prevention and progression of otherwise life-changing haemarthropathy.

Author contributions

M. Kajiwara treated the patients and wrote the article. M. Shima and A. Yoshioka treated the patients and reviewed the manuscript. All authors have approved the final submitted version.

Disclosures

M. Kajiwara received a fee for preparing materials for a Novo Nordisk Symposium. M. Shima and A. Yoshioka have received consultant and speaker's fees from Novo Nordisk and Baxter. Editorial assistance to the authors during the preparation of this manuscript was provided by Sharon Eastwood (medical writer, PAREXEL) and financially supported by Novo Nordisk in compliance with international guidelines for good publication practice.

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Towards standardization of clot waveform analysis and recommendations for its clinical applications

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Introduction

Automated coagulation analyzers can provide a wealth of information in addition to that provided by conventional clotting tests such as the prothrombin time (PT) and the activated partial thromboplastin time (APTT), which are often considered of limited use for clinical purposes [1]. One particular type of analysis, the clot waveform, which was originally described using the Multichannel Discrete Analyzer (MDA series; Organon, Technika, Durham, NC, USA), defines changes in light transmittance that occur during the process of clot formation. A number of recent reports have described the use of this type of automated clotting instrument for clot waveform analysis (CWA), and there appears to be significant advantages in using this assay for the assessment of global coagulation function. In this communication, we propose standardization of methods for the CWA using currently available clotting analyzers and overview the potential clinical applications.

Principles of clot waveform analysis

Visualization of clot waveforms

Changes in light transmittance or absorbance are determined by continuous measurements during the APTT and are designated the clot waveform (CW) (Fig. 1). This clotting process is categorized into three parts: the pre-coagulation, coagulation and post-coagulation phases. Pre-coagulation is described as the first segment of the trace, from the beginning of the signal to the onset of coagulation. After the onset of coagulation, light transmittance is decreased or absorbance is increased by the formation of fibrin, and this is defined by a slope in the waveform. At the end of coagulation, light transmittance or absorbance tends to stabilize and is characterized again by a linear segment. If fibrinolysis is enhanced due to acquired or congenital abnormalities of hemostasis, light transmittance may increase or absorbance may decrease again in the post-coagulation phase.

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Coagulation analyzer

There are two types of clotting machines for CWA. One utilizes a system to detect transmittance during the APTT clotting reaction, and is represented by the MDA-II or CS series. In this type, transmittance is decreased after initiation of clotting (Fig. 1A). The other type monitors the absorbance, and is represented by the ACL series. In this type, 0% absorbance defines the pre-coagulation phase, and the absorbance increases after the initiation of clotting (Fig. 1B). Other analyzers having similar features should also be able to provide CWA data easily, particularly if manufacturers incorporate the relevant software. Even if an automated CWA is not available, there are several analyzers that are able to provide adequate raw data (Table S1). CWA is possible using such analyzers by statistical evaluation of this raw data of transmittance or absorbance (Fig. S1).

Recommended method for standardization of CWA

APTT assay

Plasma should be prepared from fresh citrated whole blood as for the standard APTT assay. Pooled plasma from nor-

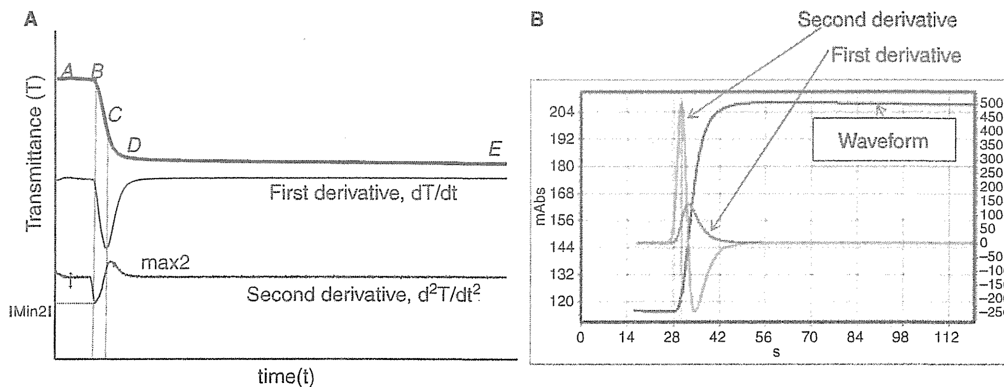

Fig. 1. APTT clot waveforms (A) Clot waveform of normal plasma by monitoring of transmittance (CS2000i). The upper trace shows the changes in light transmittance observed during the performance of APTT with normal reference plasma by CS200i (Sysmex Kobe, Japan). Point 'A' marks the beginning of the recording by the instrument, which occurs 8 s after the addition of CaCl2. Point 'B' indicates the initiation of coagulation, namely fibrin formation. The clot waveform is separated into the pre-coagulation phase (A–B), the coagulation phase (B–D) and the post-coagulation phase (D–E). (B) Clot waveform of normal plasma by monitoring of absorbance (ACL-Top). The upper blue colored trace shows the changes in absorbance observed during the performance of APTT with normal reference plasma by ACL-Top (Instrumentation Laboratory). The red colored curve is the first derivative of the absorbance corresponding to the coagulation velocity. The light blue colored biphasic curve is the second derivative of the absorbance corresponding to the coagulation acceleration.

mal individuals or commercial normal plasma is available as reference plasma. Colorless APTT reagents, without opacity, are recommended to detect sensitive changes in transmittance or absorbance, and for subsequent precise measurements of the various parameters. Although any APTT reagent that fulfills this criteria should be usable for CWA, among the reagents tested so far (Table S1), Thrombocheck APTT-SLA/0.02M $CaCl_2$ is suitable for MDA-II and CS series and HemosIL SynthASil for ACL-Top series. Other instrument and reagent combinations should also be possible to use but these need to be tested. APTT reagents used for the detection of anti-phospholipid antibodies are not recommended for the CWA because their sensitivity for assessing low clotting function is not sufficiently high. The APTT for CWA is performed in a similar manner to that for the standard APTT assay.

CWA parameters

The first derivative of the transmittance reflects coagulation velocity, and the second derivative reflects coagulation acceleration. Clotting time (CT), maximum coagulation velocity (Min1), maximum coagulation acceleration (Min2) and maximum coagulation deceleration (Max2) are common basic parameters. Among these measurements, Min2 has been reported to be correlated with clotting function in hemophilia [2].

Clinical applications of CWA

Clotting function of various bleeding disorders

Initial evaluation of clotting function by CWA is undertaken by qualitative assessment of the CW pattern. In particular, two characteristic CW patterns are observed in various coagulation abnormalities compared with normal reference plasma (Fig. S2). In normal plasma, the pre-coagulation phase is short and the slope, reflecting the coagulation phase, is steep. In factor (F) XII, X, IX, VIII, V and II deficiencies, the pre-coagulation phase is prolonged but the changes in slope are different [1]. Changes in slope are more evident in FVIII and FIX deficiencies than in other deficiencies. Thus, qualitative analysis of CW may have diagnostic value in various clinical settings of impaired clotting function.

Evaluation of clotting function in hemophilia A and B

While assays of FVIII:C and FIX:C are most important for the clinical management of hemostasis in patients with hemophilia, CWA provides a potentially widely available platform for assessment of global hemostasis in these patients [3] (Figs S3 and S4). This assay could then also provide a novel method not only for diagnosis and correlations with the bleeding phenotype but also for monitoring of hemostasis in cases of replacement therapy for serious hemorrhage or surgery.

Furthermore, the aPTT CWA is also useful for assessing very low levels of FVIII or FIX, for example less than 1 IU dL^{-1}. Studies in a number of patients with severe HA diagnosed by conventional clotting assays, demonstrated that CWA patterns differed from patient to patient. The APTT clotting time was prolonged in all patients with severe HA, but there was variation in the slope [2]. Using mixtures of severe HA plasma and exogenous FVIII ranging from zero to 1.0 IU dL^{-1}, the slope and the APTT clot time and the min2 appeared to change in a dose-dependent manner. Similarly, in further studies

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of 36 patients with severe HA, significant correlations between min2 and very low levels of FVIII:C were confirmed [4]. These results indicated that in some patients, the presence of trace amounts of FVIII mediated higher coagulation acceleration, characterized by the steeper slope, although it was possible that factors other than FVIII:C alone may have influenced clotting kinetics reflected in the waveform profile. Nevertheless, the data suggested that CWA could discriminate between different levels of FVIII:C in this critical category of severe HA, defined as having < 1.0 IU dL^{-1} FVIII:C by conventional assays (Figs S3 and S4). The evidence suggests that CWA can provide more specific data on global hemostasis in such patients, which could correlate better with the clinical phenotype.

Correlation between clinical severity and CWA parameters

Some HA patients, classified as severe on the basis of standard coagulation assays, exhibit milder clinical symptoms. It appeared possible, therefore, that CWA might provide valuable data for evaluating *in vivo* clotting function in various types of hemophilia A. To investigate this possibility, severe hemophilia A patients based on < 1 IU dL^{-1} of FVIII:C were divided into clinically severe and non- severe groups [4]. Clinically severe patients were characterized by the presence of spontaneous bleeding episodes at the age of < 1 year, the onset of joint or muscular bleeding before the age of 3 years old, or the presence of severe bleeding such as intracranial bleeding or refractory oral bleeding. The differences between the severe and the non-severe phenotype were significant for four CW parameters: clot time, maximal coagulation velocity (Min1), maximal coagulation acceleration (Min2) and maximal coagulation deceleration (Max2), These results strongly suggested, therefore, that CW parameters reflect clinical severity (Fig. S5).

Monitoring hemostatic therapy in the patients with inhibitors

The hemostatic benefits of various agents used for bypassing therapy, including activated prothrombin complex concentrates (APCC) and recombinant factor VIIa (rFVIIa), can be monitored by CWA [4,5]. In addition, CWA was also utilized effectively in a recent clinical phase 1 study for the assessment of a new bypassing agent based on mixtures of plasma-derived FVIIa and X [6]. In two hemophilia A patients with high responding inhibitors, CWA demonstrated improved hemostasis. Moreover, CWA was shown to reflect the prophylactic effect of regular infusions of FVIII during immune tolerance induction therapy (ITI) [7]. The findings confirmed that CWA is very sensitive to low levels of clotting factors, and suggested that the technique could also be useful for monitoring therapy using FVIII or FIX concentrates in patients with inhibitor.

Clotting function of acquired hemophilia

FVIII:C levels do not reflect clinical severity in many cases of acquired hemophilia A, and it may be difficult to determine clotting function precisely in these patients. CWA illustrates severely impaired patterns in these cases, however, characterized by a remarkably prolonged pre-coagulation phase and low values for maximum coagulation velocity and acceleration [8]. Assessment of clotting function by aPTT CWA, in addition to the measurement of FVIII activity, can be useful, therefore, to confirm decisions on hemostatic treatment and the monitoring of bypass therapy in these complicated clinical circumstances.

Advantages and limitations

There are several advantages to the use of CWA. The method has broad utility as a simple global test of hemostasis and is capable of providing sensitive, quantitative parameters as well as qualitative waveform patterns. Furthermore, CWA can be usefully applied in various difficult clinical settings. Not all current coagulation analyzers can be used for CWA, however, although the number of appropriate analyzers is increasing. Finally, the CWA is based on APTT-based coagulation mechanisms using an 'intrinsic' trigger. A modified CWA using trace amounts of tissue factor may extend the application of this technique.

Among the global hemostasis tests, CWA is perhaps the simplest to establish and standardize. It therefore needs to be tested more widely using standardized methods in different clinical situations to decide its place in the assessment of hemostasis and its disorders.

Addendum

M. Shima chaired the working party, performed the research, analyzed the data and wrote the manuscript. J. Thachil and S.C. Nair performed research and collected data. A. Srivastava supervised the study.

Disclosure of Conflict of Interests

M. Shima is supported for APTT reagents from Sysmex.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Data sheet of the transmittance and presentation by waveform.

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Figure S2. APTT clot waveforms of various clotting factor deficiencies.

Figure S3. Dose-dependent waveform changes in plasma containing various concentrations of FVIII.

Figure S4. Waveform changes in hemophilia A with various levels of FVIII.

Figure S5. CWA parameters and clinical severity of severe hemophilia A.

Table S1. Coagulation analyzers and APTT reagents for clot waveform analysis.

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Coagulation potential of immobilised factor VIII in flow-dependent fibrin generation on platelet surfaces

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Summary

Coagulation factor VIII (FVIII) plays an essential role in haemostasis. To date, physiologic activity of FVIII circulating in the bloodstream (S-FVIII) is evaluated by classic coagulation assays. However, the functional relevance of FVIII (-von Willebrand factor complex) immobilised on thrombogenic surfaces (I-FVIII) remains unclear. We used an *in vitro* perfusion chamber system to evaluate the function of I-FVIII in the process of mural thrombus formation under whole blood flow conditions. In perfusion of either control or synthetic haemophilic blood, the intra-thrombus fibrin generation on platelet surfaces significantly increased as a function of I-FVIII, independent of S-FVIII, under high shear rate conditions. This I-FVIII effect was unvarying regardless of anti-FVIII inhibitor levels in synthetic haemophilic blood. Thus, our results illustrate coagulation potentials of immobilised clotting factors, distinct from those in the bloodstream, under physiologic flow conditions and may give a clue for novel therapeutic approaches for haemophilic patients with anti-FVIII inhibitors.

Keywords

Factor VIII, von Willebrand factor, flow, fibrin generation, haemophilia

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Introduction

Haemostatic plug formation at sites of injured vascular walls is a critical human defense response that ensures blood flow to vital organs (1). Following platelet adhesion and aggregation, blood coagulation mechanisms lead to the fibrin network formation within platelet thrombi to stabilise the haemostatic plug (1-3). Coagulation factor VIII (FVIII) plays a pivotal role as a cofactor in factor X activation by activated factor IX, thus drastically amplifying thrombin generation in the coagulation process (4-6). Indeed, patients with congenital deficiency in this factor, known as haemophiliacs, exhibit serious bleedings throughout their life (5, 6).

To date, physiologic activity of FVIII is evaluated mostly by plasma coagulation assays that determine the capability of fibrin clot formation in closed stirring systems *in vitro*. However, the experimental conditions of such soluble-phase assays differ considerably from the *in vivo* haemostatic conditions, in which solid-phase blood coagulation occurs on platelet surfaces under whole blood flow (3). In this regard, we have focused on FVIII-von Willebrand factor (VWF) complex immobilised to thrombogenic surfaces as a solid-phase source of FVIII (immobilised FVIII; I-FVIII). We were able to discriminate between I-FVIII and those circulating in the bloodstream (soluble FVIII; S-FVIII).

Using a perfusion chamber system, we show that I-FVIII, independent of S-FVIII, plays a role in the intra-thrombus fibrin-network formation in mural thrombus generation under high shear rate conditions. In the absence of S-FVIII, I-FVIII normalised in a concentration-dependent manner the reduced fibrin deposition in synthetic haemophilic blood regardless of the circulating anti-FVIII inhibitor titre. Our results may imply the alternative therapeutic potentials of targeting I-FVIII for patients with haemophilia and high titre anti-FVIII inhibitors.

Materials and methods

Blood collection

This work was approved by the institutional review board of Nara Medical University. Blood was collected from five non-smoking healthy volunteers, who had not taken any medications in the previous two weeks. Two different ways of blood collection (anticoagulation by 1/10th volume of 3.8% sodium citrate or 20 μM of argatroban; Tanabe-Mitsubishi Co., Tokyo, Japan) were employed for evaluation of intra-thrombus fibrin generation under flow. For the citrated blood, 50 μg/ml of corn trypsin inhibitor (CTI; Haematologic Technologies Inc, Essex Junction, VT, USA) was added

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into the blood sample to minimise the contact activation of blood, then 8 mM of $CaCl_2$ was added to initiate blood coagulation just prior to perfusion. With regards to the argatroban-treated blood, this relatively low argatroban concentration was determined to allow gradual thrombin generation without the flow path occlusion by gross clot formation during blood perfusion, which makes it suitable for the evaluation of fibrin generation, as well as platelet adhesion and aggregation, when the time lag from blood drawing to the perfusion start was strictly adjusted among experiments, as described (7, 8).

Preparation of VWF-coated glass surfaces containing varying concentration of FVIII

Human native VWF (FVIII-VWF complex) was purified from cryoprecipitate as previously described (9). FVIII-free VWF was obtained by the rechromatography of purified FVIII-VWF complex with Separose-CL6B in the presence of 0.35 M $CaCl_2$ as described (10), and the complete depletion of FVIII was confirmed by ELISA assay for FVIII as previously described (11). Glass plates which had been coated with purified FVIII-free VWF as described (8, 12) were reacted with recombinant FVIII (Kogenate FS provided by Bayer Pharmaceutical Co., Osaka, Japan) at varying concentrations (0 as a negative control, 0.1, 0.5, 1, 2.5, 5, and 10 units (IU)/mL) for 2 hours (h) at room temperature. After non-adherent proteins were washed out, the amount of FVIII immobilised to the glass-bound VWF was quantified by ELISA-based assay. Briefly, a rubber ring (diameter: 8 mm) was placed on a VWF-coated glass on which various amounts of FVIII was immobilised. A peroxidase-conjugated anti-FVIII human polyclonal antibody previously described (11) was then reacted to a glass surface inside the rubber ring, followed by the routine ELISA assay procedures. The final reactant with enzyme activity inside the ring was collected, transferred to an ELISA-plate, and the enzyme intensity was determined at the wave length of 492 nm, reflecting the amount of surface-immobilised FVIII.

In vitro perfusion studies

In perfusion studies for the evaluation of platelet adhesion and aggregation, whole blood anticoagulated with argatroban was immediately incubated with the fluorescent dye DiOC6 (1 μM; Molecular Probes Inc., Eugene, OR, USA) for 10 minutes (min) at 37°C to label platelets, allowing visualisation of platelet-surface interactions by confocal laser scanning microscopy (CLSM, FV300; Olympus Co., Tokyo, Japan), as described (7, 13-15). DiOC6-labelled platelets were aspirated through the chamber by a syringe pump (Model CFV-3200, Nihon Kohden Co., Ltd., Tokyo, Japan), producing a 250 (typical low) or 1500 (typical high) shear rate at the 37°C situation, as described (7, 13-16). Fluorescent images were viewed by CLSM at 1-μm intervals from the VWF surface to a height of 60 μm from the surface, and used to calculate the percentage of the area covered by adhering platelets (surface coverage) and each thrombus volume in a defined area at the indicated time points as described (7, 15). Briefly, surface coverage of platelet thrombi was evaluated based on sliced images at 2-μm height from the VWF surface, and total volume of platelet thrombi in a defined area was calculated by summing all sliced images of identical portions using the image-analysing computer software (Image Pro Plus version 4.5; Planetron, Tokyo, Japan).

In experiments for the intra-thrombus fibrin generation, whole blood without DiOC6-labelling of platelets was perfused. Intra-thrombus fibrin generation was evaluated by image analysis of thrombi immunostained with an anti-fibrin specific antibody (7, 8). In brief, thrombi generated on a coverslip were fixed, reacted with a mixture of mouse anti-fibrin antibody (15 μg/ml; NYB-T2G1, which does not recognise fibrinogen, from Accurate Chemical., Westbury, NY, USA) and rabbit anti-fibrinogen antibody (15 μg/ml; DAKO Cytomation; Kyoto, Japan) for 90 min at 37°C, stained with a mixture of Cy3-conjugated anti-mouse IgG (5 μg/ml; Sigma-Aldrich Co., Tokyo, Japan) and FITC-conjugated anti-rabbit IgG (5.7 μg/ml; Biosource, Camarillo, CA, USA), and viewed by CLSM. The extent of intra-thrombus fibrin increase was evaluated as a "fibrin/fibrinogen" ratio of intensity of fibrin-fluorescence relative to that of fibrinogen-fluorescence. Three-dimensional (3D) images of thrombi were constructed by the image-analysing system of CLSM based on successive horizontal slices as previously described (7, 15).

Preparation of synthetic "acquired" haemophilic blood

After informed consent, plasma samples were obtained from a Japanese patient with severe haemophilia A and a high-titre inhibitor, and the anti-FVIII inhibitor IgG (human alloantibody) was puri-

Table 1: Synthetic "acquired" haemophilic blood prepared by incubating control whole blood with varying concentrations of purified anti-FVIII inhibitor IgG.

Synthetic haemophilic blood	Inhibitor titre in whole blood (BU/ml)	Plasma FVIII:C (%)	Remaining inhibitor titre in plasma (BU/ml)	aPTT (sec)	aPTT with CTI (sec)
#1	5	1.1	12.2	108.2 ± 3.2	166.2 ± 5.2
#2	10	<1.0	22.0	111.7 ± 4.3	178.4 ± 7.1
#3	20	<1.0	44.0	112.5 ± 4.5	172.4 ± 4.8
Control	0	100	0	38.5±2.3	84.2±2.5

aPTT; activated partial thromboplastin time, BU; Bethesda units, CTI; corn trypsin inhibitor (50 □g/ml).

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fied from the patient's plasma by Protein A-Sepharose chromatography as previously described (11). Synthetic "acquired" haemophilic blood were prepared by incubating control whole blood with varying concentrations of purified inhibitor IgG, at final inhibitor IgG titres of 5 (#1), 10 (#2), and 20 (#3) Bethesda U/ml (▶Table 1). The remaining FVIII clotting activities and inhibitor titres in corresponding plasma samples, measured by activated partial thromboplastin time (aPTT)-based assay, are also included in the ▶Table 1.

Statistical analysis

Statistical differences between two groups of data were evaluated by Student's t-test. In case of multiple comparisons, two-way factorial ANOVA was employed. P-values < 0.05 were considered to denote statistical significance.

Results

Preparation of VWF-coated glass surfaces containing varying concentrations of FVIII

Various concentrations of recombinant FVIII were incubated with a glass plate which had been coated with FVIII-free VWF. After non-adherent proteins were extensively washed out, amounts of FVIII immobilised onto VWF-coated glass surface (I-FVIII) were determined by the ELISA-based assay. Thus, I-FVIII increased as a function of recombinant FVIII added to a VWF-coated surface, reaching plateau at the FVIII concentrations greater than 5 U/ml (▶Figure 1). As a result, various VWF-coated glass plates with different I-FVIII density (del-FVIII as a control, #A, #B, #C and #D as indicated in the ▶Figure 1) were successfully prepared.

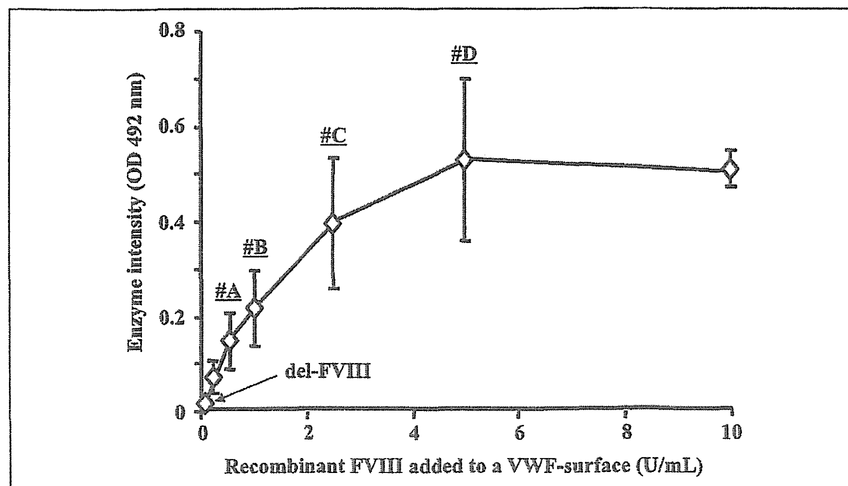

Figure 1: Preparation of VWF-coated glass surfaces containing varying concentrations of FVIII. A glass plate was coated with FVIII-free VWF and recombinant FVIII (0, 0.1, 0.5, 1, 2.5, 5, or 10 U/ml). Each data point represents mean ± standard deviation (SD) of three independent experiments. Note that I-FVIII as determined by the enzyme activity at optical density 492 nm increased as a function of recombinant FVIII added to a VWF-coated surface, reaching plateau at the FVIII concentrations greater than 5 U/ml. Thus, various VWF-coated glass plates with different I-FVIII density (del-FVIII as a control, #A, #B, #C and #D as indicated in the figure) were prepared.

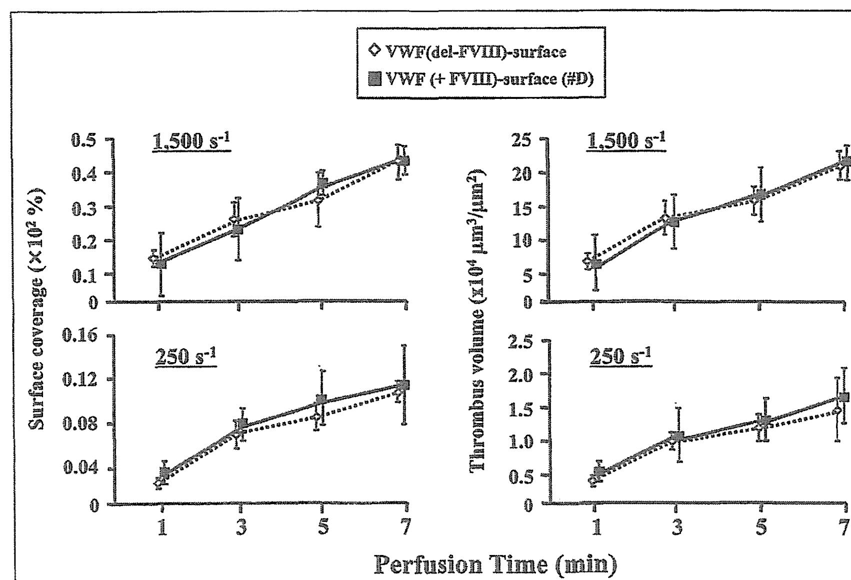

Figure 2: Time course of platelet adhesion and aggregation on a VWF-coated surface in the presence or absence of I-FVIII under high or low shear rate condition. Whole blood from healthy volunteers containing DiOC6 (1 µM)-labelled platelets, mildly anticoagulated with argatroban, was perfused over a VWF-coated glass surface with (#D) or without (del-FVIII) I-FVIII under high (1,500 s^{-1}) or low (250 s^{-1}) shear rate. The process of platelet adhesion and aggregation was evaluated by the surface coverage of thrombi generated at the time points indicated in the figure. Each data point represents mean ± SD of three independent perfusions using blood from three individual donors.

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Effects of I-FVIII on platelet adhesion and aggregation on VWF-coated surface under high or low shear rate condition

To evaluate the effects of I-FVIII on basic platelet functions under flow conditions, whole blood was perfused over a VWF-coated glass surface in the presence (#D-plate; ▶Figure 1) or absence (del-FVIII) of I-FVIII under a high (1,500 s^{-1}) or low (250 s^{-1}) shear rate condition. The process of platelet adhesion and aggregation was evaluated by the time-course of surface coverage or volume of platelet thrombi generated on a VWF-coated glass surface. No significant differences in thrombus size were confirmed in those two groups (with or without I-FVIII) under both high and low shear rate conditions (▶Figure 2). Thus, I-FVIII does not seem critically involved in the platelet adhesion and aggregation under flow conditions.

Effects of I-FVIII on fibrin generation within thrombi generated on VWF-coated surface under high or low shear rate condition

Intra-thrombus fibrin generation was evaluated under flow conditions. In contrast to the basic platelet functions, the fluorescent 3D

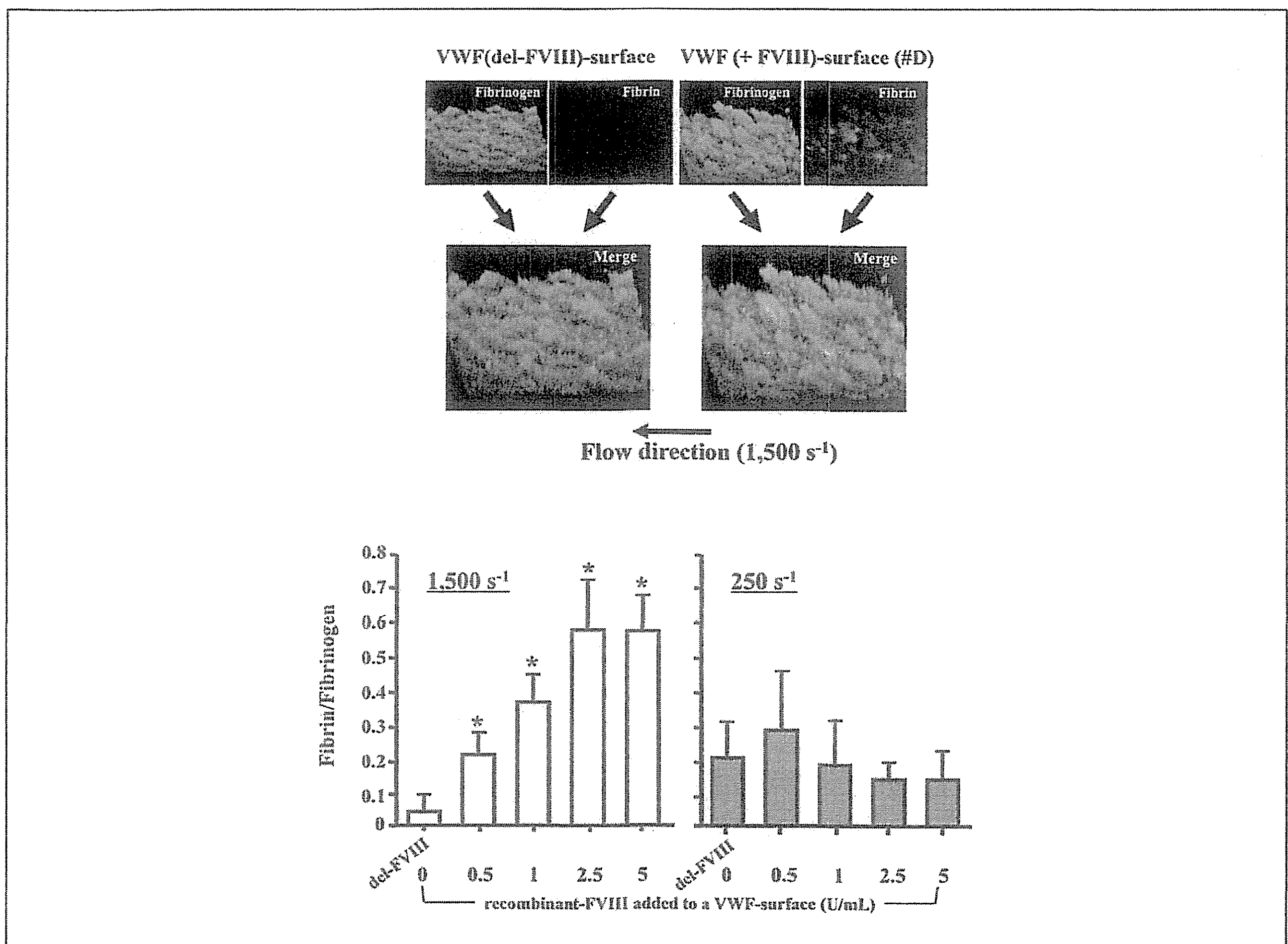

Figure 3: Functional evaluation of FVIII bound to VWF immobilised on a glass surface (I-FVIII). A) Visual evaluation of I-FVIII on fibrin generation within thrombi generated on VWF-coated surface under high shear rate condition. Citrated whole blood from healthy volunteers was perfused over a VWF-coated glass surface with or without I-FVIII under high (1,500 s^{-1}) shear rate. Just prior to perfusion, $CaCl_2$ was added to the sample blood (8 mM) to initiate blood coagulation responses. Thrombi generated on VWF-coated glass surface at 7 min-perfusion in the presence (D) or absence (del-FVIII) of I-FVIII under 1,500 s^{-1} shear were fixed, double-stained (FITC-fibrinogen: green and Cy3-fibrin: red) and viewed by CLSM. The 3D images of thrombi were representative of five independent flow experiments (original magnifications: X 600). Merged 3D images, obtained by superimposing two images of the identical portion, indicate that I-FVIII enhances the intra-thrombus fibrin deposition under high shear rate condition. B) Effects of I-FVIII on fibrin generation within thrombi generated on VWF-coated surface under high or low shear rate condition. Experimental conditions were as described in the legend to panel A. Thrombi generated on various VWF-coated glass surfaces at 7 min after perfusion were fixed, double-stained and viewed by CLSM. Bars represent mean (+ SD) fibrin/fibrinogen ratio in 25 defined areas (each 133 x 100 mm) examined (5 areas randomly selected in 5 independent perfusions of blood from 5 individual donors). Note that the intra-thrombus fibrin generation, as a function of I-FVIII, significantly (*$p < 0.01$) increased as compared to those generated in the absence of I-FVIII (del-FVIII) under high shear rate, while no effects of I-FVIII were observed under low shear rate.

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images indicate that I-FVIII enhances the intra-thrombus fibrin deposition under high shear rate condition (▶Figure 3A). Statistical analysis also confirmed that the intra-thrombus fibrin generation, as a function of I-FVIII, significantly increased as compared to those generated in the absence of I-FVIII (del-FVIII) under high shear rate, while no effects of I-FVIII were observed under low shear rate (▶Figure 3B).

Effects of I-FVIII or S-FVIII on intra-thrombus fibrin generation in perfusion of synthetic "acquired" haemophilic blood under high shear rate condition

Synthetic "acquired" haemophilic blood (see ▶Table 1) was perfused over a VWF-surface in the presence or absence of I-FVIII under high shear rate condition (1,500 s^{-1}). In some experiments to evaluate S-FVIII, recombinant FVIII was added in sample synthetic haemophilic blood 30 min prior to perfusion. As shown in the ▶Figure 4, I-FVIII significantly increased fibrin generation within synthetic haemophilic thrombi in the absence of S-FVIII. The fibrin/fibrinogen ratios of haemophilic thrombi in the presence of I-FVIII are nearly equal to that of control thrombi in the absence of I-FVIII (▶Figure 4). Note also that these I-FVIII effects are unvarying regardless of anti-FVIII inhibitor levels in synthetic haemophilic blood, while the effects of S-FVIII was totally abolished at the higher inhibitor levels.

Discussion

The blood coagulation process, essential for thrombosis and haemostasis, is a solid-phase event that occurs on cell surfaces of activated platelets or endothelium (3, 17). Under rapid blood flow *in vivo*, immobilisation of clotting factors on a thrombogenic surface could be crucial for the proper coagulation responses in such solid-phase blood coagulation. In this context, we here proposed a novel concept of "I-FVIII" (FVIII-VWF complex immobilised to a surface), and evaluated the functional relevance of I-FVIII, discriminating from S-FVIII, under experimental whole blood flow conditions.

To evaluate physiologic relevance of I-FVIII, we first compared the overall process of mural thrombus formation on FVIII-free VWF immobilised to a glass surface with that on native FVIII-

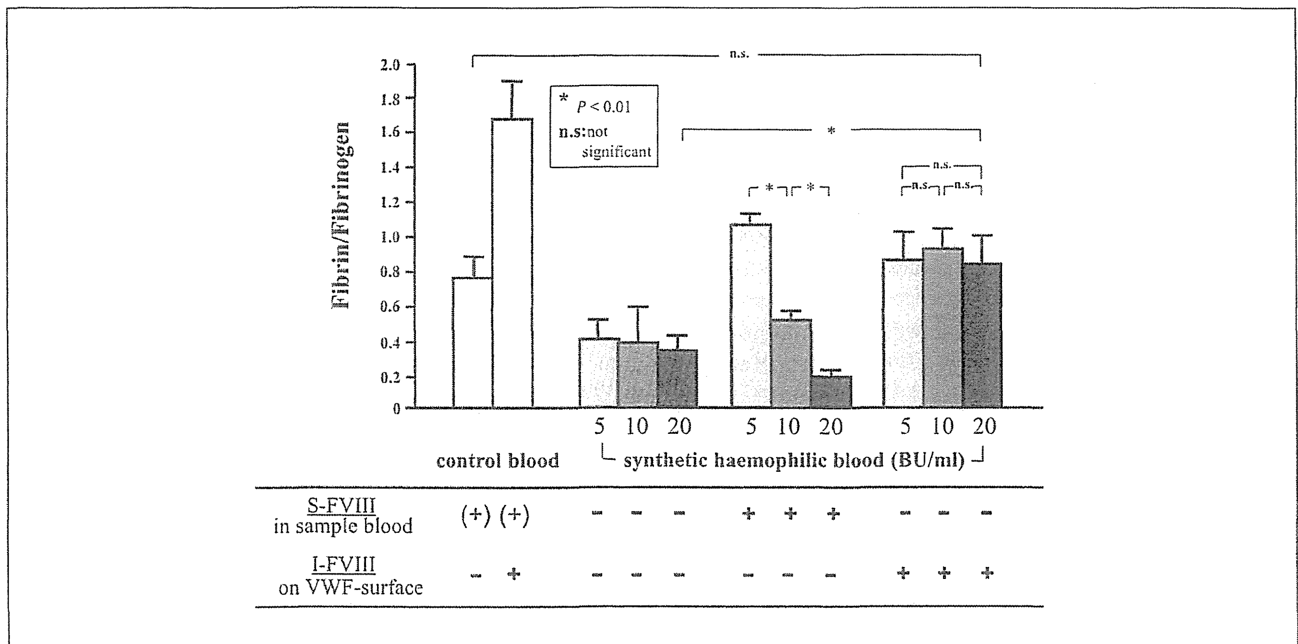

Figure 4: Effects of I-FVIII or S-FVIII on intra-thrombus fibrin generation in perfusion of synthetic "acquired" haemophilic blood under high shear rate condition. Experimental conditions are as described in the legend to Figure 3. Citrated whole blood from healthy donors rendered haemophilic by anti-FVIII human antibody was perfused over a VWF-surface in the presence (#D; indicated as "+ (plus)" in the I-FVIII column) or absence (del-FVIII; indicated as " – (minus) ") of I-FVIII under high shear rate condition (1,500 s^{-1}). Such synthetic "acquired" haemophilic blood was prepared by incubating control whole blood with varying concentrations of purified inhibitor IgG (see Table 1). In some experiments to evaluate S-FVIII, recombinant FVIII (3 U/ml) was added in sample synthetic haemophilic blood 30 min prior to perfusion (indicated as "+" in the S-FVIII column; the "+" symbol in parenthesis represents inherent FVIII present in normal blood). Bars represent mean (+ SD) fibrin/fibrinogen ratio of thrombi generated at 7-min perfusion in 15 defined areas (each 133 x 100 μm) examined (5 areas randomly selected in 3 independent sets of experiment using blood from 3 individual donors). Note that I-FVIII significantly (*$p < 0.01$) increased fibrin generation within synthetic haemophilic thrombi in the absence of S-FVIII. The fibrin/fibrinogen ratios of haemophilic thrombi in the presence of I-FVIII are nearly equal (n.s., not significant) to that of control thrombi in the absence of I-FVIII (see the right end and left end bars). Note also that these I-FVIII effects are unvarying (n.s., not significant) regardless of anti-FVIII inhibitor levels in synthetic haemophilic blood, while the effects of S-FVIII was totally abolished at the higher inhibitor levels.

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VWF complex under flow conditions. In terms of the size of platelet thrombi as well as intra-thrombus fibrin generation, no significant differences were confirmed among those two surfaces under both high and low shear rate conditions (results not shown), suggesting the limited contribution of native I-FVIII in this regard. This consequence is not surprising because only one out of over 50 VWF subunits is presumably occupied by FVIII molecule in native FVIII-VWF complex (6, 18, 19). In order to further explore the functional relevance of I-FVIII, we therefore exploited this FVIII-binding capacity of native VWF immobilised to the surface. We added an excess amount of exogenous FVIII to immobilised VWF and successfully prepared several VWF-surfaces with different I-FVIII density (▶Figure 1).

Despite sufficient levels of S-FVIII inherently present in normal control blood, I-FVIII significantly enhanced blood coagulation in a concentration-dependent manner within platelet thrombi under high shear rate conditions (▶Figure 3), albeit with no appreciable effects on basic platelet functions (▶Figure 2). In general, the increase of fibrin generation is associated with the increase of final thrombus volume under such high shear rate conditions (7). These discrepant results may be due at least in part to the different anticoagulation of sample blood; i.e. a small amount of thrombin inhibitor argatroban was used for the evaluation of platelet adhesion and aggregation, while the intra-thrombus fibrin generation was evaluated with recalcified citrated blood.

Unlike classic coagulation assays such as aPTT that evaluate fibrin clot formation in soluble phase, the intra-thrombus fibrin generation in our experimental approach reflects solid-phase blood coagulation on platelet surfaces and may be more representative of *in vivo* haemostasis (3, 17). Thus, it is assumed that local concentrations of several clotting factors must be sustained for the proper protease-substrate reactions under blood rheological situations *in vivo* (20-22). In this regard, when clotting factors are tightly immobilised on local thrombogenic sites, they may work better under blood flow conditions than those flowing in the bloodstream. Indeed, this scenario may be consistent with our observations that the effects of I-FVIII on solid-phase blood coagulation are very profound under high shear rate conditions (▶Figure 3B), where blood flow is so rapid that soluble blood clotting proteins could be easily washed out from the local thrombogenic sites. In contrast, S-FVIII may be able to contribute more efficiently to flow-dependent fibrin generation in the absence of I-FVIII under low shear rate conditions where blood flow is relatively slow.

In light of recent modelling studies incorporating the coagulation cascade and platelet deposition under flow, thrombus growth is assumed to be limited by the transport of clotting factor zymogens into the interior of thrombus (20, 22). I-FVIII fixed at the central core of generating thrombi could be apparently advantageous for such coagulation responses under flow, as compared to S-FVIII which must bind first to platelet surfaces and penetrate into thrombus against blood flow. Thus, an unusually high density of I-FVIII bound to VWF on the basal layer of a thrombogenic surface can sufficiently compensate for the complete lack of S-FVIII in the bloodstream, as seen in the synthetic "acquired" haemophilic blood (▶Table 1, ▶Figure 4). Interestingly, the effects of I-FVIII on synthetic haemophilic blood, unlike S-FVIII, were unvarying regardless of the anti-FVIII inhibitor titre in the blood under high shear flow conditions (▶Figure 4). Presumably, anti-FVIII IgGs in the bloodstream cannot easily interact with and neutralise I-FVIII when blood flow is quite fast as is the case under high shear rate conditions.

Taken together, these findings may give a clue for a novel therapeutic approach against patients with haemophilia and high titer of anti-FVIII inhibitors. Since I-FVIII bound to VWF at sites of vessel injury is more resistant to inhibitor attack compared to S-FVIII, I-FVIII could effectively enhance the coagulation potentials of blood from such haemophilic patients.

What is known about this topic?

- Coagulation factor VIII (FVIII) plays a pivotal role as a cofactor in factor X activation by activated factor IX, thus drastically amplifying thrombin generation in the coagulation process.
- Physiologic activity of FVIII is so far evaluated mostly by plasma coagulation assays that determine the capability of fibrin clot formation in closed stirring systems *in vitro*.
- However, experimental conditions of such soluble-phase assays differ considerably from *in vivo* haemostatic conditions, in which solid-phase blood coagulation occurs on platelet surfaces under whole blood flow.

What does this paper add?

- We focused on FVIII-von Willebrand factor (VWF) complex immobilised to thrombogenic surfaces as a solid-phase source of FVIII (immobilised FVIII; I-FVIII), and were able to discriminate between I-FVIII and those circulating in the bloodstream (soluble FVIII; S-FVIII).
- Using a perfusion chamber system, we show that I-FVIII, independent of S-FVIII, plays a role in the intra-thrombus fibrin-network formation in mural thrombus generation under high shear rate conditions. In the absence of S-FVIII, I-FVIII normalised in a dose-dependent manner the reduced fibrin deposition in synthetic haemophilic blood regardless of the circulating anti-FVIII inhibitor titre.
- Our results may imply the alternative therapeutic potentials of targeting I-FVIII for patients with haemophilia and high titre anti-FVIII inhibitors.

Conflicts of interest
None declared.

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特 集
臨床血液学 今後の展望（2013年版）―血小板・凝固・線溶系疾患―

血友病

嶋 緑倫

Key words : Hemophilia A, Hemophilia B, Inhibitor

はじめに

定期補充療法の普及に伴い，わが国における血友病患者のQOLは確実に向上している。特に小児期の患者では非血友病者と同等の活動性を維持することが可能になりつつある。しかしながら，頻回の製剤投与には多大な精神的・身体的苦痛を与え血友病患者をささえる家族や支援者にも負担をしいている現状がある。また，抗第VIII因子あるいは抗第IX因子同種抗体（インヒビター）が発生すると血友病補充療法の止血効果は激減～消失するために，患者の止血管理に難渋することになる。したがって，現在の血友病止血治療の課題は，より長時間作用する治療とインヒビター保有例の対策である。

2012年度の血友病に関する文献を総括すると，長時間型製剤の開発と定期補充療法の有用性，医療経済的側面に関する論文が多い。さらにインヒビターの発生要因と製剤に関する問題が再提起されている。また，新たな止血治療のコンセプトや将来の「血友病を治癒する」遺伝子治療関連の研究成果が注目される。

I 長時間型作用補充療法製剤の開発と課題

長時間作用型第VIII因子（FVIII）あるいは第IX因子（FIX）製剤の臨床試験は2012年に入って急速に進んでいる[1]。2013年には各長時間作用型製剤の第3相の臨床試験の成績が明らかにされると思われる。さらに，最近，Fc蛋白融合FIX因子製剤およびFVIII製剤の第3相試験の結果がプレスリリースされた（http://www.biogenidec.com/PRESS_RELEASE_DETAILS.aspx?ID=5981&ReqId=1738359, http://www.biogenidec.com/PRESS_RELEASE_DETAILS.aspx?ID=5981&ReqId=1752097）。Fc融合FIX製剤では115名が治験を終了し，半減期は82時間（対照rFIX製剤は34時間），100単位/kg，10～14日毎の投与で年間出血回数1.38回であったことから2週間に1回の投与でも予防効果があることが発表されている。Fc融合FVIII製剤では153例が治験を終了し半減期の中央値は19時間（対照rFVIII製剤は12.4時間）で，25～65単位/kg 3～5日の投与で年間出血回数1.6回，65単位/kg 1回/週投与で年間出血回数は3.6回であった。したがって，週1回の投与はやや予防効果は劣るが，週2回の投与で十分予防効果を期待できるものと思われる。

II 定期補充療法の動向

1．定期補充療法の目標と医療経済的側面

血友病治療の基本は血漿由来高純度製剤や遺伝子組み換え型製剤の導入や早期定期補充療法が血友病性関節症の発症を抑制するエビデンス[2]の集積により，オンデマンド止血治療から「出血を予防する」定期補充療法に確実にシフトしつつある。この傾向は，20歳までの若年患者で特に顕著で，最新の全国調査[3]によると70%の血友病A患者が定期補充療法を実施している。20歳以降の成人患者の実施率は40%と低いものの，ちょうど，5年前の全国調査では20%であり，この5年間で成人患者の定期補充の実施率は倍増している。血友病B患者では，血友病A患者と比較して定期補充療法の実施率は低いが，この5年間に20歳までの実施率が増加している。長時間作用型製剤の導入後，さらにこの傾向が強くなるものと予想される。長時間作用型製剤の導入により，より高いトラフレベルを長期間維持できる。例えば，通常，定期補充療法の基本的概念はトラフを>1%に維持することであるが，長時間作用型rFIX製剤では投与量によっては週1回投与でも軽症レベル（>5%）や止血レベル（>20%）を維持できる（図1）。したがって，今後，トラフレベルをどこに設定するのかが重要な課題となる。これは，血友病患者のQOLの向上と，医

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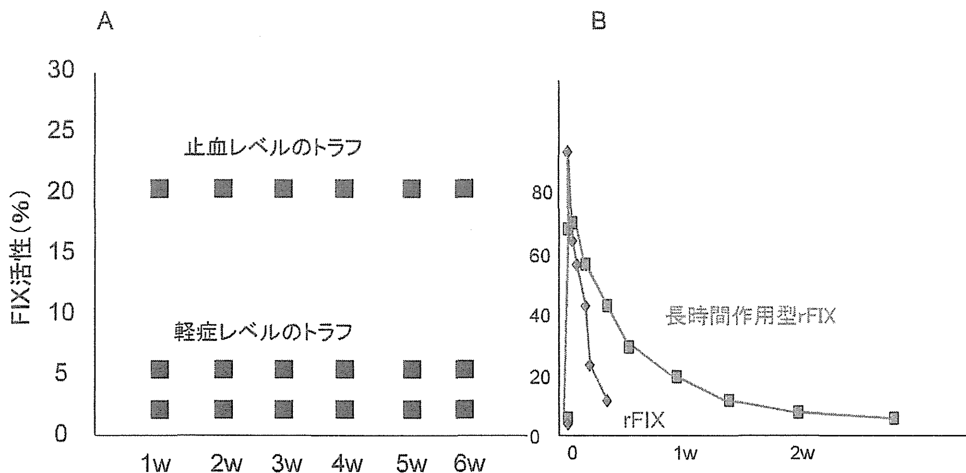

図1　長時間作用型 rFIX 製剤の PK と目標トラフ
A　定期補充療法における現在のトラフ目標は凝固因子活性を＞1%に維持することである。長時間作用型製剤の導入により，トラフを軽症レベル以上＞5%，あるいは止血レベルである＞20%を維持することも可能である。
B　長時間作用型製剤では半減期が伸びるために投与量によりトラフ値を上昇することが可能になる。

療経済のバランスというきわめてむつかしい課題に直面することになる。医療経済的資源は無限ではない。血友病診療における医療コストの 90%以上が高額な治療製剤費である。インヒビターの治療製剤はさらに高額で，わが国でも，毎年の高額レセプトの上位を血友病患者が占めている。 国際的にみると，定期補充療法が実施されている国は欧米諸国を中心で多くの国はいまだにオンデマンド療法が中心である。それぞれの国の財政に応じた治療選択が実施されているのが現状である。

2.　定期補充療法個別化の必要性

血友病医療においても限られた医療資源の中で“cost effectiveness”を考慮することは重要である。定期補充療法の投与量の決定においても，画一的な投与量の設定より，患者毎に様々な因子を総合評価して投与レジメの個別化をはかることはきわめて重要であり，今後，長時間作用型製剤が導入される場合，ますます必要となろう。実際，体重あたり同じ投与量でも，出血予防効果は患者により異なる。また，定期補充療法の基本はトラフを＞1%に維持して，重症を中等症にすることであるが，＞1%では予防効果が不十分な例もあるし，＜1%でも十分な予防効果が得られる場合もある。したがって，トラフや投与量のみではなく，より凝固機能，年齢，活動性，標的関節の有無などを総合的に考慮すべきである[4]。

3.　成人患者の定期補充療法

成人患者の定期補充は出血回数はオンデマンド治療の患者と比較して激減するものの関節症の進行には無効であるというのが定説であり，成人患者の定期補充の有効性に関するエビデンスは少ない。しかしながら，近年，成人患者における定期補充療法の有用性に関する報告が散見されるようになった[5~7]。わが国の全国調査においても定期補充療法を実施している成人の患者が増加している。Noone らは 18～35 歳の重症血友病患者計 124 例を対象に比較検討を行った。対象患者の内訳はいつもオンデマンド群（26 例），生涯の＜50%定期補充群（26 例），生涯の＞50%定期補充群（35 例），いつも定期補充群（15 例）で，出血回数，可動性，重篤な出血，出血に基づく疼痛，就労状況などについて各群別に評価を行った。長期間の定期補充療法群では，概ね，標的関節が少ない（$p<0.001$），重篤な出血が少ない（$p<0.05$），反復出血が少ない（$p<0.01$），外科手術が必要ではない（$p<0.05$）という結果であった。さらに，移動性，日常の活動性，疼痛や不安感などを評価する健康に関する有用性においてはオンデマンド群が明らかに低かった（$p<0.01$）。したがって，成人患者においても定期補充療法は，出血回数を減少させるのみならず，日常生活の QOL も向上することが示唆される[8]。

はたして，成人の重症血友病患者全員に定期補充療法をすすめるべきかについては議論の余地がある。患者の活動性や医療経済などについて考慮する必要がある。また，定期補充を継続することが困難な場合も多い。その理由の上位は，出血回数の減少，症状が消失すること，忘れること，時間が少ない等である[9]。Fischer らは定

期補充を実施していた患者が成人期に達したとき，関節の状況が良好でほとんど出血がみられていない場合は投与量や投与間隔を減少させて中止することもひとつの選択であることを勧めている[10]。ただし，長時間作用型製剤により自己注射の遵守率が高くなる可能性もあり，定期補充の継続を希望する患者が増加することも十分考えられる。

III インヒビター陽性例の動向

インヒビターの発生は血友病の止血治療における重大な問題であり，課題でもある。インヒビターの発生率は重症血友病A患者で20～30%，重症血友病Bで3～5%と血友病Aの方が多い。インヒビター陽性例の治療目標は，インヒビターの発生を防ぐこと，インヒビターの消失をはかること，インヒビター陽性例にも有効な止血治療を確立することである。

1. インヒビター発生要因と予防

インヒビターの発生を防ぐためには，インヒビターの発生要因を明らかにしてインヒビターの発生リスクを評価することがまず必要である。インヒビターの発生リスクは大きく遺伝的因子と非遺伝的因子に分けられる。遺伝的因子としては，第VIII因子あるいは第IX因子の遺伝子異常が重要である。特に，欠失，イントロン22由来逆位，ノンセンス点変異などのいわゆるnull遺伝子異常におけるインヒビター発生率は高くハイリスク遺伝子異常と考えられている。しかしながら，血友病の兄弟を対象とした調査研究によると，必ずしも遺伝子異常とインヒビター発生が一致しない兄弟例もあり，その他の遺伝的なリスクの存在も示唆される。Astermarkらは，IL-10，TNFα，CTLA-4などの遺伝子がインヒビターの発生に関連することを報告している[11]。

早期の定期補充開始によるインヒビターの発生予防効果については，最近の話題のひとつである。この概念は定期補充群がオンデマンド群よりインヒビターの発生率が低かったことがCanal研究により発表されたこと[12]，組織傷害や感染等の炎症のようないわゆるdanger signalを基盤としたインヒビター発生機序の仮説[13]に基づいている。実際，出血症状が出現する10ヶ月ごろから25単位/kg週1回で開始してインヒビターの発生リスクの高い50投与日数までFVIII刺激やdanger signalを防ぎ，以後投与回数を増やして関節出血を防ぐプロトコールでインヒビターの発生率が従来の1次的補充療法群より低下したことが報告された[13]。しかしながらこれは単一施設の報告で，多施設による追試が必要である。

遺伝子組み換え型FVIII製剤（rFVIII）と血漿由来FVIII製剤（pdFVIII）との間でインヒビターの発生率について差があるのかについては未だに結論は出ていない[14～17]。Mancusoらは製剤別，製剤の純度別のインヒビター発生率に関するコホート調査を実施した[18]。対象患者数は721例である。報告によるとインヒビター発生集積率は重症血友病A 565例中27%で，pdFVIII，rFVIIIではそれぞれ22%，49%であった。さらに，high responding例は22%で，製剤別では18%，44%と全インヒビターおよびhigh responding inhibitorともにrFVIII>pdFVIIIであった。また，製剤の純度別では低中間純度製剤，高純度pdFVIII製剤，rFVIII製剤でのインヒビター発生率はそれぞれ18%，34%，43%と純度によりインヒビター発生率が左右されていることが示唆された。最近発表された2000～2010年に生まれた重症血友病A患者574名を対象としたRODIN studyの調査報告によると，インヒビターの発生率は血漿由来製剤と遺伝子組み換え型製剤との間では有意な差がないこと，von Willebrand因子の含有の程度とも関連しないこと，さらに，第2世代のrFVIII製剤のインヒビター発生率が第3世代のrFVIIIより有意に高いという結果であった[19]。製剤とインヒビターの発生率についてはいまだに議論の多いところであり，結論は出ていない。より明らかにするためには前向き無作為調査が必要である。現在，高純度pdFVIII製剤とrFVIII製剤による前向き調査（SIPPET study; Survey of Inhibitors in Plasama Product Exposed Toddlers）が実施されている。わが国でも厚生労働省エイズ対策研究事業血友病の治療とその合併症の克服に関する研究班においてインヒビター発生患者の実態調査が実施された。41施設から116例のインヒビター症例が登録された。インヒビター発生郡と非発生群で有意差のある背景因子は単編量解析では血友病の重症度（p 0.0243），血友病Aインヒビターの家族歴（p 0.0001），FVIII製剤初回投与時の年齢で，ロジスティック回帰解析ではインヒビターの家族歴のみ有意であった。pdFVIII製剤のみを使用した群とrFVIII製剤のみを使用した群との間でインヒビターの発生率に差は見られなかった[20]。

2. 今までに治療歴のある患者（PTPs）におけるインヒビター発生

製剤変更後のインヒビター発生に関して，これまで十分なエビデンスは報告されていない。製剤変更後のインヒビター発生リスクを評価するためには，今までに治療歴のある患者（PTPs: Previously Treated Patients）でのインヒビター発生率に関するデータが必要である。Alfonsoらの総説[21]によるとPTPsにおけるインヒビター発生率は1995年のColvinらの報告[22]では1.5%，Darbyら（2004年）の報告[23]では15歳以上で2.0%，5～14歳で2.9%，Kempton（2006年）らの報告[24]では

2.1%，最近の Hay らの報告（2011 年）[25] では 10～49 歳で 5.3%，50～59 歳で 5.2%である。したがって，2～5%が概ね PTPs におけるインヒビター発生率といえる。興味深いことには年々，PTPs のインヒビター発生率が上昇していることである。実際，1995 年と 2011 年では 3 倍の差がある。PTPs におけるインヒビターの発生に関する認識が高まってきたこと，インヒビターの測定がより頻回になっていることが，インヒビター発生率の増加の原因と考えられるが，一方で定期補充療法の普及により製剤の投与量が増加していることや製剤の変更がより多くなってきたことも否定はできない。

Aledort らは全長型の rFVIII 製剤（FL-rFVIII）と B ドメイン除去 rFVIII（BDD-rFVIII）の投与を受けた PTPs におけるインヒビター発生についてメタ解析を行った[26]。解析対象となった調査研究は 29 で計 3,012 例の PTPs が含まれている。そのうち，インヒビターの発生例は 35 例で発生率は 1.25%であった。BDD-rFVIII に関連したインヒビター発生率は FL-rFVIII より高値であった。したがって，著者らは BDD-rFVIII の免疫原性が FL-rFVIII より高いことを述べている。しかしながら，本研究については BDD-rFVIII に変更しなかったコントロール群の欠如による統計的弱点が指摘されている[27, 28]。

いずれにしても，現時点では製剤変更とインヒビター発生との関連性に関する明らかなエビデンスはない。PTP におけるインヒビター発生率に関する基礎的データと十分なコントロールを考慮した調査研究が必要である。現在 2 つの調査研究プロジェクト（EUHASS: European Hemophilia Surveillance Scheme[29]），（The National Institutes of Health inhibitor study[30]）が実施されている。

3. 免疫寛容導入療法の動向と展望

インヒビターの消失をはかる免疫寛容療法（ITI: Immune tolerance induction）の有用性は国際的に認知され，ITI は我が国でもインヒビター陽性例の重要な治療法になっている。しかしながら，開始のタイミング，投与量，製剤の選択などはいまだに標準化されていない。ITI の有効性と製剤の投与量や種類との関連性について，2002 年から国際共同研究が開始された。低用量群の出血症状が多かったことが原因で本研究は中止となったが，最近，これまでの研究結果が発表された[31]。

1. 免疫寛容導入誘導療法国際研究の結果

本研究は 17 か国，計 70 施設が参加した前向き無作為調査でエビデンスレベルは高い。我が国も参加している。患者の基準は，重症血友病 A，インヒビター力価の過去最高値が 5～200 BU/m*l*，登録時のインヒビター力価が 10 BU/m*l* 未満，インヒビター力価が 12 か月以内に 10 BU/m*l* 未満になること，登録時の年齢が 8 歳未満である。ITI のプロトコールは，高用量群（200 単位/kg 連日投与），低用量群（50 単位/kg 3 回/週）で，計 115 症例が無作為別に両治療群に振り分けられた。終了した症例は 78 例で寛容成功例は 37 例，部分成功例 3 例であった。

1) ITI 成功因子

単変量解析によると有意な ITI 成功因子は，今までのインヒビター最高値（p 0.026）と ITI 中の最高値（p 0.002）であった。しかしながら，多変量解析では経過中のインヒビター力価最高値のみが有意であった。従来，ITI 開始時のインヒビター力価が有意な成功因子と考えられているが（IITR: International Immune Tolerance Registry[32], NAITR: North American Immune Tolerance Registry[33]），本研究ではすでに ITI 開始時のインヒビター力価が<10 BU/m*l* の患者の基準があるため，成功因子としての有意性は明らかでなかった。しかしながら，この結果は，ITI 開始時のインヒビターは <10 BU/m*l* であればその範囲内でのインヒビター力価は成功率に関係しないことを示している。

2) 各治療段階における成功までの期間（表 1）

インヒビター消失およびインヒビター消失から正常回収率までの期間では高用量（HD）群が統計学的有意にと低用量（LD）群で比較して短縮していたが，正常回収率までの全 ITI 期間において有意差はみられなかった。

3) ITI 治療期間中の出血回数（表 2）

ITI 期間中の出血回数は LD 群のほうが有意に多かった（p 0.019）。各治療段階別に評価すると，出血回数の有意差はインヒビター消失までの期間で明らかであったが，消失後，回収率が正常になるまでの期間において有意差はみられなかった。

4) 製剤と ITI 成功率

VWF 含有製剤の成功率が 91%であるのに対して，高純度 FVIII 製剤の成功率は 29%と，vWF 含有 FVIII 製剤がより有効であることを後ろ向き調査で報告されている[34]。しかしながら，ITI 成功率と製剤との関連性についてはいまだに議論の多いところである[35]。今回の国際研究では，90%が rFVIII 製剤を選択しており，この問題に関して明らかなエビデンスをもたらすことはできなかった。最近，ITI 未実施の不良リスク群（過去のインヒビター力価最高値>200 BU/m*l*, ITI 開始時のインヒビター力価>10 BU/m*l*, インヒビター診断から>5 年経過）を対象に vWF 含有製剤あるいは rFVIII 製剤 200 単位/kg 投与による ITI の前向き調査（RESIST 研究）が実施されている[36, 37]。

表1 両治療群における ITI 各段階までの期間

	中央値		P value
	低用量群	高用量群	
BU 陰性化までの期間（n＝60）	n＝29 9.2（4.9～17.0）	n＝31 4.6（2.8～13.7）	0.017
回収率正常化までの期間（n＝50）	n＝27 13.6（9.7～18.9）	n＝23 6.9（3.5～11.9）	0.001
半減期正常化まで（n＝47）	n＝25 15.6（10.8～22.0）	n＝22 10.6（5.9～20.5）	0.096

表2 各治療群と研究段階における出血率（全出血数/月）

ITI の各段階	治療群	平均	中央値	IQ Range	P value
BU 陰性化まで	低用量群	0.63	0.56	0.09～0.89	0.0001
	高用量群	0.28	0.00	0.00～0.44	
回収率正常化まで	低用量群	0.157	0.00	0.00～0.07	0.283
	高用量群	0.087	0.00	0.00～0.00	
半減期正常化まで	低用量群	0.150	0.00	0.00～0.15	0.552
	高用量群	0.033	0.00	0.00～0.00	
定期補充投与期間	低用量群	0.175	0.151	0.00～0.22	0.112
	高用量群	0.102	0.000	0.00～0.23	

2. 血友病Bインヒビターの ITI

血友病Bインヒビターの ITI については症例数が少なく，明らかな推奨プロトコールが存在しない。NAITR では成功率は 5/16 例（31%）であった。また，血友病Bインヒビターの ITI 実施においてはアレルギー症状やネフローゼ症候群の発生リスクを十分に念頭をおく必要がある[34]。特に，アレルギー歴のある患者についてはあらかじめ脱感作を検討すべきである[38]。

3. 今後の ITI の課題

国際研究の結果から投与量については最終的な成功率には差がないことが明らかになったが，インヒビター消失までの期間が HD 群では有意に短く，また，出血回数も少ないことが判明した。実際，これらの結果をわが国の ITI 治療にどのように反映するかがきわめて重要である。年齢，投与の実行性，医療経済的側面など様々な要因を考慮して決定する必要がある。幼少期の患者では比較的投与量が少なく HD の ITI も考慮してもよいかもしれない。ただ，ITI の成功率を現行の治療法でさらに向上させるには限界があり，新たな免疫的アプローチの確立が必要と思われる。

IV 新規血友病止血治療製剤の開発

理想的な血友病の止血療法治療製剤の条件として，長時間作用すること，投与が平易であること，インヒビター保有例にも有効であることなどが挙げられる。現状では，これらの条件を満たす製剤はないが，最近，まったく新たな概念の止血治療製剤が開発されている。

1. 抗 TFPI 療法

現在の凝固反応は Monroe らの提唱した細胞基盤型凝固モデルで理解されている。本モデルによると，出血がおこると，組織因子（TF: Tissue factor）が活性型第 VII 因子（FVIIa）と結合して第 X 因子（FX）が活性型第 X 因子（FXa）に変換される（*Initiation*）。FXa は微量のトロンビンを産生させるが，この段階ではフィブリンを形成するには不十分であるが血小板，FVIII，第 V 因子（FV）を活性化することにより凝固反応は増幅し（*Propagation*），結果的にトロンビンが爆発的に産生され（*Thrombin burst*）安定したフィブリンが形成される。Initiation 相の FVIIa/TF の機能は，組織因子経路インヒビター（TFPI: Tissue factor pathway inhibitor）により制

御されている。したがって，TFPI をブロックすることにより，止血効果を期待することができる。このコンセプトで，抗 TFPI 物質，アプタマー，抗体，ペプチドなどが開発されているが，最近，ヒト型抗 TFPI 抗体の第1相臨床試験が開始された。血友病 A の家兎モデルに本抗体を投与すると APTT は正常化し，出血症状を抑制，予防できることが報告された[39]。

2. FVIII 代替バイスペシフィック抗体

FVIII は FIXa による FX 活性化反応の補因子として機能していると理解されてきたが，その補因子機能の発現機序はわかっていなかった。FVIII は FXa 生成反応における Km を低下させ，Vmax を約2万倍増加させる。したがって，FVIII は凝固反応系の律速段階である FXa 生成反応のアクセルの役目を有する。FVIII が FIXa と FX との間に介在することにより両分子が作用しやすい立体的位置に維持することが FVIII 補因子作用であるとの仮説のもとに，一方の手が FIXa，もう一方が FX と結合する2重特異性のバイスペシフィック抗体が創製された（図2）本抗体はヒト型遺伝子組み換え型抗体として改変され血友病 A 患者血漿の APTT やトロンビン生成を改善すること，抗 FVIII 抗体を投与して作製されたサル後天性血友病 A モデルに投与すると出血症状が抑制することが発表された（図3）[38]。

本製剤は抗体製剤であり，皮下投与が可能である。さらに，半減期も長く，1～2週毎の投与で出血予防レベルを維持できる可能性がある。さらに，抗 TFPI 抗体と同様，本抗体の作用機序に FVIII 自体は関与しておらず，インヒビターの存在には全く影響されない。すなわちインヒビター保有患者においても非保有患者と全く同等の作用を期待することができるメリットがある。

V 血友病遺伝子治療に関する動向

遺伝子治療は将来の血友病治療として最も期待されている。基礎研究に関しては20年前から現在までに1,000編もの論文が発表されている[40]。FIX 遺伝子サイズが小さいことから血友病 B の遺伝子治療に関する研究が血友病 A より進んでいる。Adeno-associated Virus（AAV）が病原性はなく染色体に取り込まれないためにもっとも有用なベクターとして考えられてきた。当初は筋肉内注射による投与法が主体であったが，発現は一過性であり，また，ベクターに対する免疫反応のために投与8週後に FIX 遺伝子がトランスフェクトされた肝細胞は消失することが判明し，臨床応用は困難と考えられた。St Jude Children Hospital と University College London の

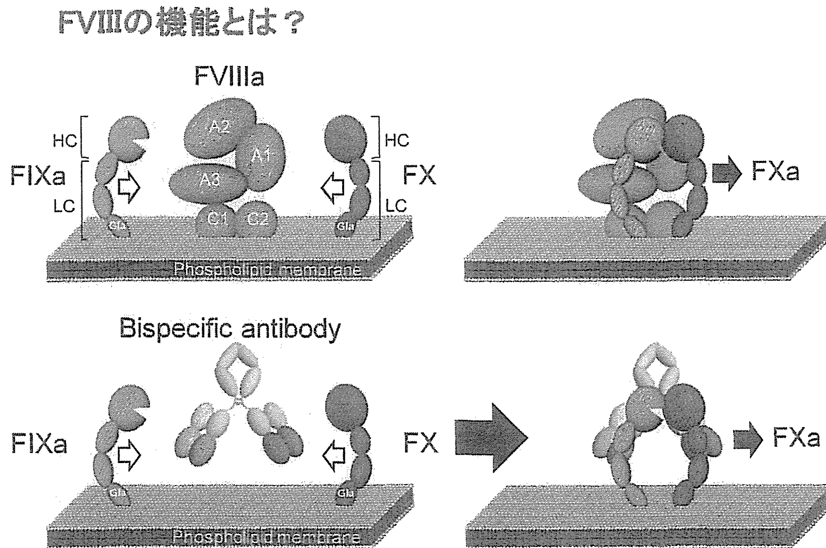

図2 バイスペシフィック抗体の FVIII 代替作用機序
活性化された第 VIII 因子（FVIIIa）は活性型第 IX 因子（FIXa）と第 X 因子（FX）の間に介在して両者がいいい位置関係になるようにサポートする。そのために，セリンプロテアーゼである FIXa が基質の FX に結合・開裂させ活性型第 X 因子（FXa）が生成される。バイスペシフィック抗体は，一方の手が FIXa，もう一方手が FX を認識するために本抗体は FVIIIa と同様に FIXa が FX に反応しやすい位置関係をもたらすことにより FVIIIa と同様の作用を発揮する。

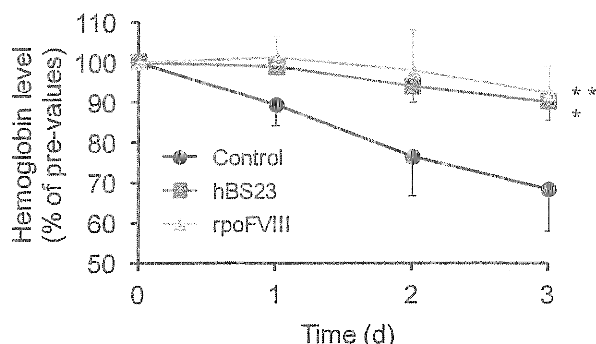

図3 バイスペシフィック抗体の出血惹起血友病Aサルモデルにおける止血効果
抗FVIII抗体を投して確立したサル血友病Aモデルに出血刺激を行うとヘモグロビンレベルは低下するが，あらかじめバイスペシフィック抗体（hBS23）を投与するとヘモグロビンの低下は抑制された。また，その効果はブタFVIII（rpoFVIII）と同等であった。

研究チームは生理的な凝固因子の産生部位である肝臓に特異的な遺伝子発現が可能となるAAV8ベクターを新たに開発した。本ベクターは，安全性が高く，マウスやサルでの実験では従来のベクターより100倍発現効果が高く，系静脈投与が可能である[41,42]。最近，6名の血友病B患者を対象に米国と英国で本ベクターによる臨床試験が実施された[43]。6名はベクターの投与量別に，低用量（LD: 2×10^{11} vg/kg），中等量（ID: 6×10^{11} vg/kg），高用量（HD: 2×10^{12}）の3群に分けられた。第一例（LD）はベクター投与後2年間にわたり2%のFIX活性を維持し定期補充療法を中止することができた。第2例（LD）は第一例と同様2%のFIX活性を維持できたが，重症の関節症のために定期補充療法の投与の継続が必要であった。第3例（ID）も2%を維持したが，定期補充の続行が必要であった。症例4（ID）は4%を15か月にわたり維持し，定期補充の必要はなくなった。症例5（HD）は5～7%まで上昇したが，肝酵素ALTの上昇とともに発現レベルは3%まで低下した。酵素レベルはステロイド投与で低下し，オンデマンドで追加投与を実施している。症例6（HD）ではFIXレベルは8～10%に増加したが，投与8週後にALTの上昇がみられFIXの発現レベルは一過性に低下した。本症例もステロイド投与でALTは低下し，FIXも5%で維持し，現在出血もなく予防投与も不要な状態で維持している。ALTの上昇がみられたのはいずれもHD群で，ベクターによる肝機能異常はベクターの投与量に関係することが示唆される。

今回の対象患者はHIV陰性，HCV RNA陰性，抗AAV8抗体陰性とかなり限定されている。今後，広範囲の患者に適応できるか，血友病Aの遺伝子治療についても応用が可能であるかなどが課題であるが，血友病Bの遺伝子治療の臨床応用は着実に近づいている[44]。

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