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Elevation of B cell-activating factor belonging to the tumour necrosis family family (BAFF) in haemophilia A patients with inhibitor

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Dear Sir,
The development of factor VIII (FVIII) neutralizing antibodies (inhibitors) is one of the serious complications in the clinical management of haemophilia A. As replacement therapy with FVIII concentrates is ineffective or markedly impaired in this setting, the management of inhibitor patients is full of difficulty. Although FVIII inhibitor will develop by both B cell- and T cell-dependent immune system (1, 2), the precise immune regulatory mechanism of inhibitors has not been fully addressed.

Recently, new ligands, BAFF (B cell-activating factor belonging to the tumour necrosis factor [TNF] family, also known as BlyS) and APRIL (a proliferation inducing ligand), have been found to regulate immune system. BAFF is a member of the TNF superfamily of ligands and is involved in the survival and maturation of B cells (3). Another member of the superfamily, APRIL, also stimulates B- and T-cell proliferation and triggers humoral immune responses (4). BAFF binds to the TNF-related receptors such as B-cell maturation antigen (BCMA), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and BAFF receptor (BAFFR), whereas APRIL binds to TACI and BCMA and to heparan-sulfate proteoglycans (5). Such BAFF/APRIL ligand-TNF related receptor interaction comprises a complex network that is critically involved in the induction and regulation of humoral immunity. We hypothesized that BAFF and/or APRIL would participate in the development and preservation of inhibitors. To test the hypothesis, we measured BAFF and APRIL levels in haemophilia A patients with and without inhibitors.

Citrated plasma samples were obtained from 25 healthy individuals and 21 haemophilia A patients without inhibitors. Sixteen samples were from eight haemophilia A patients with inhibitors at different times. Mean (± standard deviation [SD]) historical peak inhibitor titers were 420.2 ± 877.8 BU/ml (range 8.2–2586). Inhibitors had been present at least for nine months when sample plasma was taken. All haemophilia A patients with and without inhibitors were diagnosed in our laboratory as severe or moderate type haemophilia A. Since BAFF level is significantly increased in patients with chronic hepatitis C virus (HCV) (6), haemophilia A patients with HCV infection were not enrolled in this study.

Plasma BAFF and APRIL levels were measured using specific ELISA kits (R&D Systems, Minneapolis, MN, USA and Bender MedSystems, Burlingame, CA, USA, respectively). Each sample was tested in duplicate. Total plasma IgG was also measured with a standard immunoturbidimetric test. Statistical analysis was performed using unpaired t-tests between groups.

Mean (± SD) inhibitor titer in haemophilia A patients with inhibitors was 104.0 ± 191.9 BU/ml (range 1.2–742). Mean (± SD) age of healthy individuals was 16.7 ± 14.0 years (range 1–42), that of haemophilia A patients without inhibitors was 15.0 ± 6.1 years (range 4–26), and that of haemophilia A patients with inhibitors was 9.4 ± 10.6 years (range 3–44). There was no significant difference in age among these groups.

In non-haemophiliac individuals (n=19), the plasma levels of BAFF and APRIL correlated well with those of serum ($p<0.001$, $R^2=0.92$; $p<0.001$, $R^2=0.94$, respectively), confirming the validation of these assays using citrated plasma samples.

Mean plasma BAFF levels (± SD) were significantly higher in haemophilia A patients with inhibitors (896 ± 191.4 pg/ml, range 594–1,399) than in healthy controls (746 ± 220 pg/ml, range 375–1,269) ($p<0.05$) and in haemophilia A without inhibitors (751 ± 236 pg/ml, range 352–1,056) ($p<0.05$) (Fig. 1, left). There was no significance between BAFF levels in healthy controls and in haemophilia A patients without inhibitors ($p=0.938$). In APRIL levels, no significance was observed between in healthy controls (10.0 ± 7.5 ng/ml, range 0.4–24.0) and in haemophilia A patients without inhibitors (8.2 ± 6.1 ng/ml, range 0.0–17.4) ($p=0.415$), between in healthy controls and in haemophilia A patients with inhibitors (15.1 ± 15.6 ng/ml, range 0.1–51.3) ($p=0.169$), and between in haemophilia A patients without inhibitors and those with inhibitors ($p=0.101$). These results suggest that BAFF is involved in the regulation of immunological response toward preservation of inhibitor. Meanwhile, significant differences of BAFF/APRIL levels were not observed between severe type and moderate type of haemophilia A (data not shown). No evident correlation between BAFF/APRIL

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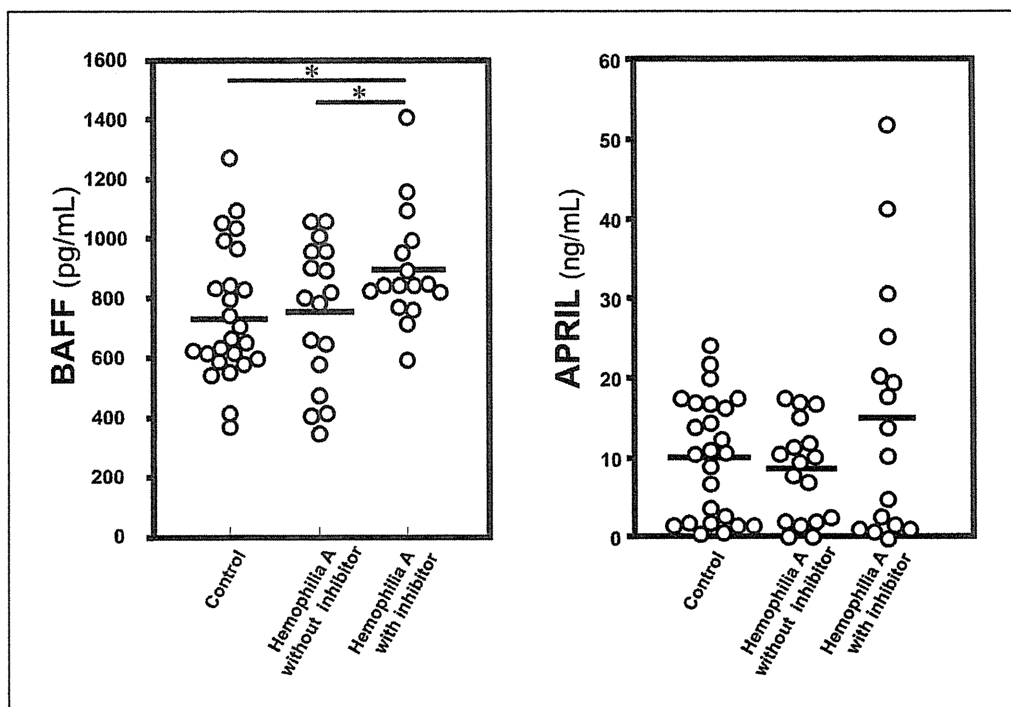

Figure 1: Plasma BAFF and APRIL levels in healthy controls, haemophilia A patients without inhibitors, and haemophilia A patients with inhibitors. Bars represent mean. Left: Plasma BAFF levels. Right: Plasma APRIL levels. *: $p<0.05$.

level and inhibitor titer, historical peak, and duration of inhibitor was confirmed (data not shown). Total IgG levels were within normal range consistent with age in all haemophilia A patients (data not shown). No correlation was observed between BAFF and IgG levels in haemophilia A patients with and without inhibitors (data not shown), suggesting that elevated BAFF levels in haemophilia A patients with inhibitors were insufficient for aberrant elevation of total IgG. Further IgG subclass analysis will be helpful to investigate the role of BAFF in inhibitor preservation as BAFF might induce IgG subclass switch.

Previous mouse studies demonstrated that constitutive BAFF overexpression results in the survival of autoreactive B cells (3, 7, 8), leading to a breakdown of peripheral tolerance. In this setting, autoimmune disorders develop in mice through aberrant activation of B cells, spontaneous production of multiple autoantibodies and polyclonal hypergammaglobulinaemia. In humans, elevated BAFF levels are correlated with hypergammaglobulinaemia and several B cell-mediated autoimmune diseases (9–11). Previous studies showed that anti-FVIII antibodies in the circulation and anti-FVIII antibody-secreting cells in the spleen and bone marrow persist for a long time even after termination of FVIII treatment (12). In haemophilia A patients with inhibitors, elevated BAFF levels would allow anti-FVIII antibody-secreting plasma cells to survive and produce inhibitors.

On the other hand, APRIL levels showed little difference among healthy controls, haemophilia A patients without inhibitors, and those with inhibitors. Although APRIL promotes IgA and IgG1 class switching in mouse $IgM^{+}IgD^{+}$ B cells (13), elevated APRIL levels in some haemophilia A patients with inhibitors remains of unknown significance. Further investigations will be required to address the role of APRIL in the haemophilia A patients with inhibitors.

Hitherto, congenital haemophilia A patients with inhibitors have received fewer B cell-targeted therapies such as rituximab therapy (14, 15) than acquired haemophilia (16). Treatment with a BAFF antagonist such as belimumab, a fully human monoclonal antibody that specifically binds to and neutralizes BAFF, was started in rheumatoid arthritis patients and demonstrated safety and efficacy (17). Targeting BAFF may represent a new therapeutic strategy in a subset of haemophilia A patients with refractory inhibitors presenting elevated BAFF levels.

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ORIGINAL ARTICLE

A modified thrombin generation test for investigating very low levels of factor VIII activity in hemophilia A

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Abstract Discrepancies between low levels of FVIII:C and clinical symptoms in severe hemophilia A are well-known. We have recently demonstrated that levels of FVIII:C < 0.2 IU/dl were consistent with clinical phenotype by clot waveform analysis, suggesting that precise measurement of very low levels of FVIII:C was clinically important. Thrombin generation tests (TGTs) triggered by tissue factor (TF) have been recently utilized to monitor coagulation function in hemophilia A. We examined whether TGT was useful for evaluating hemophilia A patients with very low levels of FVIII:C. TGTs in 40 hemophilia A plasmas with FVIII:C < 0.2–17 IU/dl (measured by clot waveform analysis using MDA-II^{TM}) were performed using TF and/or ellagic acid (ELG). The lagtime in ELG-TGT at very low levels of FVIII:C was shortened dose-dependently, whilst this parameter in TF-TGT was not significantly affected. Other parameters (endogenous thrombin potential, peak thrombin, time to peak) correlated with FVIII:C levels to some extent in both assays ($r = 0.4$–0.7). Using a TF/ELG mixture in TGT, however, the correlation coefficients increased to ~ 0.85. TGT parameters correlated well with levels of FVIII:C > 0.2 IU/dl, although the lagtime was not especially informative. We conclude that modified TGT, using a TF/ELG mixture as the trigger, is useful for monitoring coagulation function at very low levels of FVIII:C in hemophilia A.

Keywords Hemophilia A · FVIII activity · TGT

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1 Introduction

Hemophilia A results from a deficiency or defect in the plasma protein, factor (F)VIII and is the most common of the severe, inherited bleeding disorders. The clinical severity of the disorder generally correlates well with the level of FVIII activity (FVIII:C), and by convention, patients are classified into three clinical categories on the basis of circulating procoagulant activity; severe (FVIII:C < 1.0 IU/dl), moderate (1.0–5.0 IU/dl), and mild (>5.0 IU/dl). The majority of patients in the severe category (undetectable FVIII:C by conventional clotting assays) have frequent spontaneous bleeding episodes unless treated with prophylactic FVIII replacement. Coagulation function in hemophilia A is generally assessed by the measurement of FVIII:C in an activated partial thromboplastin time-assay (aPTT). Some puzzling discrepancies between FVIII:C and bleeding symptoms are well documented, however [1, 2]. In this context, we have previously reported that clot waveform analysis, based on the aPTT, provided additional information on real-time coagulation function in hemophilia A. The temporal parameters in clot waveform analysis, including coagulation velocity and acceleration, appeared to be more relevant to the clinical status than the conventional aPTT alone [3].

More recently, thrombin generation tests (TGT) have been developed to monitor coagulation function in hemophilia A [4, 5]. The TGT provides data on global coagulation by the temporal measurement of thrombin generation, and is generally accepted to be more clinically relevant than specific assays of clotting factors. The concept is based on a cell-based coagulation model [6] in which an enzyme complex of activated FVII (FVIIa) and tissue factor (TF) activates FX (and FIX) and generates of a trace amount of thrombin on cell surfaces at local sites of

endothelial injury (termed the initiation phase). This generated thrombin activates FVIII, FV, and platelets, leading to the formation of tenase and prothrombinase complexes and resulting in a thrombin burst for the conversion of soluble fibrinogen to insoluble fibrin (termed the propagation phase). In hemophilia A, therefore, the reduction in FVIII:C markedly decelerates the thrombin burst during the propagation phase, causing the hemostatic abnormality. In the TGT, the initiation phase is reflected by the lagtime (LT) and the propagation phase is reflected by the parameters peak thrombin (PeakTh), time to peak (ttPeak), and endogenous thrombin potential (ETP) [7, 8]. Thrombin generation in the TGT is usually initiated by the addition of TF/phospholipid (PL) complex. [9].

We have recently reported that although FVIII:-C < 1.0 IU/dl is generally used to define severe hemophilia A, precise low levels of FVIII:C (<0.2 IU/dl) were more consistent with this clinical phenotype, suggesting that measurements of very low levels of FVIII:C could be very important to classify clinical status [10]. We anticipated that if the TGT accurately reflects very low levels of FVIII:C, the assay could complement the diagnosis of clinical severity and assist in clinical management. In the present study, therefore, we attempted to optimize the TGT technique for the monitoring of coagulation function in hemophilia A patients with very low levels of FVIII:C.

2 Materials and methods

2.1 Reagents

The reagents, recombinant human TF (rTF; Innovin®, Dade, Marburg, Germany), and ellagic acid (ELG, Sysmex, Kobe, Japan), a thrombin-specific fluorogenic substrate (Z-Gly-Gly-Arg-AMC, Bachem, Bubendorf, Switzerland) were obtained from the indicated vendors. PL vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, and 30% phosphatidylethanolamine were prepared as previously described [11]. The thrombin calibrator was obtained from Thrombinoscope (Maastricht, Netherlands). Recombinant FVIII (rFVIII) was a generous gift from Bayer Corp. Japan (Osaka, Japan).

2.2 Plasma samples

Normal pooled plasma was prepared from ten normal healthy individuals. Blood was drawn into evacuated anticoagulant tubes [blood:3.8% (w/v) trisodium citrate, 9:1]. After centrifugation for 15 min at 1,500*g*, the plasmas were stored at −80°C, and thawed at 37°C immediately prior to the assays. Patients' plasmas were obtained from 40 hemophilia A patients. FVIII:C levels in 12 patients were <0.2 IU/dl, 6 patients were 0.2–1.0 IU/dl, and 22 patients were 1.0–17.0 IU/dl. FVIII:C levels were measured by clot waveform analysis using MDA-II™ Hemostasis System. The present studies were performed using blood samples obtained during routine follow-up of patients in the Nara Medical University Hemophilia Program. All samples were obtained with informed consent following local ethical guidelines. Standard samples containing very low levels of FVIII (ranging between 0 and 1.0 IU/dl) were prepared by the addition of known concentrations of rFVIII to congenital FVIII-deficient plasma (George King INC, Overland Park, KS, USA).

2.3 FVIII:C assays

FVIII:C was measured by a one-stage aPTT clotting assay (Thrombocheck APTT-SLA, Sysmex) on the MDA-II™ Hemostasis System (Trinity Biotech, CW, Ireland) [12]. FVIII-deficient plasma was used as the substrate (Thrombocheck FVIII, Sysmex). A standard curve was prepared using serial dilutions of Coagtrol N plasma (Sysmex) (1:10–1:5, 120) in 50 mM imidazole saline buffer (pH 7.3). Each test sample was diluted 1:10 and 1:20 in imidazole saline buffer.

2.4 Thrombin generation assay

The TGT was measured using a modification of the method reported by Hemker et al. [9]. Briefly, 20 μl trigger reagent (TF/PL or ELG) and 80 μl test plasma were mixed in 96-well round-bottom microtiter plates (Immulon2HB U bottom plate; ThermoLab System, Helsinki, Finland). The plate was placed in the fluorometer and allowed to warm to 37°C for 10 min. The dispenser of the fluorometer was flushed with 20 μl warmed 100 mM $CaCl_2$ and 5 mM fluorogenic substrate Z-Gly-Gly-Arg-AMC. At the start of the assay, 20 μl of $CaCl_2$ and fluorogenic substrate was dispensed to all wells to be measured. The development of a fluorescent signal was monitored at 8-s intervals using a Fluoroskan Ascent microplate reader (Thermo Electron Co., Waltham, MA, USA) with a 390-nm (excitation) and 460-nm (emission) filter set. Thrombin generation (in nM) was calculated from Fluorescent signals corrected by reference to the thrombin calibrator samples. Data analyses were performed using Thrombinoscope software. The parameters, LT, ETP, PeakTh, and ttPeak, were recorded.

2.5 Data analyses

Measurements were obtained in several separate assays as indicated, and the mean and standard deviation are shown. Correlations between the four parameters in the TGT and

FVIII:C were determined using Spearman's correlation test.

3 Results

3.1 TF-trigger TGT (TF-TGT) in hemophilia A plasmas

For assessing coagulation function in hemophilia A, the TGT has been generally performed using a mixture of TF/PL complex as the initiation trigger [9]. In the present study we utilized a mixture of TF/PL at concentrations of 0.5 pM and 4 μM, respectively. Figure 1a illustrates a representative thrombogram observed in normal plasma and hemophilia A plasma (FVIII:C < 0.2 IU/dl). The TGT parameters obtained from normal plasma were LT, 6.3 ± 1.1 min; ETP, 2,771 ± 1,124 nM min; PeakTh, 203 ± 53 nM; and ttPeak, 15.2 ± 2.9 min. In hemophilia A, these parameters were 6.2 ± 1.5 min, 677 ± 403 nM min, 62.0 ± 16.7 nM, and 26.8 ± 4.4 min, respectively, showing significant differences in thrombin generation but no difference in LT. We further examined the relationship between FVIII:C levels (by one-stage clotting assay) and TF-TGT in hemophilia A. FVIII:C levels in the 40 hemophilia A plasmas ranged from <0.2 to 17.0 IU/dl. The ETP (Fig. 1, panel C) and PeakTh (panel D) correlated with FVIII:C levels to some extent ($r = 0.689$ and 0.628, respectively). The LT (panel B) and ttPeak (panel E) correlated less well with FVII:C ($r = 0.220$ and 0.417, respectively).

In order to evaluate the relationship between very low levels of FVIII:C and the TF-TGT, serial dilutions of rFVIII were added to commercial FVIII-deficient plasma. Significant differences between ascending concentration pairs were determined by analysis described in Sect. 2. There were no significant differences in adjoining values at any concentrations <1.6 IU/dl (Table 1A). The lowest limit of FVIII:C detected by this method was 3.2 IU/dl, confirming the relative insensitivity of the method to reflect low levels of FVIII:C.

3.2 ELG-trigger TGT (ELG-TGT) in hemophilia A plasmas

The contact activation factor, ELG, was used as an alternative initiation trigger in TGT. ELG is known to replicate glass activation of FIXa in the intrinsic coagulation system [13]. In control experiments, thrombin generation obtained in the presence of ELG was dose-dependent, and the reaction was shown to be saturated and optimal at a concentration of 0.3 μM (data not shown). ELG was used in the present study, therefore, at a concentration of 0.3 μM. Figure 2a illustrates a representative thrombogram observed in normal plasma and hemophilia A plasma (FVIII:C < 0.2 IU/dl). TGT parameters using normal plasma were LT, 9.9 ± 1.6 min; ETP, 3,197 ± 545 nM min; PeakTh, 425 ± 48 nM; and ttPeak, 13.0 ± 2.4 min, indicating that thrombin generation in the ELG-TGT was greater than that in the TF-TGT. In contrast, the ELG-TGT failed to respond to this low level of FVIII:C.

Analyses of ELG-TGT and FVIII:C in hemophilia A demonstrated that ETP (Fig. 2, 0 C), PeakTh (panel D), and ttPeak (panel E) correlated with FVIII:C to similar or greater degree ($r = 0.645$, 0.690, and 0.669, respectively) compared to the data obtained in the TF-TGT. The LT (panel B) also correlated reasonably well with FVIII:C

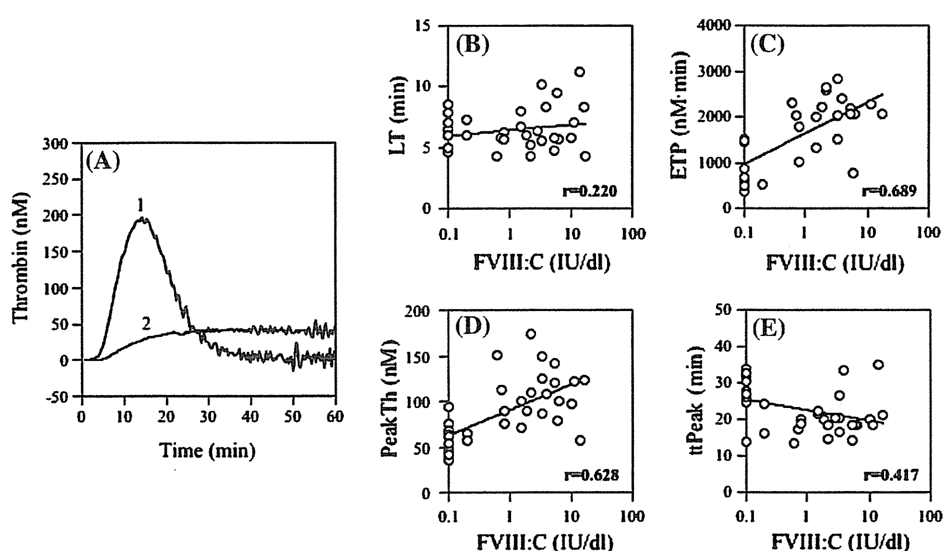

Fig. 1 TF-TGT in hemophilia A. **a** Mixtures of TF and PL (f.c. 0.5 pM and 4 μM) were added to normal plasma (*line* 1) or hemophilia A plasma (FVIII:C < 0.2 IU/dl, *line* 2) prior to evaluating thrombin generation. Representative thrombograms are illustrated in **a**. **b–e** Correlations between various FVIII:C levels and TGT parameters (LT, ETP, PeakTh, and ttPeak, respectively) obtained in 40 hemophilia A patients. The coefficient variation (CV) of all parameters obtained by this assay showed <10%

Table 1 Parameters of TF-TGT or ELG-TGT at various concentrations of rFVIII

(A) TF-TGT

TGT parameters	FVIII:C (IU/dl) 0	0.1	0.2	0.4	0.8	1.6	3.2
LT (min)	5.8 ± 1.0	5.9 ± 1.1	5.9 ± 1.1	5.9 ± 1.1	5.9 ± 1.1	5.7 ± 0.6	5.8 ± 0.6
P value		←n.d. →	←n.d. →	←n.d. →	←n.d. →	←n.d. →	←n.d. →
ETP (nM·min)	1768 ± 92	1790 ± 165	1822 ± 115	1834 ± 101	1903 ± 108	2016 ± 86	2222 ± 159
P value		←n.d. →	←n.d. →	←n.d. →	←n.d. →	←n.d. →	←n.d. →
PeakTh (nM)	81.5 ± 6.7	76.8 ± 5.2	79.6 ± 4.4	81.8 ± 6.1	85.5 ± 6.0	89.1 ± 2.8	95.8 ± 7.2
P value		←n.d. →	←n.d. →	←n.d. →	←n.d. →	←n.d. →	←p<0.05 →
ttPeak (min)	27.0 ± 2.5	27.0 ± 2.3	26.0 ± 1.7	25.6 ± 1.7	24.4 ± 1.5	23.4 ± 1.6	20.2 ± 1.8
P value		←n.d. →	←n.d. →	←n.d. →	←n.d. →	←n.d. →	←p<0.05 →

(B) ELG-TGT

TGT parameters	FVIII:C (IU/dl) 0	0.1	0.2	0.4	0.8
LT (min)	>60	24.5 ± 3.5	19.3 ± 1.6	16.1 ± 1.0	14.2 ± 1.0
P value		←p<0.01 →	←p<0.05 →	←p<0.05 →	←p<0.05 →
ETP (nM·min)	0	180 ± 28	540 ± 98	1626 ± 191	2302 ± 162
P value		←p<0.01 →	←p<0.01 →	←p<0.01 →	←p<0.01 →
PeakTh (nM)	0	22.5 ± 5.3	47.5 ± 9.3	82.2 ± 12.7	127 ± 14
P value		←p<0.01 →	←p<0.01 →	←p<0.01 →	←p<0.01 →
ttPeak (min)	>60	47.4 ± 4.8	37.9 ± 2.9	29.9 ± 2.1	25.2 ± 1.4
P value		←p<0.01 →	←p<0.01 →	←p<0.01 →	←p<0.01 →

Various concentrations of rFVIII were added to FVIII-deficient plasmas, and TGT of samples were performed as described in Sect. 2. The measurements were performed at ten separate times, and the average and standard deviation values were shown

n.d. No difference

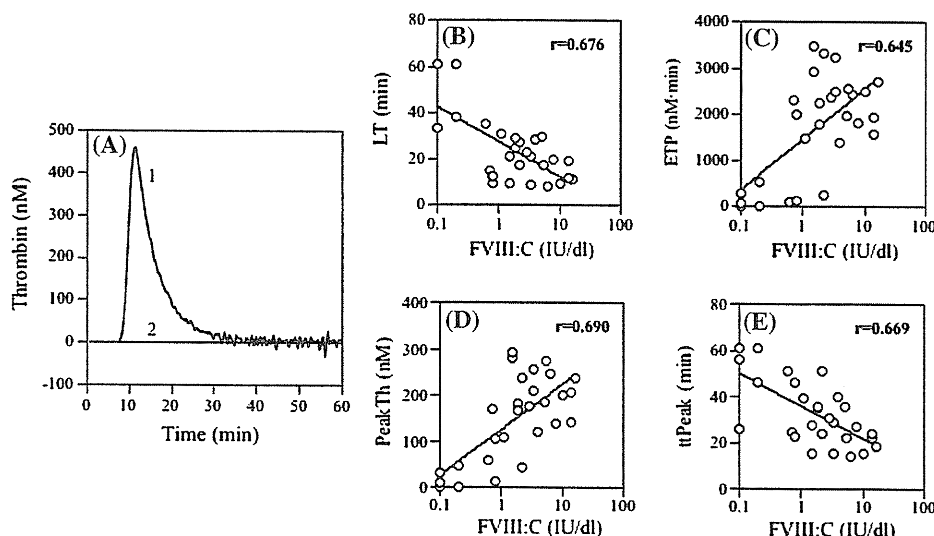

Fig. 2 ELG-TGT in hemophilia A. **a** ELG (f.c. 0.3 μM) was added to normal plasma (*line* 1) or hemophilia A plasma (FVIII:C < 0.2 IU/dl, *line* 2) prior to evaluating thrombin generation. Representative thrombograms are illustrated in **a**. **b–e** Correlations between FVIII:C levels and TGT parameters (LT, ETP, PeakTh, and ttPeak, respectively) obtained in 40 hemophilia A patients. The CV of all parameters obtained by this assay showed <10%

level ($r = 0.676$). Significant differences of adjoining values were demonstrated in all ELG-TGT parameters at low levels of FVIII:C even at concentrations <0.1 IU/dl (Table 1B). The lowest limit of sensitivity of FVIII:C by this method was <0.1 IU/dl. In addition, the LT strongly depended on FVIII:C levels indicating that this method

poorly reflected the initiation phase on the coagulation process in the cell-based model.

3.3 TF/ELG-trigger TGT (TF/ELG-TGT) in hemophilia A

The above results suggested that neither the TF-TGT nor the ELG-TGT was likely to be informative in the clinical management patients with very low levels of FVIII:C. We assessed, therefore, the TGT using a mixture of TF/PL (0.5 pM/4 μM) and ELG (0.3 μM) in order to compensate for the apparent disadvantages of the separate assays. Figure 3a illustrates a representative thrombogram observed in normal plasma and severe hemophilia A. The TGT parameters obtained in normal plasma were 4.5 ± 0.3 min (LT), 3,140 ± 480 nM min (ETP), 370 ± 33 nM (PeakTh), and 8.2 ± 0.9 min (ttPeak). In hemophilia A, the parameters were 4.6 ± 0.8 min, 1,095 ± 464 nM min, 64.0 ± 14.1 nM, and 27.3 ± 9.8 min, respectively. The results indicated that the range of generated thrombin was greater than that in TF-TGT and that the LT was unchanged and PeakTh was moderated in severe hemophilia A patient.

Analyses of the TGT parameters and FVIII:C levels in hemophilia A demonstrated that ETP (Fig. 3, panel C), PeakTh (panel D), and ttPeak (panel E) correlated with FVIII:C levels to a much greater extent ($r = 0.858$, 0.857, and 0.849, respectively) than that obtained in the separate assays. Of particular note, the LT (panel B) was poorly related to FVIII:C. On the limit for low level of FVIII:C, evaluated by TF/ELG-TGT, significant differences of the adjoining values were not observed at LT and ETP at concentration of <0.8 IU/dl (Table 2), while they were observed at PeakTh ($P < 0.01$) and ttPeak ($P < 0.05$) at >0.2 IU/dl. The lowest limit of sensitivity for FVIII:C in this assay was 0.2 IU/dl and was similar to that for clot waveform analysis reported by Matsumoto et al. [14]. Furthermore, the presence of corn tripsin inhibitor (CTI) in this assay did not show significant differences ($P > 0.05$) for all parameters, compared to its absence (data not shown).

We previously demonstrated that very low levels of FVIII:C (lowest limit 0.2 IU/dl) could be evaluated by clot waveform analysis and FVIII:C < 0.2 IU/dl was significantly consistent with clinical severe phenotype of hemophilia A [14]. Therefore, we compared with the parameters of TF/ELG-TGT and clot waveform analysis in hemophilia A patients with FVIII:C < 1.0 IU/dl. Both parameters of PeakTh and ttPeak in TGT highly ($r = 0.73$–0.83) correlated with both parameters of clot time and |min 2| in clot waveform analysis (Table 3), supporting that even TF/ELG-TGT could fully evaluate very low levels of FVIII:C and clinical severity in hemophilia A patients.

Taken together, these findings demonstrated that the TF/ELG-TGT could provide valuable information for monitoring the coagulation mechanism based on cell-based reactions in patients with very low levels of FVIII:C (>0.2 IU/dl).

4 Discussion

We have demonstrated that a modification of the TGT assay to include TF/ELG to initiate thrombin generation accurately reflects coagulation function in hemophilia A patients with very low levels of FVIII:C. Several groups [5, 15, 16] have previously reported that TGT assays using low concentrations of TF (0.5–1.0 pM) and PL (4 μM) generally correlate with FVIII:C, although the effectiveness of

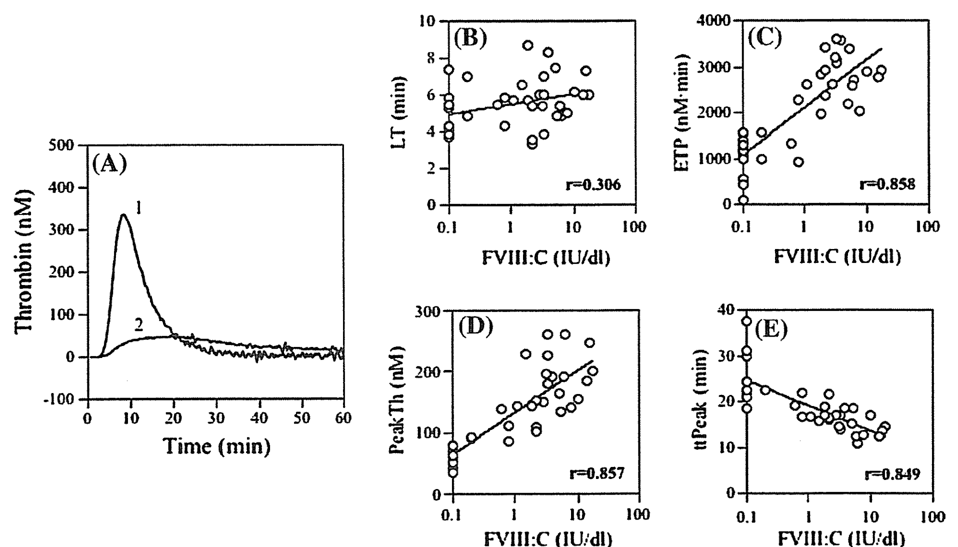

Fig. 3 TF/ELG-TGT in hemophilia A. **a** Mixtures of TF/PL and ELG (f.c. 0.5 pM/4 and 0.3 μM) were added to normal plasma (*line* 1) or hemophilia A plasma (FVIII:C < 0.2 IU/dl, *line* 2) prior to evaluating thrombin generation. Representative thrombograms are illustrated in **a**. **b–e** Correlations between FVIII:C levels in and TGT parameters (LT, ETP, PeakTh, and ttPeak, respectively) obtained in hemophilia A patients. The CV of all parameters obtained by this assay showed <10%

Table 2 Parameters of TF/ELG-TGT at various concentrations of rFVIII

TGT parameters	FVIII:C (IU/dl)				
	0	0.1	0.2	0.4	0.8
LT (min)	4.5 ± 0.1	4.6 ± 0.2	4.7 ± 0.2	4.8 ± 0.3	4.9 ± 0.3
P value	←n.d. →	←n.d. →	←n.d. →	←n.d. →	
ETP (nM·min)	2571 ± 109	2572 ± 74	2572 ± 63	2581 ± 49	2606 ± 24
P value	←n.d. →	←n.d. →	←n.d. →	←n.d. →	
PeakTh (nM)	139 ± 8	140 ± 8	142 ± 9	157 ± 7	176 ± 2
P value	←n.d. →	←n.d. →	←$p<0.01$ →	←$p<0.01$ →	
ttPeak (min)	15.2 ± 0.5	15.8 ± 0.8	15.7 ± 0.9	14.6 ± 0.6	13.8 ± 0.4
P value	←n.d. →	←n.d. →	←$p<0.05$ →	←$p<0.05$ →	

Various concentrations of rFVIII were added to FVIII-deficiency plasmas, and TGT of samples were performed as described in Sect. 2. The measurements were performed at ten separate times, and the average and standard deviation values were shown

n.d. No difference

Table 3 Correlation between the parameters of TF/ELG-TGT and clot waveform analysis in hemophilia A patients with FVIII:-C < 1.0 IU/dl

TF/ELG-TGT	Clot waveform analysis Clot time (sec)	|min 2|
LT (min)	0.050	0.053
ETP (nM min)	−0.532	0.598
PeakTh (nM)	−0.768	0.734
ttPeak (min)	0.829	−0.798

Values show the correlation coefficient between each parameter obtained in both assays

the method in the presence of very low levels of FVIII:C remains to be established. In the present study, low concentrations of TF mediated modest thrombin generation in normal plasma, and differences in PeakTh and ETP (reflecting the propagation phase) between normal and hemophilia A plasma were not markedly significant. It is generally accepted that the spontaneous hemorrhagic symptoms in severe hemophilia A are caused by relatively minor amounts of TF in joints and muscles [17] resulting in defective activation of the initiation phase by FVIIa/TF complex. In the present investigation, however, the LT, reflecting the initiation phase, was not especially sensitive to very low levels of FVIII:C (<1.0 IU/dl) and this parameter was not useful for assessing precise coagulation function in these circumstances.

Several studies have utilized the TF-TGT to evaluate replacement therapy in hemophilia A. Variable TGT parameters have been described in patients with similar levels of FVIII:C, and different ranges of ETP values have been demonstrated even in control individuals with normal levels of FVIII:C [4, 18]. Furthermore, Beltran-Miranda et al. [5] reported that although there was a reasonably good correlation between clinical severity and PeakTh in hemophilia A, overall, the TF-TGT did not offer any advantages for the evaluation of clinical severity compared to the measurement of FVIII:C by one-stage clotting assay. In addition, as discussed earlier, little information is available on the use of the TGT for assessing very low levels of FVIII:C.

McIntosh et al. [13] reported that the TGT triggered by FIXa reflected coagulation function at low levels of FVIII:C. In aPTT-based assays, soluble ELG is widely used as an activator of intrinsic coagulation. In the present study we also used ELG for the following reasons: (1) thrombin generation using ELG gave similar results to those using FIXa (data not shown), (2) other coagulation disorders as well as hemophilia A can be evaluated, and (3) it is cost-effective. We demonstrated that the ELG-TGT provided a sensitive index of coagulation function even at very low levels of FVIII:C. This assay showed the difference of lowest limit of FVIII:C (0.2 and <0.1 IU/dl, respectively) in plasmas of hemophilia A patients and samples prepared by the addition of rFVIII to FVIII-deficient plasma. This discrepancy may be explained by the difference of amounts of other blood coagulation factors except for FVIII containing in plasmas of hemophilia A patients. However, in ELG-TGT, the LT was again dependent on the presence of FVIII:C, and this parameter failed to quantify coagulation function in the cell-based coagulation model.

The aforementioned findings prompted us, therefore, to design a method based on a mixture of TF/PL and ELG. Under these conditions, the LT was little affected independently of FVIII:C, and differences in thrombin generation between normal individuals and hemophilia A was strikingly more evident than that in TF-TGT. Significantly higher correlations ($r > 0.85$) between FVIII:C and TGT parameters were observed in hemophilia A patients and the

findings indicated that the TF/ELG-TGT could be a useful technique to examine both the initiation phase and the propagation phase of cell-based coagulation function. Moreover, the sensitivity of the method to very low levels of FVIII:C was approximately 0.2 IU/dl, similar to that observed using clot waveform analysis [14]. The TGT parameters were more variable than those seen in the aPTT-based assay, however, and may have depended on the concentration of coagulation factors other than FVIII:C in the plasma samples. Nevertheless, the TF/ELG-TGT provided good data for the classification of clinical severity in patients with very low levels of FVIII:C.

The CTI is often used in TF-TGT to block the activation of contact pathway and to have little influence of parameter LT, reflecting the initiation phase [19]. Although, in particular, the addition of CTI appears to be preferred in TF-TGT using a low concentration (1 pM) of TF [20], the effects of CTI by TF-TGT at very low levels of FVIII:C have remained to be investigated. The presence of CTI in TF/ELG-TGT, however, did not significantly affect all parameters, compared to its absence (data not shown), supporting that CTI would not be required for evaluation of coagulation function on very low levels of FVIII:C in this assay, although this reason is unclear at this present. Therefore, we conclude that in TF/ELG-TGT, the initiation trigger is due to TF/FVIIa, based on little change of LT, and the presence of ELG causes more enhancing activation of intrinsic coagulation; consequently, this assay can possess the higher sensitivity for coagulation function of hemophilia A with very low levels of FVIII:C.

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血栓止血誌 20(1)：56～65, 2009

◆原　著◆

化膿性股関節炎の術後重篤な出血をきたし，インヒビターが一過性に出現した異常第Ⅷ因子（Thr1774Asn）を有する軽症血友病A（CRM⁺）の一例

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過去に股関節手術を行い，多量出血を認めた患者の左化膿性股関節炎の術前検査で，APTT が 53.8 秒と延長し，第Ⅷ因子活性は 72%，von Willebrand 因子抗原は 326% と乖離が見られた．APTT 延長を説明できる原因は他になく，骨掻爬術を施行したが，大量出血し出血性ショックとなった．遺伝子組み換え第Ⅷ因子製剤 3000 単位静注により，APTT は短縮し，止血された．1ヵ月後にインヒビターが発生したが，遺伝子組み換え活性型第Ⅶ因子製剤に変更後，インヒビターは自然消滅した．凝固能が安定した状態でも第Ⅷ因子活性は 50% 前後で同抗原の半分以下と乖離していた．遺伝子検査にて，第Ⅷ因子のエクソン 16 に cytosine が adenine に置換される塩基配列異常を認め，1774 番の threonine が asparagine に置換した異常第Ⅷ因子を有する軽症血友病 A（CRM⁺）と診断した．

Key words: mild hemophilia A, inhibitor, CRM⁺, nucleotide substitution

1．緒言

軽症血友病は外傷や手術時に止血異常で発見されることが多い．今回，我々は過去 3 回の股関節手術を経験し，いずれも出血量は多かったが通常の方法で止血した症例の，四度目の術前検査において，第Ⅷ因子活性（FVIII：C）測定値 72% にも拘らず APTT は 53.8 秒と延長を認めた．本症例は，術後出血性ショックを 2 回経験し，遺伝子組み換え第Ⅷ因子製剤（rFVIII）が著効して APTT の短縮と止血効果が得られたが，rFVIII 投与中にインヒビターが生じたため，診断および治療に苦慮した．多施設の協力の下に特殊な血液凝固学的検査と遺伝子検査を行い，本症例を軽症血友病 A，cross reacting material positive（CRM⁺）と診断するに至ったので報告する．

2．対象および方法と成績

2.1 症例

42 歳男性で，血液型は B 型 RhD+ であった．
家族歴：母が 35 歳で子宮癌手術時，輸血を要

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したが，出血量は不明であった．

既往歴：左側先天性股関節脱臼．中学時，抜歯後止血不良で縫合を要した．今まで3回の股関節手術を受け，いずれも出血量は多かったが通常の方法で止血した．(1) 34歳，左股関節臼蓋回転骨きり術を施行した際，出血量4700mlでヘモグロビン（Hb）が4.3g/dlまで低下してプレショック状態になるも，濃厚赤血球（MAP）10単位を輸血し回復した．術前の凝固検査でAPTTは35.4秒と正常であった．(2) 37歳，左股関節固定術を施行時，出血量1300mlでHb 6.8g/dlまで低下したが，自己血と回収血で対処できた．術前の凝固検査でAPTTは41.5秒と軽度延長を認めた．(3) 39歳，左股関節の抜鋲を行うが偽関節状態となった．出血量は2010mlでHbが8.1g/dlまで低下した．術前の凝固検査でAPTTは59.1秒と延長を認めた．

現病歴：悪寒，発熱と左股関節の腫脹と疼痛があり，近医にて抗生剤点滴静注を行うも軽快せず，紹介入院となった．入院後のMRI所見と関節液の穿刺培養検査によりStreptococcus sanguisが検出され左化膿性股関節炎（骨髄炎）と診断．速やかな手術の施行が必要であったが，入院時の検査でAPTTが延長していたため，精査のため内科へ紹介（第1病日）となった．

入院時身体所見：体重81kg．体温38度．結膜に貧血，黄疸なし．リンパ節腫脹なし．心音清，呼吸音清．肝脾腫なし．左股関節の腫脹と圧痛あり．

入院後検査所見（**Table1,2**）：血小板形態は正常，出血時間も正常だがAPTTは53.8秒と延長していた．好中球増加，フィブリノーゲン高値，赤沈とCRP高値などの炎症所見が見られた．ALPはアイソザイム検査で肝由来であった．術前のFVIII：C 72%（一段法）とvon Willebrand因子抗原（VWF：Ag）326%との著明な乖離がみられ，第Ⅷ因子（FVIII）の異常が疑われたが，凝固因子測定ではAPTT延長の原因は不明であった．また，ループスアンチコアグラント，フォスファチジルセリン依存性抗プロトロンビン抗体も陰性であった．

Table1 Laboratory findings on admission

WBC	13700/μl	FIX activity	123%
neut	72.0%	FVIII activity	72%
eo	0.2%	VWF activity	174%
ba	0.2%	VWF antigen	326%
ly	18.4%	FIX inhibitor	(-)
mo	9.2%	FVIII inhibitor	(-)
RBC	$427 \times 10^4/\mu l$	FXII activity	65%
Hb	14.3g/dl	FXI activity	142%
Ht	42.1%	FX activity	126%
Plt	$42.3 \times 10^4/\mu l$	FV activity	162%
ESR	78mm/h	FII activity	147%
CRP	38.5mg/dl	FXIII activity	74%
Fib	902mg/dl	FVII activity	101%
FDP	20μg/ml	TAT	7.8ng/ml
PT	11.7sec	PIC	0.9μg/dl
APTT	53.8sec	VWFmultimer	normal
TT	73%	ProteinC activity	134%
ATIII	>25mg/dl	Prekallikrein activity	103%
Bleeding time	2min	Antiplasmin activity	110%
platelet shape	normal		

Table2 Laboratory findings on admission

T-Bil	0.79 mg/dl	RF	2 U/ml
AST	38 U/l	ANA	× 80 (nucl)
ALT	93 U/l	anti-DNA antibody	4.9 IU/ml
LDH	221 U/l	anti-RNP antibody	(-)
ALP	1378 U/l	anti-mitochondrial antibody	< × 20
γ-GTP	404 U/l	PR3-ANCA	<1.3 U/ml
CK	22 U/l	MPO-ANCA	<1.3 U/ml
BUN	11.8 mg/dl	LAC (PLI)	-1.6 sec (-)
Cr	0.67 mg/dl	LAC (DRVVT)	1.2 (-)
UA	3.2 mg/dl	CL-β_2GP I	<1.3 U/ml
AMY	88 U/l	anti-prothrombin antibody	11 U/ml
TP	7.6 g/dl	sIL2R	142 U/ml
Alb	3.5 g/dl	AFP	3.0 ng/ml
Na	141 mEq/l	PIVKAII	24 mAU/ml
K	4.2 mEq/l	CEA	1.1 ng/ml
Cl	100 mEq/l	CA19-9	10.0 U/ml
Ca	9.3 mg/dl	TSH	1.5 μIU/ml
FBS	117 mg/dl	FT3	3.1 pg/ml
Urinalysis	clear	FT4	0.9 ng/dl

CL-β_2GP I : anti-CL-β_2GP I complex antibody

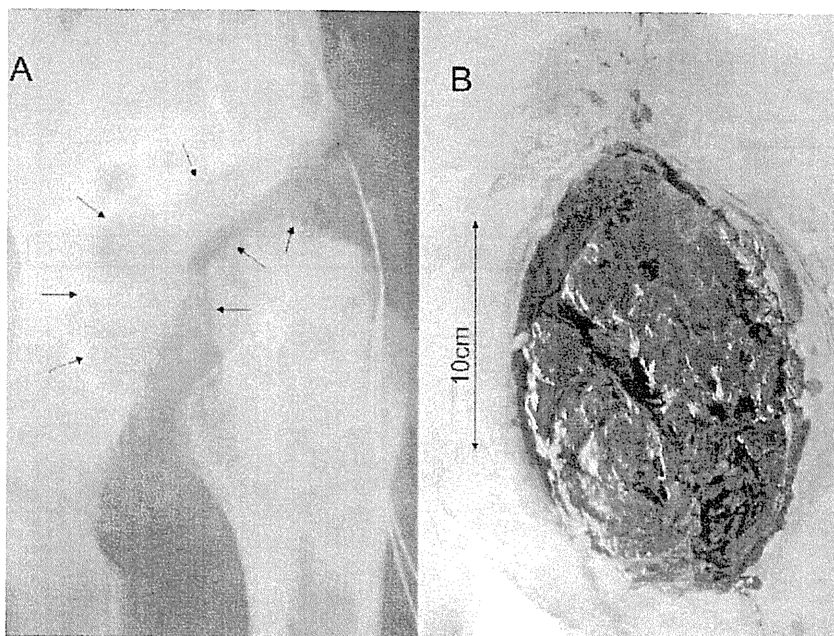


Fig.1 : A : His X-ray photo of postoperative hip joint and drain tube. By this operation, antero-inferior iliac bone, acetabulum and head of femur were shaved (→ mark).
B : Massive hematom of his left hip joint.

2.2 手術経過

患者の状況から早急に骨掻爬を行う必要性があり，第4病日，**Fig.1A**に示す如く左股関節の骨掻爬後、持続環流を行った．術前，術中，術後に新鮮凍結血漿（FFP）を各5単位補充した．出血量は2970mlでMAPを7単位輸血した．**Fig.2**にその後の経過を示す．術後2日目ドレーンより大量の出血がありHbが4.7g/dlと低下し，出血性ショック状態となった．そのため，ドレーンを抜去し，MAP10単位，FFP15単位を輸血してショック状態を脱した．しかし，第18病日，安静度を緩和した際に再出血し，左股関節に巨大な血腫を形成した．Hbも10.4g/dlから7.5g/dlと低下した．仙台医療センターに搬送し，軽症血友病Aも否定できずとの判断でrFVIII 3000単位を静注したところ，APTTは52.0秒から1時間後に32.8秒まで短縮し，止血するとともに血圧も回復した．その後，rFVIII 2000単位を一日2回静注し，輸血はせずに徐々に貧血改善，当院に帰院した．出血により創部には巨大血腫（**Fig.1B**）が形成されており，帰院後はrFVIIIを使用しながら外科的に少しずつ血腫を除去し，徐々にrFVIIIの投与量を減量した．

2.3 FVIII インヒビターの発生と経過

第40病日（rFVIII開始22日目）頃よりrFVIIIを使用中にも拘らず，FVIII：Cレベルが低下し始め，第44病日にはAPTT60.8秒，FVIII：C 16%となり，1BU/mlのインヒビターが出現した．第47病日にrFVIIIの使用を中止し，翌日からは遺伝子組換え型活性化第Ⅶ因子製剤（rFVIIa）7.2mgの静注を処置時に行い，3日間継続した後はトランサミンの点滴静注のみで対処した．インヒビターは第49病日の6BU/mlを最高に低下傾向になり，FVIII：Cも同日の3%を最低値に，上昇へ転じた．副腎皮質ステロイド剤はこの間使用しなかった．

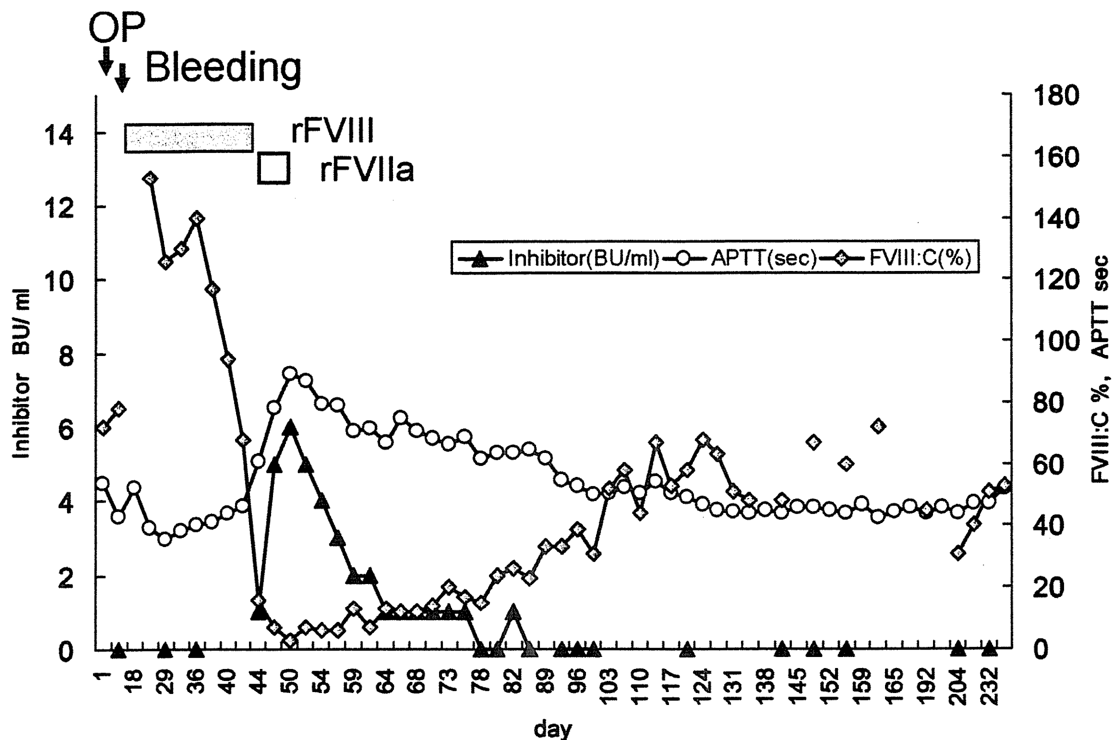


Fig.2：Clinical course of this case. Graph shows APTT (Nishitaga National Hospital), FVIII activity and FVIII inhibitor (SRL). OP：operation

Table3 Coagulative data at 3 hospitals after the appearance of inhibitor to FVIII

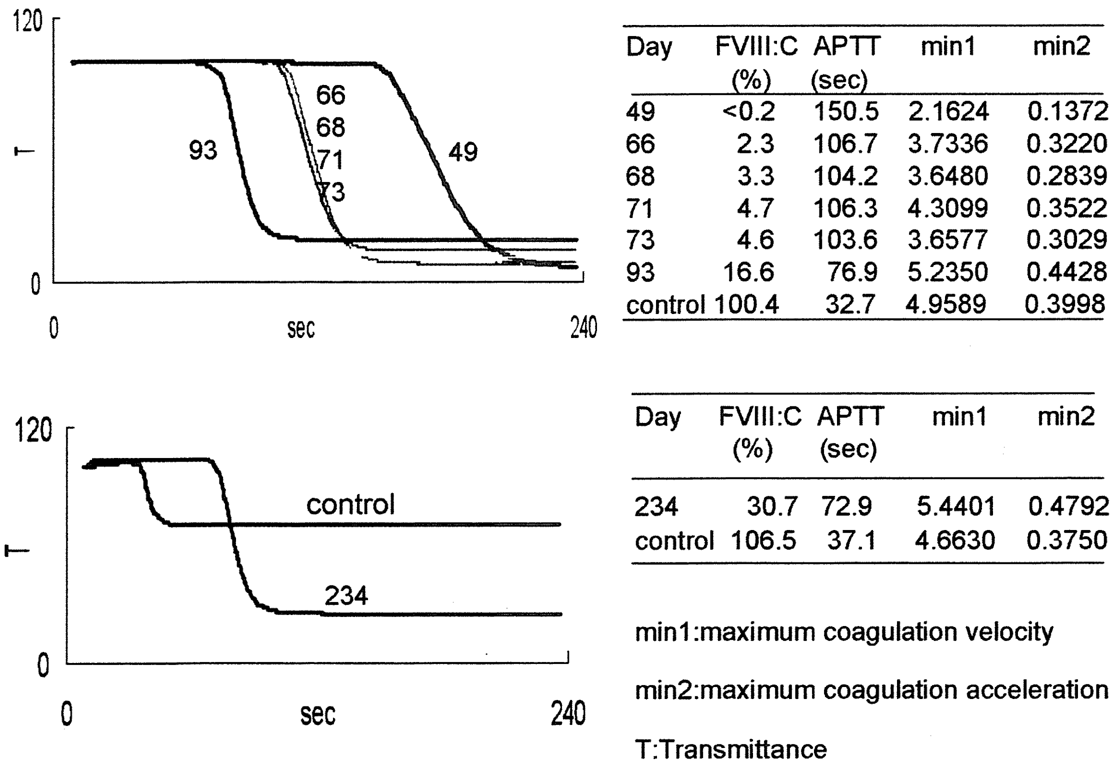
Day	Labo.	49	66	73	93	234	358	508
APTT sec	1*	89.4	74.8	66.2	54.8	47.3	43.7	43.4
	2	150.5	106.7	103.6	76.9	72.9		78.1
	3					71.2		73.8
FVIII:C %	1	3	12	20	33	51	58	42
	2	<0.2	2.3	4.6	16.6	30.7		21.3
	3					54.6		45.2
FVIII:C % Chromo.	3					72.7		42.5
FVIII:Ag %	2				24.0	68.6		78.8
Inhibitor BU/ml	1	6	1	1	(-)	(-)	(-)	(-)
	2	8.7			<0.3	<0.3		<0.3

Methods
Instruments ; reagents for APTT and FVIII:C ; FVIII deficient plasma
1* Nishitaga National Hospital CA-500 (Sysmex) ; Thrombocheck APTT (Sysmex)
1 SRL BCS (Dade Behring) ; Pathromtin SL (Dade Behring) ; GK-08 (George King Bio-Medical)
2 Nara Med. Univ. MDA- II (Trinity Biotech); Thrombocheck APTT-SLA (Sysmex); Thrombocheck FVIII (Sysmex)
3 Tokyo Med. Univ. ACL9000 (IL); Hemos IL APTT-SP(IL); GK-08 (George King Bio-Medical)
Chromogenic 2-stage method Chromogenic substrate Coatest SP FVIII (Chromogenics)
FVIII:Ag assay Sandwich ELISA method [1]
Inhibitor assay
1 SRL standard Bethesda method
2 Nara Med. Univ. standard Bethesda method

2.4 血液凝固学的検討

インヒビター発生後、奈良県立医科大学小児科にて、当院と平行して凝固検査を随時行った（**Table3**）．各施設間でデータの差はあるものの，rFVIII を中止後約 3 日でインヒビターはピークに達し、その後約一ヶ月半を経過した時点ではインヒビターは感度以下となった．さらに一ヶ月後には APTT, FVIII:C ともに安定した状態となった（**Fig.2**）．退院前（第 234 病日）の検査でインヒビターは感度以下、FVIII：C も 30.7〜72.7 % だが APTT は 47.3〜72.9 秒と入院時同様に FVIII：C に見合わない延長を示していた．さらに **Table3** に示した如く、本症例の FVIII：C は FVIII 抗原量（FVIII：Ag）（Sandwich ELISA 法[1]）の約 1/2〜1/4 であった．また凝固波形解析[2]（**Fig.3**）では、FVIII：C の回復に伴い最大凝固速度（min1）、最大凝固加速度（min2）は速やかに回復したが、FVIII：C が plateau に達した後でも、依然として凝固前相は延長していた．トロンビン生成試験[3]（**Fig.4**）でも FVIII：C の回復に伴い徐々に改善したが、同様に FVIII：C が plateau に達した後でも正常とはならず，トロンビン生成能も Peak Th が 261.9nM（正常血漿 355.6nM）と正常血漿に比し軽度低下している状態でありながら、lag time 20.0 分（コントロール 10.3 分）や tt Peak 26.5 分（コントロール 15.2 分）は著明に延長していた．

血友病 A の鑑別診断として問題になるのは，FVIII：C の半減期が著減する von Willebrand disease（VWD）type2N である．デスモプレシンを 0.4 μg/kg 静注後、内因性 FVIII：C 値を片対数グラフの対数軸にプロットし（静注前 45%, 静注 1 時間後 89%、3.5 時間後 84%、6.5 時間後 72%、24 時間後 55%）、その半減期を求めたとこ



Day	FVIII:C (%)	APTT (sec)	min1	min2
49	<0.2	150.5	2.1624	0.1372
66	2.3	106.7	3.7336	0.3220
68	3.3	104.2	3.6480	0.2839
71	4.7	106.3	4.3099	0.3522
73	4.6	103.6	3.6577	0.3029
93	16.6	76.9	5.2350	0.4428
control	100.4	32.7	4.9589	0.3998

Day	FVIII:C (%)	APTT (sec)	min1	min2
234	30.7	72.9	5.4401	0.4792
control	106.5	37.1	4.6630	0.3750

Fig.3：Clot waveform analysis. According to the recovery of FVIII activity, maximum coagulation velocity and maximum coagulation acceleration normalized, but pre-coagulation phase did not normalized.

ろ7時間から14時間の間にあり，本症例についてVWD type2Nは完全に否定された．

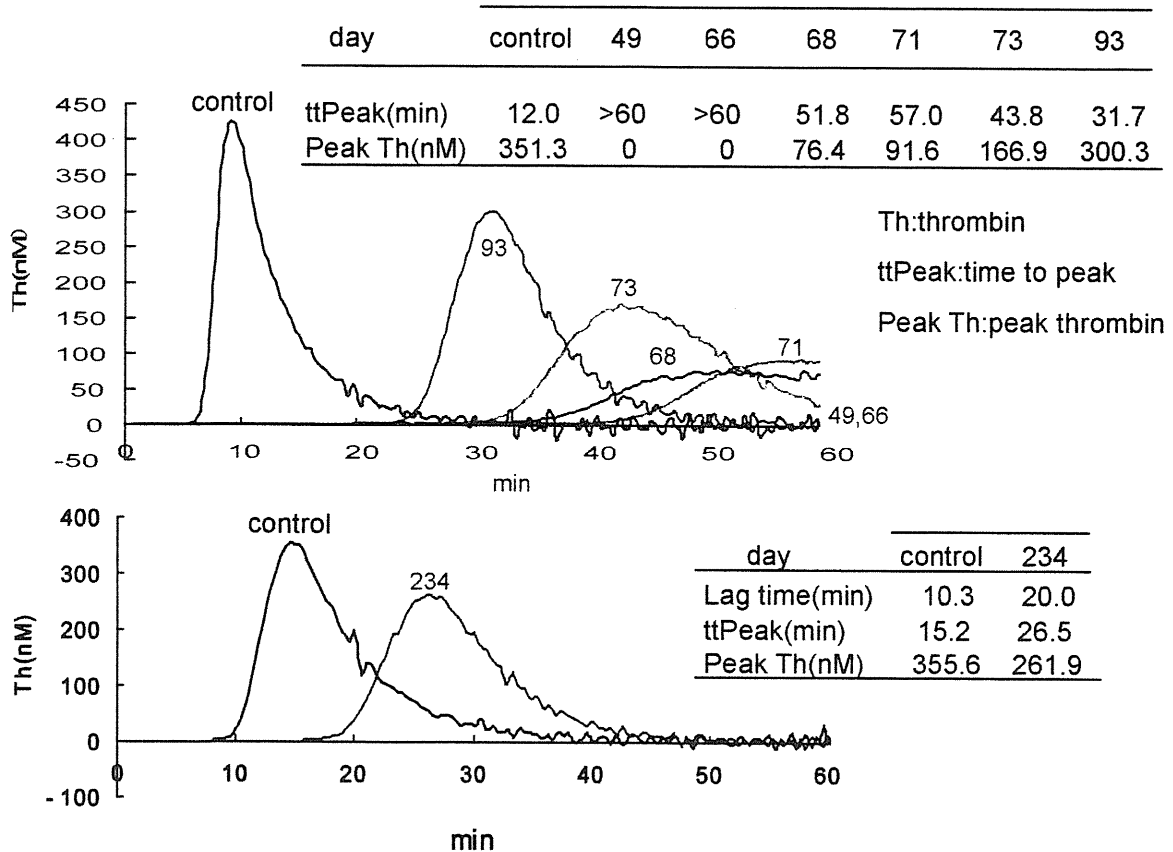
なお，母親のFVIII：Cは190%，VWF：Agは398%で，共に高値ではあるが，Zimmermanら[4]の保因者検索法に当てはめれば，両者の比は保因者の可能性を否定するものではなかった．

2.5 遺伝学的検討

本症例の診断を遺伝学的に検討するために東京医科大学臨床検査医学講座において，第Ⅷ因子遺伝子（*F8*）解析を行った．この解析は世界医師会によるヘルシンキ宣言に基づいて，東京医科大学医学倫理委員会の審査を経て，同大学学長により承認されている「先天性血液凝固線溶因子欠損（欠乏）および異常症に関する遺伝子研究」の研究計画に従い，患者には文書による説明と同意を取得したうえで行った．*F8*解析には末梢血白血球から抽出したゲノムDNAを用いた．*F8*を構成する26のエクソンとそのイントロン境界領域，およびプロモーター領域を，34対のプライマーペアー（エクソン14を8分割したため）を用いてそれぞれPCRにて増幅した．PCR増幅産物はアガロースゲル電気泳動後，ジデオキシ法によるダイレクトシークエンスを行い全塩基配列を決定した．その結果，*F8*のエクソン16内にcytosineがadenineに置換される一塩基置換を認めた．この結果，この症例ではFVIIIの1774番のthreonineがasparagineに置換（Thr1774Asn）していると考えられた（**Fig.5**）．

3．考案

FVIIIはVWFと結合して血液中を循環し，FVIIIの血中濃度はVWF濃度に依存している．VWFは血管内皮細胞から比較的容易に血中に放出されるため，運動などの日常的な負荷のほか，外傷や感染症などで高値を示すことが知られている．VWFと同時にFVIIIも増加するため，中等



day	control	49	66	68	71	73	93
ttPeak(min)	12.0	>60	>60	51.8	57.0	43.8	31.7
Peak Th(nM)	351.3	0	0	76.4	91.6	166.9	300.3

day	control	234
Lag time(min)	10.3	20.0
ttPeak(min)	15.2	26.5
Peak Th(nM)	355.6	261.9

Fig.4：Thrombin generation assay. According to the recovery of FVIII activity, thrombin generation was also gradually recovered but not normalized. Despite the recover of Peak thrombin (PaekTh), the shortening of lag time and time to peak (ttPeak) was not enough.

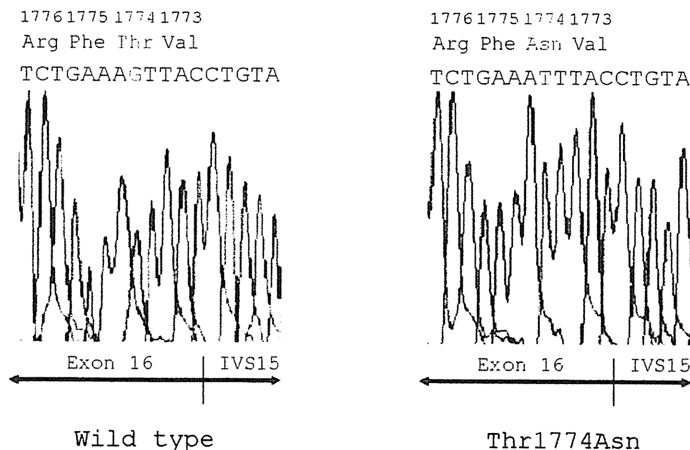


Fig.5：Nucleotide sequence analysis of exon 16 of the *F8*. Exon 16 of the *F8* was amplified by PCR. The nucleotide sequence of the antisense strand was directly sequenced by using the PCR product as template. The sequencing revealed a cytosine to adenine transversion in exon 16. The nucleotide substitution resulted in a threonine 1774 to asparagine in A3 domain.

症や軽症血友病患者においてもFVIII：Cの変動原因となり，軽症血友病を診断する際の障害となる可能性がある．一般的に血友病AのFVIII：Cは一段法で40%以下とされるが[5]，本症例では術前検査でFVIII：C測定値は72%と正常域にあったため血友病Aとは診断できなかった．しかし，同時に測定したVWF：Agは326%と高値を示し，FVIII：Cとの間に著明な乖離がみられた．VWF：Agの増加は患者への身体的ストレスによって上昇していたと考えられるが，FVIII：Cの増加が72%に止まった乖離は本症を疑う手掛かりであった．(1) 二度目の出血性ショック時に仙台医療センターで投与されたrFVIIIが劇的に奏効し，APTTの正常化に伴って止血した事は，軽症血友病Aの可能性を強く疑う根拠となった．(2) インヒビターは軽症血友病でも発生するが重症例に比し低頻度である[6]．しかし本症例はrFVIII使用中にインヒビターを発生した．更に (3) FVIII：CがFVIII：Agの1/2から1/4と明らかな活性／抗原比の低下が認められ，本症例のFVIIIが機能異常を有する異常FVIIIであることが示唆された．(4) 遺伝子解析を行った結果，*F8*のエクソン16内にcytosineがadenineに置換される塩基配列異常を認め，FVIIIの1774番のthreonineがasparagineに置換している異常FVIII（Thr1774Asn）であると考えられた．この置換は*F8*のpolymorphismとしてはSeattleSNPs[7]やViel KR *et al*[8]の文献にも登録されておらず，日本人の約70本のX染色体からも検出されなかった．この変異がこの症例のFVIIIにおいてどのような異常を引き起こしているのかは，更なる検討が必要ではあるが，稲葉ら[9]は同じ遺伝子変異を持つ血友病Aの症例を経験しており，その症例は抜歯後に止血困難をきたし診断されたFVIII：Cが52%の軽症例であった．この事からThr1774Asnは本症例においても同じく，軽症血友病Aの原因と思われた．以上の (1) から (4) の結果をもとに我々は本症例をFVIII：Cの測定結果にとらわれず異常FVIII（Thr1774Asn）による軽症血友病A,CRM+と診断した．鑑別すべき病態として，本症例はFVIIIの活性／抗原比が低いCRM+の病型であることから可能性は低いが，VWD type2Nとの鑑別診断をデスモプレシン負荷を行い否定した．

血友病Aの病型にはFVIII：Agの量から，重症血友病Aに見られる抗原が検出されないcross reacting material negative（CRM-），抗原量が減少しているcross reacting material reduced（CRM^R），本症例のように抗原量が正常に存在するCRM+に分類される．インヒビターは重症例のCRM-に頻発するが，発生率は3～13%と重症例より低いものの中等症・軽症血友病Aにも発生する．これは中等症・軽症では遺伝子変異によりFVIIIの構造変化をきたし野生型FVIIIと異なる抗原性を示すためと考えられる．実際，FVIIIの構造機能上重要なA2ドメインやC1/C2ドメインにおける点変異はインヒビターの発生リスクとされる[6]．本症例はA3ドメインの点変異であるが，同様なメカニズムでインヒビターが発生した，まれな例と思われる．このインヒビターはFVIIIを含まないrFVIIaに切り替えることにより免疫抑制療法を行うことなく速やかに低下し，やがて自然消滅したことから，自己抗体ではなく同種抗体の可能性が高いと考えられた．本症例ではインヒビターによって一過性に本症例のFVIII：Cも低下したことから，自己の内因性FVIIIにも交差反応したものと思われた．

軽症血友病Aでは，遺伝子異常の種類によっては，使用する機器や試薬，方法（一段法，合成基質二段法）によりFVIII：Cが大きく異なる例が存在する[9-11]．当院では凝固因子関連検査はSRLに依頼し，FVIII：Cは一段法で測定された．奈良医大，東京医大でも同じ検体でAPTT，FVIII：C等を測定した結果，測定値にかなりの違いが見られた（**Table3**）．この原因としてはAPTTの測定法や測定試薬が各施設で統一されておらず，特に機能異常を有する異常FVIIIの活性測定値に測定法間差が出たものと考えられる．稲葉ら[11]は軽症血友病A 16症例のFVIII：Cと*F8*を解析した結果，用いるAPTT試薬によってはFVIII：C測定値に約2倍の乖離が見られること，さらにその乖離がFVIII高次構造の変化を伴う遺伝子異常と関連性があることを報告している．本症例のFVIII：Cについて，退院後（第508病日）に再度測定しているが，奈良医大の結果ではFVIII:Cは21.3%と他施設の約1/2であっ

た．以上から本症例は初診時FVIII：C 72%と高値に測定されたが，測定試薬を変更すれば低い活性値を示した可能性も示唆された．APTTが延長し，FVIII：CとVWF：Agが乖離する例では，たとえFVIII：Cが正常範囲であっても，本症例のような軽症血友病も考慮して精査することが重要と考えられた．

凝固反応の始まりには出血部位に露出される組織因子（TF）がFVIIa/TF複合体を形成し第IX因子（FIX）や第X因子が活性化され微量のトロンビンが形成される．この微量のトロンビンがFVIII，第XI因子，第V因子や血小板を活性化させ，これにより活性化血小板上で凝固因子の活性化が起こり，FVIIIやFIXが反応増幅機転の要となり，トロンビン生成反応は爆発的に増大する[12]．この血小板依存性のThrombin burstこそ安定したフィブリン形成（止血）に必要である．一方，FVIII：Cの低下でAPTTに影響を及ぼすのは教科書的には30%以下とされており[13]，本症例のFVIII：C測定値ではAPTTの延長を説明できない．血漿混合試験でもループスアンチコアグラントは否定的で，フォスファチジルセリン依存性抗プロトロンビン抗体も陰性であった．APTT延長の理由としては，FVIII：C測定法の問題により，本来低値のFVIII：Cが偽高値を示していた可能性や第XII因子の低下などの複合的な要因の可能性もある．しかし，(1) 系時的な凝固波形解析で，凝固速度や凝固加速度は凝固状態安定後に正常化したが，凝固前相は長いままであり，(2) 系時的なトロンビン生成試験でも，凝固状態安定後もPeak Thの回復に比しlag timeやttPeakが長い傾向にあることから，FVIII：C測定値のみでは説明困難であったAPTTの延長や重篤な出血傾向は，異常FVIIIに起因するThrombin burstの著しい遅延に起因するものと考えられた．

近年，血友病Aの臨床的重症度が必ずしもFVIII：Cと相関しない症例が存在することが報告されている[14]．また，FVIII：Cが>20%と一般的には止血レベルを有する軽症患者でも，強い外力が働いた際や観血的処置においては止血困難を呈することがある．APTTの延長が軽度であっても，構造・機能異常を有する軽症血友病A CRM^{+}病型ではFVIII：Cのみで凝固機能を評価することは困難であり，トロンビン生成試験など微細な凝固能の違いを反映できる検査法が，病態を推定するために有用であると考えられた．

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