Informed consent was obtained from all subjects.

Preparation of CSP and CP

FFP was prepared at Nara Red Cross Blood Center and stored in inventory at –30°C. However, FFP preparations beyond 1 year for the inventory were provided to us. These FFP preparations were then kept frozen at –80°C in our institution. Outdated FFP was then thawed overnight at 4°C, followed by centrifugation at 7000 × *g* for 30 minutes at 4°C. After centrifugation, the CSP was separated and kept frozen in aliquots at –80°C. For analysis of ADAMTS13 activity and VWF antigen, the CP was dissolved in one-fifth volume of 20 mmol/L Tris-buffered saline (TBS, pH 7.4) without rinsing.

For H-SIPA, the CP was rinsed with cold TBS containing 0.38% Na_3-citrate, 2 mmol/L benzamidine-HCl, 20 mmol/L 6-amino-*n*-caproic acid, and 0.02% NaN_3 and then centrifuged at 4°C. This procedure was repeated twice. Ultimately, the CP was dissolved in 1/10 volume of TBS containing 0.38% Na_3-citrate and then stored in aliquots at –80°C.

Purified plasma VWF and ADAMTS13

Purification of plasma VWF was performed essentially as previously described:[13] cryoprecipitation of outdated pooled FFP collected from normal volunteers, removal of fibronectin by gelatin-agarose affinity chromatography, precipitation with 40% saturated $(NH_4)_2SO_4$, and finally purification by size-exclusion chromatography in Sepharose 4B gel. Fractions eluted in the anterior half of the void volume of the Sepharose 4B column were pooled; the resulting protein consisted of higher VWF multimers and migrated as a single 250-kDa band on a sodium dodecyl sulfate (SDS)-5% polyacrylamide gel under reducing conditions.[13] After dialysis against TBS, the purified plasma-derived (pd)-VWF was kept frozen in aliquots at –80°C until use.

Purification of plasma ADAMTS13 was achieved using anti-ADAMTS13 monoclonal antibody (MoAb) A10 (IgG2b-κ)–coupled beads as recently described.[14] The epitope of A10 resides on the disintegrin-like domain of ADAMTS13.[15] Briefly, the CSP was prepared essentially as described above, from outdated FFP in the presence of two protease inhibitors (2 mmol/L benzamidine-HCl and 20 mmol/L 6-amino-*n*-caproic acid) and 0.02% NaN_3. The CSP was then applied to an A10-coupled column at 4°C and washed extensively. The ADAMTS13 bound to the column was eluted in two steps, first with 10% dimethyl sulfoxide (DMSO) and then with 40% DMSO. The ADAMTS13 eluted with 40% DMSO was pooled and concentrated and then purified by size-exclusion chromatography on a Superdex HR10 column. The purified

pd-ADAMTS13 migrated on a SDS-5% polyacrylamide gel as a single 170-kDa band before reduction and a single 190-kDa band after reduction; specific activity was 300 units/mg.[14] One unit of ADAMTS13 activity was defined as the amount contained in 1 mL of pooled normal plasma.

Assays for ADAMTS13 and VWF

The ADAMTS13 activity and antigen were measured with a chromogenic ADAMTS13-act-enzyme-linked immunosorbent assay (ELISA)[16] and an in-house sandwich ELISA using two MoAbs, respectively.[17] The VWF antigen was determined with a sandwich ELISA using a rabbit polyclonal anti-human VWF antibody (Dako Cytomation, Kyoto, Japan).[18] A value of 100% of the ADAMTS13 activity and antigen were defined as the amount in the pooled normal human plasmas, which were prepared from a total of 40 normal volunteers, consisting of 10 individuals from each ABO blood group.

Preparation of ADAMTS13-depleted plasma

The ADAMTS13-depleted (dp) plasma was prepared from the whole FFP using an A10-agarose column equilibrated with TBS containing a cocktail of protease inhibitors (5 mmol/L benzamidine, 2 mmol/L phenylmethanesulfonyl fluoride, and 20 mmol/L 6-amino-*n*-caproic acid, each final concentration) at room temperature. Flowthrough fractions were monitored with ADAMTS13 activity and antigen; both the values indicated less than 0.5 and 0.1% of the normal, respectively and were dialyzed with TBS containing 0.38% Na_3-citrate and stored in aliquots at –80°C.

IEF using an agarose-acrylamide composite gel

IEF gel plate was assembled with two glass plates and 1-mm-thick plastic spacers. Four grams of sucrose and 0.3 g of agarose (final 0.75%, agarose IEF, GE Healthcare Bio-Science AB, Uppsala, Sweden) were mixed with 34.2 mL of distilled water. The mixture was dissolved by microwave oven and kept at 56°C. Then, 1.67 mL of 30% acrylamide-bisacrylamide (final 1.25%), 1.67 mL of distilled water, 2.5 mL of 40% carrier ampholyte (Pharmalyte 3-10, GE Healthcare Bio-Science AB), 0.27 mL of ammonium peroxodisulfate (22.8 mg/mL), and 0.01 mL of *N,N,N′,N′*-tetramethylethylenediamine were added to this mixture. The mixture was poured into the IEF gel plate quickly and left for more than 1 hour at room temperature followed by 4°C overnight.

The IEF gel was placed on the Multiphor apparatus (GE Healthcare Bio-Science AB) equilibrated at 10°C. The electrode strips were prepared using 0.5 mol/L acetic acid at the anode and 0.5 mol/L sodium hydroxide at the cathode. The electrical conditions used for IEF were the first 30 minutes at a maximum of 100 V, 5 mA, and 15 W; then 60 minutes at a maximum of 200 V, 10 mA, and 6 W; and finally 90 minutes at a maximum of 1500 V, 15 mA, and 6 W. After IEF, the isolated proteins were electrophoretically transferred to nitrocellulose membrane.

Iodoacetamide effect on complex of ADAMTS13 and VWF in plasma milieu

Recent studies have indicated that free thiols exposed in ADAMTS13 play an important role to regulate thiol-disulfide exchange of VWF under a high shear stress. Furthermore, blocking these active thiols decreases ADAMTS13 activity in cleaving UL-VWFM under flow conditions.[19] We evaluated the effect of iodoacetamide (IAA), which blocks the free thiols and prevents the formation of a covalent complex through disulfide bonds. For this experiment, each reagent of ADAMTS13-dp plasma, purified pd-ADAMTS13, and pd-VWF was separately treated with or without 100 mmol/L IAA before mixing for 30 minutes at room temperature. The mixture of these three reagents was exposed to a high shear stress generated by a vortex mixer at 3200 rpm for 5 minutes. The final concentration of each reagent in this mixture (a total of 130 μL) was 60 μg/mL for pd-VWF, 2.3 μg/mL for purified pd-ADAMTS13, and 65 μL for ADAMTS13-dp plasma.

Two-dimensional gel electrophoresis using either polyacrylamide gel electrophoresis or agarose

In some experiments, after IEF the two-dimensional gel electrophoresis was performed using either SDS-5% polyacrylamide gel electrophoresis (PAGE) under reducing conditions or SDS-0.9% agarose gel electrophoresis under nonreducing conditions. The former was used for an analysis of ADAMTS13 antigen and the latter for VWF multimer patterns. In both instances, the separated proteins were electrophoretically transferred to polyvinylidene fluoride membrane or nitrocellulose membrane, and then the blot proteins were immunoreacted with anti-ADAMTS13 MoAb (WH2-11-1, an epitope residing on the fourth thrombospondin Type 1 domain of ADAMTS13)[20] or rabbit polyclonal anti-human VWF antibody and then visualized by chemiluminescent detection kits (Perkin-Elmer Life Science, Inc., Boston, MA).

H-SIPA in the absence of ADAMTS13

To reproduce PLT aggregation assumed to be occurring in TTP patients, H-SIPA at a constant shear rate of 108 dynes/cm^2 was measured with an argon laser-assisted cone PLT aggregometer (Toray Medical, Tokyo, Japan),[21] using a mixture of normal washed PLTs (300×10^9/L, final), ADAMTS13-dp plasma (29% vol/vol, final), and the purified pd-VWF (250% of the normal plasma, final).

For this assay, normal washed PLTs were prepared and suspended in a Hepes-Tyrode buffer (pH 7.3) containing 1.8 mmol/L $CaCl_2$.[22] The mixture with a total volume of

400 μL was preincubated at 37°C for 5 minutes, and then H-SIPA was measured for 6 minutes. The maximum PLT aggregation was seen in the absence of any additives, and the minimum or nonspecific PLT aggregation was determined in the presence of anti-VWF MoAb NMC-4 (10 μg IgG/mL, final), which totally blocks the VWF binding to PLT GPIb.[13]

For assessment of the inhibitory effect of various forms of pd-ADAMTS13 to H-SIPA, they were spiked into the above-mentioned assay mixtures and incubated for 5 minutes at 37°C before measurement. H-SIPA was measured at room temperature and completed within 2.5 hours after blood collection. The inhibition rate of H-SIPA was calculated in the following formula: Inhibition rate (%) = [1 − (% light transmittance of tested sample/% light transmittance of control)] × 100. These data were expressed as the mean ± SD. We calculated the inhibition rate in two points at 140 and 340 seconds after the initiation of H-SIPA. Comparison between these two points was tested for significance using paired t test using computer software (StatView, SAS Institute, Inc., Cary, NC). A p value of less than 0.05 was considered significant.

RESULTS

ADAMTS13 and VWF on IEF agarose-acrylamide composite gels

We detected the purified pd-ADAMTS13 (15 ng) as one band at pI 4.9 to 5.6 (median, 5.4) using anti-ADAMTS13 MoAb (WH2-11-1) on IEF agarose-acrylamide composite gels (Fig. 1, left panel). Next, we analyzed various amounts (1-10 μL) of normal citrated plasma and found that ADAMTS13 antigen in the plasma milieu could also be detected as a major band at pI 4.9 to 5.6, as in the case of purified pd-ADAMTS13. In plasma, however, two additional bands of ADAMTS13 antigen were also detected: one was composed of a cluster of blurred bands at pI 5.8 to 6.7, and the other consisted of two clear bands at pI 7.0 or 7.5. In T3-VWD plasma, two groups of ADAMTS13 bands, pI 4.9 to 5.6 and 5.8 to 6.7, were detected, but the bands at pI 7.0 or 7.5 were totally absent (Fig. 1, right panel). T3-VWD plasma lacks VWF antigen; therefore, the two groups of bands at pI 4.9 to 5.6 and 5.8 to 6.7 appear to exist independently of the presence of plasma VWF. Conversely, we assumed that the bands at pI 7.0 or 7.5 represented a complex with VWF that exists within the plasma milieu. The bands at pI 7.0 or 7.5 were also detected after mixing FFP with 1 mol/L NaCl (final), excluding the possibility that the complex is formed by an ionic linkage (data not shown).

Generation of the pI 7.0 of 7.5 band of ADAMTS13 complex with VWF

Next, we performed the mixing experiments shown in Fig. 2A. T3-VWD plasma spiked with purified pd-VWF yielded a new band at pI 7.5. USS-EE4 plasma initially

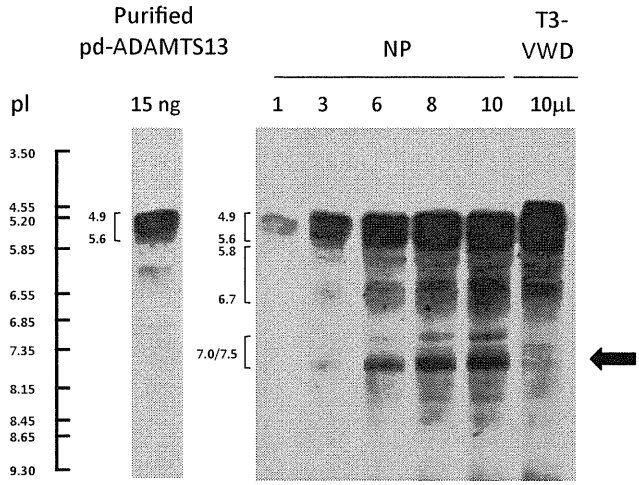

Fig. 1. Separation of plasma ADAMTS13 by IEF in healthy control and patient with T3-VWD. Purified pd-ADAMTS13, normal plasma (NP), and T3-VWD plasma were subjected to IEF and immunoblotting with anti-ADAMTS13 MoAb (WH2-11-1). Purified pd-ADAMTS13 (15 ng) was detected as one band at pI 4.9 to 5.6 (median, 5.4; left panel). In various amounts (1-10 μL) of NP, ADAMTS13 antigen was detected as a major band at pI 4.9 to 5.6. Two additional groups of bands of ADAMTS13 antigen were also detected: pI 5.8 to 6.7 and pI 7.0 or 7.5. In T3-VWD, the ADAMTS13 band groups of pI 4.9 to 5.6 and 5.8 to 6.7 were detected, but the band of pI 7.0 or 7.5 was barely detectable (right panel). Arrow indicates the VWF-ADAMTS13 complex.

lacked three groups of ADAMTS13 bands (pI 4.9-5.6, 5.8-6.7, and 7.0/7.5), but once that plasma was spiked with purified pd-ADAMTS13, the band at pI 7.5 clearly appeared. When T3-VWD and USS-EE4 plasma samples were mixed together, the band at pI 7.5 also appeared, confirming that it represents a complex of VWF and ADAMTS13.

ADAMTS13 (pI 7.5) is a noncovalent complex with VWF in the plasma milieu

We next evaluated the effects of IAA, which blocks free thiols and prevents the formation of disulfide bond–mediated covalent complexes, under high shear stress in a vortex mixer. As shown in Fig. 2B, the band at pI 7.5, representing the VWF-ADAMTS13 complex, was generated irrespective of the presence of IAA. When pd-VWF was spiked into this mixture, the density of the band at pI 7.5 increased. These results indicate that in our experiments, formation of the VWF-ADAMTS13 complex does not depend upon disulfide bond bridges.

ADAMTS13 is present in plasma in complex with a large VWFM

As shown in Fig. 3, IEF gel analysis of normal plasma revealed ADAMTS13 as three groups of bands (pI 4.9-5.6,