anti-FXIII-A autoantibodies may have fluctuated and spontaneously disappeared, as previously reported in a Japanese AH13 case [15]. Unfortunately, her plasma/serum sample was not available during that period, because she had been monitored by her original physician in another hospital.

The internal bleedings risked this patient's life. Above all, cardiac tamponade was most life-threatening. This symptom has never been observed among the remaining 84 AH13 cases in the world (unpublished data of JCRG). Interestingly, this symptom is rather common in male FXIII-A knock-out mice as reported previously [16]. Cardiac tamponade, however, is never seen in female counterparts. In contrast, female FXIII-A knock-out mice die of excessive bleeding due to spontaneous miscarriage [17]. This is consistent with the fact that female cases of congenital FXIII deficiency manifest pregnancy-related bleeding [10, 18].

The present case apparently argues against a premature hypothesis that AH13 would either die of acute hemorrhage or recover permanently [19]. Actually, we confirmed that 10 % of all AH13 patients became intractable even though they had successfully survived the life-threatening acute stage (unpublished data of JCRG). These findings reinforces that AH13 must be considered a chronic intractable disorder.

Conclusion

Although this patient clinically recovered once from AH13, she died of hemorrhage 3.5 years after admission. Therefore, AH13 should be considered as a chronic intractable life-threatening disease, and closely monitored for a prolonged period.

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Conflict of interest No authors declare conflict of interest.

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Supplemental materials

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

Supplemental materials

Fig. S1. Life-threatening bleedings revealed by computed tomography (CT).

During the acute stage (Nov 10, 2009), the patient developed cardiac tamponade due to pericardial hemorrhage (**A**) and an intrathoracic hematoma (**B**). After 3.5 years, she manifested subcortical hemorrhage in the right frontal lobe (**C**), and a severe spleen laceration with intraperitoneal hemorrhage (**D**). Lesions are enclosed by broken circles.

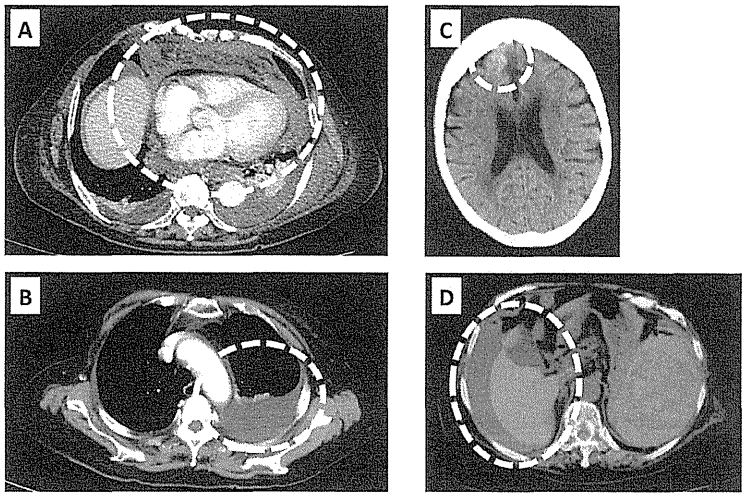

Fig. S2. JCRG detailed analyses of the patient's FXIII protein and anti-FXIII antibodies.

(**A**) A western blotting analysis was carried out using rabbit polyclonal anti-FXIII-A and anti-FXIII-B antibodies. The results showed the presence of both FXIII-A and FXIII-B proteins when diluted patient's plasma was analyzed (dilution: 1/50 to 1/200 for FXIII-A; 1/100 to 1/400 for FXIII-B; from left to right). (**B**) A fibrin cross-linking study was performed by adding 1 unit/mL thrombin and 5 mM $CaCl_2$ into the patient's plasma and normal control plasma. The clots were recovered at the indicated time intervals and subjected to SDS-PAGE analysis. A serious delay in γ-dimerization and a lack of α-polymerization were clearly shown.

(**C**) Dot blot assays were performed using rFXIII-A, rFXIII-B, and their complexes (A_2B_2) at the indicated amounts shown as antigen (ng). The results showed the presence of anti-FXIII-A antibodies. The positive controls stand for AH13 patients' plasmas identified previously. P and S stand for plasma and serum, respectively.

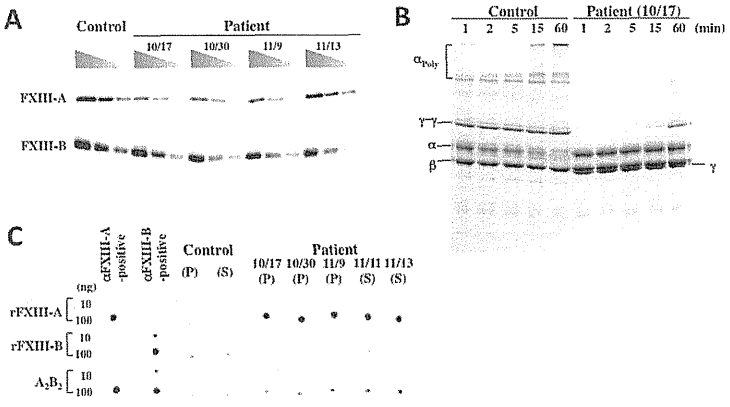

Table S1. Hematological and coagulation-fibrinolysis tests (Nov 2, 2009).

Item	value	Normal range	unit	Item	value	Normal range	unit
WBC	9.0	3.5-8.5	$x10^9$/L	Factor IX inhibitor	N.D	N.D	
RBC	2.04	3.70-50.0	$x10^{12}$/L				
Hemoglobin	64	113-150	g/L	Antithrombin	83	79-121	%
Hematocrit	20.3	33.0-45.0	%	Thrombin-Antithrombin	13.9	<3.0	ng/mL
Platelet	262	120-380	$x10^9$/L	aCardiolipin	0.7	<10.0	U/mL
PT	86	80.0-120.0	%				
APTT	21	24.3-36.0	sec	Plasminogen	75	71-128	mg/dL
Fibrinogen	2.3	1.5-4.0	g/L	α_2-Plasmin Inhibitor	97	85-115	%
Factor II	77	75-135	%	tPA/PAI-1 complex	21.2	<50.0	ng/mL
Factor V	103	70-135	%	D-dimer	2.4	<1.0	μg/mL
Factor VII	78	75-140	%				
Factor VIII	156	60-150	%				
Factor IX	157	70-130	%	CRP	1.2	<3	μg/L
Factor X	112	70-130	%				
Factor XI	110	70-135	%				
Factor XII	99	50-150	%				
VWF antigen	239	50-155	%				

N.D.; not detected, VWF; von Willebrand factor, tPA; tissue plasminogen activator, PAI; tissue plasminogen activator inhibitor, abnormal values are underlined.

Table S2. Hematological and coagulation tests (Mar 30, 2013).

Item	value	Normal range	unit	Item	value	Normal range	unit
WBC	13.0	3.5-8.5	$x10^9/L$	PT	72.5	80.0-120.0	%
RBC	2.46	3.70-5.00	$x10^{12}/L$	APTT	22.8	24.3-36.0	sec
Hemoglobin	85	113-150	g/L				
Hematocrit	25.2	33.0-45.0	%	CRP	1	<3	μg/L
Platelet	169	120-380	$x10^9/L$	Abnormal values are underlined.			

Table S3. JCRG detailed FXIII analyses.

	FXIII-A antigen		FXIII-B antigen		AI Activity		Spe. Act
	U/ml	(%)	U/ml	(%)	U/ml	(%)	U/U
Control	*1.51*	*(100)*	*1.10*	*(100)*	*1.41*	*(100)*	*0.93*
Patient							
09. 10/17	0.34	(23)	0.93	(85)	0.16	(12)	0.47
09. 10/30	0.39	(26)	0.83	(75)	0.14	(10)	0.36
09. 11/09	0.49	(32)	1.01	(92)	0.17	(12)	0.35
09. 11/13	1.68	(111)	1.19	(109)	0.80	(57)	0.48
10. 06/04	0.69	(46)	1.20	(109)	0.43	(30)	0.62
Japanese (n = 79)	*1.05 ± 0.29*		*0.94 ± 0.23*		*0.99 ± 0.29*		*0.98*

AI; amine incorporation, Spe. Act; specific activity, Abnormal values are underlined. All data for healthy controls are *italicized.*

Table S4. JCRG screening tests outsourced to a commercial service laboratory (Oct 18, 2009 and Jan 19, 2011).

	Oct. 18, 2009	Jan. 19, 2011	Normal value	unit
FXIII Act	9	19	70-140	%
FXIII Act: Control	*67*	*99*	*70-140*	*%*
Calculated FXIII Act mix	38	59	-	%
FXIII 1:1 mix	8	53	-	%
Inhibited value	30	6	(5)	%
Inhibited rate	0.79	0.10	(<0.1)	
α_2PI plasma	100	91	85-115	%
α_2PI serum	110	103	(85-115)	%
Cross-linked α_2PI	10	6.2	(15-30)	%
Cross-linked α_2PI ratio	0.083	0.057	(0.15-0.3)	
α_2PI plasma: control	*98*	*127*	*85-115*	*%*
α_2PI serum: control	*91*	*119*	*(85-115)*	*%*
Cross-linked α_2PI: control	*26.6*	*33.4*	*(15-40)*	*%*
Cross-linked α_2PI ratio: control	*0.23*	*0.22*	*(0.15-0.4)*	

	Oct. 18, 2009	Jan. 19, 2011	Normal value	unit
Lp(a) plasma	-	2.1	(<40)	%
Lp(a) serum	4.1	2.8	<40	%
Lp(a) plasma: control	*1.0*	*1.0*	*(<40)*	*%*
Lp(a) serum: control	*0.6*	*2.8*	*<40*	*%*
PIC	1.1	1.1	<0.8	μg/dL
D-dimer	16.7	1.5	<1.0	μg/dL
FDP	59	3	<4	μg/dL
Fibrinogen	-	2.15	1.5-4.0	g/L
FXIII antigen	-	125	70-140	%
FXIII specific Act	-	0.15	-	
FVIII Act	132	140	60-150	%
FIX Act	123	96	70-130	%

PI; plasmin inhibitor, PIC; plasmin-plasmin inhibitor complex, Abnormal values are underlined. All data for normal controls are *italicized.*

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ORIGINAL ARTICLE

Anti-factor XIII A subunit (FXIII-A) autoantibodies block FXIII-A_2B_2 assembly and steal FXIII-A from native FXIII-A_2B_2

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Department of Molecular Patho-Biochemistry and Patho-Biology, Yamagata University School of Medicine, Yamagata, Japan; and †The Japanese Collaborative Research Group (JCRG) on Acquired hemorrha-philia due to anti-factor XIII autoantibodies (AH13)

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Summary. *Background:* Autoimmune hemophilia-like disease (hemorrha-philia or hemorrhagic disorder) caused by anti-factor XIII antibodies (termed AH13) or 'autoimmune FXIII deficiency' is a life-threatening bleeding disorder. AH13 was thought to be rare worldwide. *Objectives:* Because the number of diagnosed AH13 cases has recently been increasing, at least in Japan, we conducted a nationwide survey supported by the Japanese Ministry of Health, Labor, and Welfare, and explored the pathologic mechanism(s) of AH13. *Methods:* We diagnosed AH13 cases during the last 11 years according to the presence of anti-FXIII autoantibodies confirmed by a dot blot assay and ELISA, and characterized 33 of these both immunologically and biochemically. *Results:* The AH13 cases were immunologically classified into three types, Aa, Ab, and B. Type Aa autoantibodies, observed in 27 cases, were directed against the native FXIII A subunit (FXIII-A), and blocked FXIII activation. The autoantibodies not only prevented assembly of new FXIII-A_2B_2 heterotetramers, but also removed FXIII-A from native FXIII-A_2B_2 heterotetramers by forming an FXIII-A–IgG complex. Type Ab autoantibodies, detected in three cases, preferentially bound to activated FXIII-A and inhibited its activity. Type Aa and Ab autoantibodies were 'neutralizing' FXIII antibodies (or FXIII inhibitors), and thus could be screened with functional assays. Type B antibodies, detected in two cases, were non-neutralizing anti-FXIII B subunit (FXIII-B) autoantibodies that possibly accelerated the clearance of FXIII, and thus could be diagnosed exclusively with immunologic methods. *Conclusion:* There are three major types of anti-FXIII autoantibody, with distinct targets and mechanisms that cause AH13.

Keywords: antibody diversity; autoimmune diseases; chronic disease; hemorrhagic disorders; immunosuppressive agents.

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Introduction

Coagulation factor XIII is a proenzyme of plasma fibrin-stabilizing factor (FSF) consisting of two catalytic A subunits (FXIII-A) and two non-enzymatic B subunits (FXIII-B) [1–3]. Activation of FXIII proceeds through several distinct steps: the activation peptide (AP) of FXIII-A is cleaved by thrombin, generating an activation intermediate (A'_2B_2), and FXIII-B dissociates from FXIII-A′ in the presence of calcium, converting A'_2B_2 to activated FXIII (FXIII-A*) [4,5]. FXIII-A* crosslinks fibrin monomers, and also fibrin to α_2-plasmin inhibitor (α_2-PI), fibronectin, etc., which ensures a regulated hemostatic response and accelerates wound healing. Its congenital deficiency therefore results in a lifelong propensity for bleeding and abnormal wound healing [1–3].

Autoimmune hemophilia-like disease (hemorrha-philia or hemorrhagic disorder) caused by anti-FXIII antibodies (AH13) or 'autoimmune FXIII deficiency' is a rare life-threatening bleeding disorder [6–8] that mainly occurs in older adults. Approximately half of AH13 cases are idiopathic, and the remaining cases are associated with autoimmune diseases, malignancies, etc.

AH13 was considered to be rare worldwide [6,9], although a number of patients may have been overlooked, owing to lack of awareness and/or the absence of proper screening tests for FXIII deficiency. In addition, FXIII activity is often not examined by clinicians.

To clarify the status of AH13 in Japan, we conducted a nationwide survey of exclusively bleeding patients who had no past history, family history, or abnormal clotting times, and reported 12 cases of AH13 as of June 2010 [9]. The number of diagnosed AH13 cases has increased to a total of 40, including the previously reported 12 cases (as of March 2014).

In this study, we performed a detailed characterization of the autoantibodies present in the 33 AH13 cases whose plasma samples were available for experimentation, in order to understand the pathogenesis of AH13 and to improve its diagnosis and treatment.

Materials and methods

Materials

Recombinant FXIII-A (rFXIII-A) was a gift from Zymogenetics (Seattle, WA, USA). Recombinant FXIII-B (rFXIII-B) was expressed by use of a baculovirus expression system and purified [10]. Anti-FXIII-A mAb was obtained from G. Reed (Massachusetts General Hospital, Boston, MA, USA). Anti-FXIII-A polyclonal antibody (pAb) was generated in-house. Anti-FXIII-B antiserum was purchased from Nordic Immunological Laboratories (AX Eindhoven, The Netherlands). Peroxidase-conjugated anti-human IgG was purchased from MP Biomedicals (Solon, OH, USA). Biotin-conjugated anti-human IgG_1 and IgG_4 antibodies, bovine thrombin, heparin, *N*,*N*-dimethylcasein and monodansylcadaverine were purchased from Sigma-Aldrich (St Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated anti-human IgG_2 and IgG_3 antibodies were purchased from Southern Biotech (Birmingham, AL, USA). HRP-conjugated streptavidin was purchased from GE Healthcare Bioscience AB (Uppsala, Sweden). Tetramethylbenzidine (TMB) peroxidase substrate kits were purchased from Bio-Rad Laboratories (Hercules, CA, USA). FXIII-deficient plasma was obtained from George-King Bio-Medical (Overland Park, KS, USA). Antithrombin was obtained from CSL Behring (Tokyo, Japan). Goat anti-α_2-PI antibody was purchased from Affinity Biologicals (Ancaster, Canada).

This study was approved by the institutional review board of the Yamagata University School of Medicine [11]. All procedures were conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants, including patients and normal healthy controls.

FXIII antigen and its activity in plasma

ELISA for FXIII antigens, amine incorporation (AI) assays, ammonia release (AR) assays and fibrin crosslinking analysis were performed as described previously [12].

ELISA for anti-FXIII autoantibodies

Recombinant A_2B_2 (rA_2B_2) was prepared by mixing equal amounts of rFXIII-A and rFXIII-B. The activation intermediates recombinant FXIII-A′ (rFXIII-A′) and recombinant A'_2B_2 (rA'_2B_2) were generated by incubation of each of rFXIII-A and rA_2B_2, respectively, with thrombin (1 U/1 μg FXIII-A) at 37 °C for 20 min, followed by the addition of heparin and antithrombin (5 U each to 1 U of thrombin). Two millimolar $CaCl_2$ was added to rFXIII-A′ and rA'_2B_2 to yield fully activated recombinant FXIII-A (rFXIII-A*) and recombinant $A^*_2B_2$ ($rA^*_2B_2$) ($rA^*_2 + B_2$), respectively. One microliter of plasma was incubated with 200 ng of recombinant FXIII at 37 °C for 2 h, and diluted 100-fold with 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl (TBS) containing 2% bovine serum albumin (BSA). Ten microliters of diluted plasma was applied to a 96-well plate coated with an anti-FXIII-A mAb or an anti-FXIII-B antibody, and incubated for 2 h at room temperature. The plate was incubated with peroxidase-conjugated anti-human IgG. After washing, TMB substrate was allowed to react for 10 min, and the reaction was terminated by addition of 0.5 M sulfuric acid. To determine IgG subclass, biotin-conjugated anti-human IgG_1 and IgG_4 antibodies coupled with HRP-conjugated streptavidin, and HRP-conjugated anti-human IgG_2 and IgG_3 antibodies, were used.

Inhibition of rFXIII-A* activity

Ten micrograms of rFXIII-A were reacted with 5 U of thrombin in a 0.5-mL mixture at 37 °C for 30 min, and the reaction was terminated by addition of 10 U of antithrombin and 100 U of heparin. Ten microliters of the rFXIII-A′ preparation were mixed with 40 μL of plasma at room temperature for 30 min. Ten-microliter aliquots of plasma and the rFXIII-A′ mixture were reacted with 0.2% *N*,*N*-dimethylcasein, 2 mM monodansylcadaverine, 5 mM $CaCl_2$, 2 mM dithiothreitol and 20 mM Tris-HCl (pH 7.5) in a 0.1-mL mixture at 37 °C for 60 min.

Cleavage of AP by thrombin

Five microliters of patient plasma diluted with FXIII-deficient plasma were incubated with 5 μL of healthy control plasma at 37 °C for 2 h. The mixed plasma was reacted with 0.5 U of thrombin in a 25-μL mixture at 37 °C for 30 min. The reaction was terminated by the addition of 0.1 mL of 8 M urea, 1% SDS, and 50 mM Tris-HCl (pH 8.0), and the reaction mixture was boiled with 0.1 mL of SDS-reducing (5% β-mercaptoethanol) buffer. Ten-microliter aliquots were electrophoresed on an 8% polyacrylamide gel containing 0.1% SDS, and this was followed by western blot analysis with an anti-FXIII-A pAb.

Thrombin-dependent and Ca^{2+}-dependent dissociation of the A_2B_2 complex

Forty nanograms of A_2B_2 complex were incubated with 4 μL of plasma in 0.2 mL of TBS at 37 °C for 1 h. Fifty-microliter aliquots were reacted with 0.05, 0.1

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or 0.2 U of thrombin and 5 mM $CaCl_2$ in a 0.1-mL mixture at 37 °C for 20 min, and diluted to 1 : 10 with TBS containing 2% BSA. The quantity of A_2B_2 complex remaining after the reaction was determined by ELISA.

A_2B_2 complex formation assay

Five nanograms of rFXIII-A were preincubated with 1 μL of plasma in 5 μL of TBS at 37 °C for 1 h. Ten nanograms of rFXIII-B in 5 μL of TBS were then added and incubated at room temperature for 20 min. The mixture was diluted with 1 mL of TBS containing 2% BSA. The quantity of A_2B_2 complex formed was measured by ELISA.

Gel-filtration analysis

One hundred microliters of plasma was applied to a Sepharose CL6B column (45 mL), and eluted with TBS. Fractions of 0.8 mL were collected. The quantities of FXIII-A, FXIII-B and A_2B_2 complexes in the fractions were measured by ELISA.

In vitro exchange of FXIII-B in the A_2B_2 complex by type Aa autoantibodies

Twenty nanograms of A_2B_2 complex were incubated with 0.4 μL of plasma in 20 μL TBS at 37 °C for 1, 2, 4, 8, 12, or 24 h. The mixture was diluted with 1 mL of TBS containing 2% BSA. The quantities of A_2B_2 complex and FXIII-A-bound IgG were measured by ELISA.

Statistical analysis

ELISA and AI activity were determined at least three times for individual samples. Comparison between groups by use of the Mann–Whitney and Kruskal–Wallis tests and correlation between parameters by the use of Spearman's coefficient were performed with the software program JMP (version 11.0.0, SAS Institute, Cary, NC, USA), and differences were determined to be statistically significant at a *P*-value of < 0.05.

Results

When plasma and/or serum samples were available, we first screened for anti-FXIII autoantibodies. Among the 88 suspected AH13 cases, 33 patients were positive for anti-FXIII antibodies (IgGs) according to our in-house dot blot assays (Table S1), and were therefore diagnosed as having AH13. The remaining 55 patients were diagnosed with hemorrhagic acquired FXIII deficiency [8], because they did not possess either anti-FXIII antibodies or FXIII inhibitors.

Classification of AH13 types by immunologic binding assays

On the basis of their immunologic properties, anti-FXIII autoantibodies could be classified into three groups. Type Aa autoantibodies, which were observed in 27 cases, bound to native (untreated) FXIII-A. Their reactivity drastically decreased when FXIII-A was converted to FXIII-A* by thrombin and calcium ions (Fig. 1A,B; Table S2). The reactivity of type Aa autoantibodies against the A_2B_2 complex was somewhat weaker than that against native FXIII-A (Fig. 1C).

Type Ab autoantibodies were explored when patients' FXIII-A-bound FXIII-B levels were not as low as their FXIII activity, and were detected in three AH13 cases (cases 8, 15, and 29). They reacted with rFXIII-A weakly as compared with type Aa cases ($P < 0.001$) (Fig. 1A), although they bound much more strongly to FXIII-A* (Fig. 1B). The conversion of FXIII-A to an activation intermediate, treated with thrombin alone, as well as to FXIII-A*, significantly enhanced the reactivity of type Ab samples to varying extents, regardless of the presence or absence of FXIII-B (rA′ and rA* or rA'_2B_2 and $rA^*_2B_2$ [A^*_2 + B_2] in Fig. 1D).

Type B autoantibodies, which were detected in two cases (cases 7 and 13), reacted to FXIII-B as well as A_2B_2, but not to FXIII-A (Fig. 1A,B; Table S2).

Abnormal FXIII parameters in AH13 cases

Both FXIII-A antigen (Fig. 2A) and AI activity (Fig. 2D) were significantly reduced in most type Aa cases ($n = 27$, $P < 0.001$), and the specific activity of FXIII (Fig. 2E), i.e. AI activity per FXIII-A antigen, in plasma was also significantly decreased ($P < 0.001$). These findings are consistent with the idea that type Aa autoantibodies are 'neutralizing' FXIII inhibitors.

Notably, essentially no A_2B_2 heterotetramer was detected in type Aa cases ($P < 0.001$; Fig. 2C), despite the presence of considerable amounts of both FXIII-A and FXIII-B antigens (Fig. 2A,B). Several type Aa cases had higher FXIII-A antigen levels than the 80 healthy controls (Fig. 2A), mainly because they had been infused with FXIII concentrates as part of their hemostatic therapy before their plasma samples were collected.

Type Ab AH13 patients ($n = 3$) also showed significantly reduced AI activity ($P < 0.01$; Fig. 2D) and low specific activity of FXIII ($P < 0.01$; Fig. 2E), whereas the levels of FXIII-A antigen and A_2B_2 heterotetramer were not decreased (Fig. 2A,C). These findings are consistent with the idea that type Ab autoantibodies are also 'neutralizing' FXIII inhibitors.

Type B AH13 cases ($n = 2$) showed reduced levels of FXIII-A antigen (Fig. 2A) and A_2B_2 heterotetramer (Fig. 2C), as well as reduced AI activity (Fig. 2D), whereas the specific activity was unchanged (Fig. 2E), indicating that the autoantibodies did not target the enzymatic activity of FXIII.

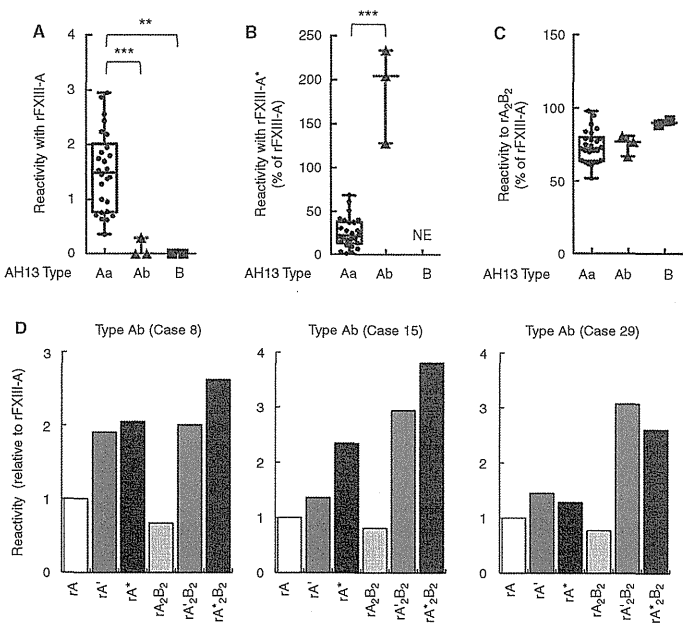

Fig. 1. Reactivity of anti-FXIII A subunit (FXIII-A) autoantibodies. (A-C) Reactivity of anti-FXIII-A autoantibodies with (A) recombinant FXIII-A (rFXIII-A), (B) activated rFXIII-A (rFXIII-A*) or (C) recombinant A_2B_2 (rA_2B_2) was determined for the three AH13 types. rFXIII-A reactivity is shown as a value relative to that of case 1, and reactivity with rFXIII-A* and rA_2B_2 is the percentage of that observed with rFXIII-A. Results are presented as box-and-whisker plots denoting the median, interquartile range, and the minimum and maximum data points. NE, not examined. ***$P < 0.001$; **$P < 0.01$. (D) The reactivity of type Ab autoantibodies in cases 8, 15 and 29 with activation intermediates (rFXIII-A′, recombinant A'_2B_2 [rA'_2B_2] and recombinant $A^*_2B_2$ [$rA^*_2B_2$] [rA^*_2 + B_2]) was determined and compared with that of rFXIII-A.

As expected, strong correlations between the levels of FXIII-A antigen, FXIII-B antigen and the A_2B_2 heterotetramer and FXIII AI activity were observed (Fig. S1) in healthy controls. No such relationships were observed in type Aa cases, especially for the A_2B_2 heterotetramer (Fig. S1B), suggesting that type Aa autoantibodies may prevent the formation of an A_2B_2 heterotetramer complex.

Differences in functional mixing assays for screening of FXIII inhibitors

Some discrepancies were observed between the AI and AR assays in the five-step mixing tests, especially for types Aa and Ab (Fig. 3A); the AR assay yielded significantly higher inhibition values than the AI assay (range 23–91% and median 80% vs. range < 1–71% and median 35%) in type Aa cases ($P < 0.001$; Fig. 3B), clearly indicating that the AR assay is more sensitive than the AI assay in screening for type Aa autoantibodies. In contrast, the arbitrary inhibitory potencies of type Ab autoantibodies obtained with the AR assay were lower than those obtained with the AI assay (Fig. 3B; Table 1). This suggests difference(s) in the inhibitory mechanisms of type Aa and type Ab autoantibodies.

Next, we employed FXIII-A* for the AI assay. This FXIII-A*-based AI assay yielded significantly higher arbitrary inhibitory potencies for type Ab autoantibodies than for type Aa autoantibodies ($P < 0.0001$; Fig. 3C; Table 1), but no significant difference was observed when the ordinal AI assay was used (Fig. 3B). Thus, type Ab autoantibodies targeted FXIII-A*, but type Aa autoantibodies did not.

No discrepancy was observed between the two functional methods in type B AH13, simply because type B autoantibodies are not neutralizing FXIII inhibitors (Fig. 3B,C).

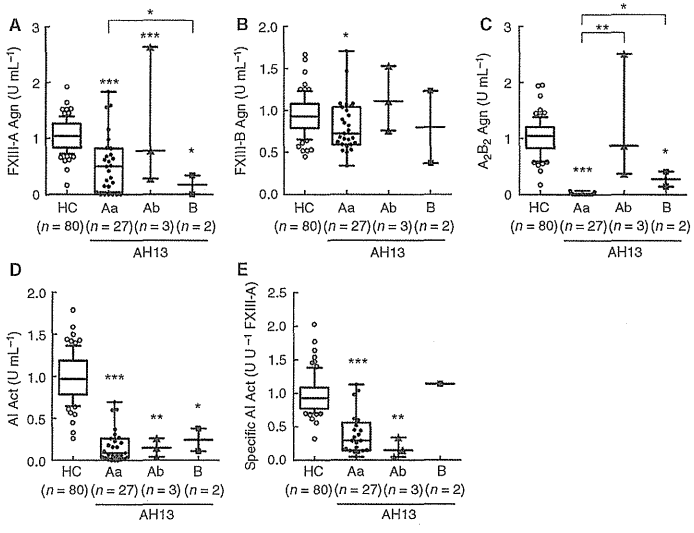

Fig. 2. FXIII parameters in plasma from healthy control and AH13 cases. (A–C) Levels of (A) FXIII A subunit (FXIII-A), (B) FXIII B subunit (FXIII-B) and (C) A_2B_2 heterotetramer antigens in plasma were measured by ELISA. (D) Amine incorporation (AI) activity was assayed with monodansylcadaverine and *N,N*-dimethylcasein. (E) The specific AI activity was calculated by dividing AI activity by FXIII-A antigen. Numerals in parentheses are numbers of individuals. Results are presented as box-and-whisker plots as defined previously for AH13 patients, and as the range from 10% to 90% for healthy controls (HCs). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. Act, activity; Agn, antigen.

Inhibition of FXIII activation by type Aa anti-FXIII autoantibodies

To directly confirm that type Aa autoantibodies are inhibitors of proteolytic activation of FXIII by thrombin [13–16], we examined the initial AP-cleavage reaction for all three types of anti-FXIII autoantibody by using a mixing study. As predicted, the addition of a patient's plasma containing type Aa autoantibodies to a control plasma clearly produced a marked blockage in the conversion of FXIII-A to FXIII-A$^{\prime}$ by thrombin (Fig. 4A). However, AP cleavage was not blocked by the addition of plasma containing either type Ab or type B autoantibodies.

The thrombin-dependent and Ca^{2+}-dependent dissociation of the A_2B_2 heterotetramer was also examined to explore the possible influence(s) of anti-FXIII autoantibodies on the later stages of FXIII activation [10,13,15,17,18]; the dissociation of the A_2B_2 heterotetramer was inhibited by anti-FXIII autoantibodies to various extents (Fig. 4B; Fig. S2; Table 1). The addition of type Ab plasma interfered with A_2B_2 dissociation by only < 1% in cases 15 and 29, but by up to 86% in case 11, whereas AP cleavage was completely blocked by plasma from case 11 but not at all by plasma from cases 15 and 29 (Table 1). However, case 8 was an exception, because A_2B_2 dissociation was partially inhibited, whereas AP cleavage was not detectably blocked.

Inhibition of fibrin-crosslinking reactions by anti-FXIII autoantibodies

In plasma of healthy controls, γ-dimerization was completed within 2 min, and α-polymerization was almost complete after 60 min, leaving only a trace amount of α-monomer (Fig. 5A). However, γ–γ dimer formation was not observed or was markedly delayed in type Aa plasma, and almost all α-monomer remained even after 60 min (Fig. 5A; Table S1). A 1 : 1 mixing test with healthy control plasma confirmed the inhibitory effects of type Aa autoantibodies on the FXIIIa-catalyzed fibrin-crosslinking reaction (Fig. 5B; Table 1).

Notably, γ-dimerization was normal in two type Ab cases (cases 8 and 15), although α-polymerization was completely blocked (Fig. 5A; Fig. S3A,C). Furthermore, plasma from cases 8 and 15 interfered with α-polymerization but not with γ-dimerization in control plasma in the mixing test (Fig. 5B; Fig. S3B). Therefore, type Ab autoantibodies inhibited α-polymerization exclusively.

Plasma containing type B autoantibodies (case 7) showed only a delay in γ-dimerization (Fig. 5A), and no severe inhibition of crosslinking was observed in the mixing test (Fig. 5B).

Inhibition of the fibrin–α_2-PI crosslinking reaction by anti-FXIII autoantibodies

Essentially all α_2-PI species recovered from fibrin clots were highly crosslinked α_2-PI (XL-α_2-PI) in the control sample (Fig. 5C). In contrast, almost no XL-α_2-PI was detected in any samples obtained from AH13 cases, except for cases 12, 13, 20, and 22. Although case 22 showed a moderately reduced quantity of XL-α_2-PI species, the remaining three cases (cases 12, 13, and 20) showed only a small amount of α-chain-monomer–XL-α_2-PI complex. These four AH13 cases retained relatively high AI activities (cases 12, 13, 20, and 22; 0.61, 0.28, 0.37 and 0.52 U mL^{-1}, respectively) (Table S1), which may have been sufficient to produce XL-α_2-PI, albeit in variably reduced quantities [11].

Although only very small amounts of α-chain-monomer–XL-α_2-PI complex and of α-polymers were detected in case 8 (type Ab), neither XL-α_2-PI with α-chain nor α-polymers were observed in case 15 (type Ab) or case 17 (type Aa) (Fig. S3D).

Inhibition of A_2B_2 heterotetramer complex formation by type Aa autoantibodies

We next examined whether FXIII-A and FXIII-B formed complexes or were present separately by using gel-filtration analysis. In plasma from a healthy control, both FXIII-B and FXIII-A were detected by ELISA in the same fractions, along with the A_2B_2 heterotetramer (Fig. 6A). This was true for both type Ab and type B cases of AH13 (Fig. S4). In contrast, in plasma obtained

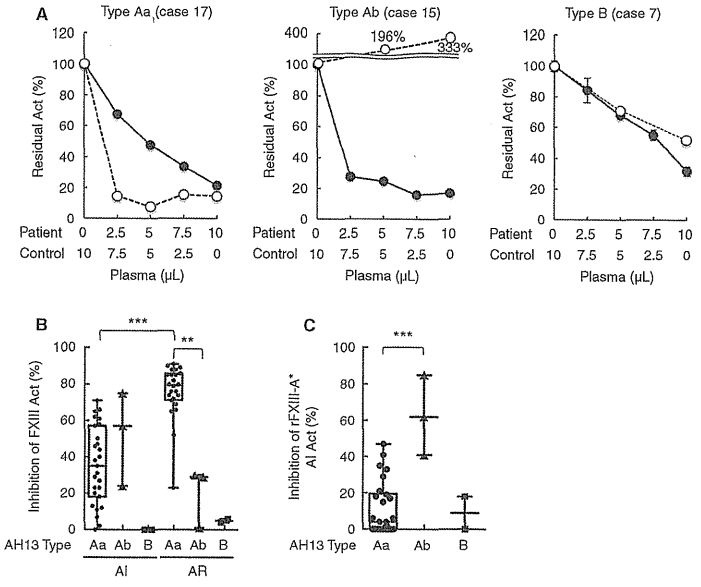

Fig. 3. Inhibition of FXIII activity by anti-FXIII autoantibodies. (A) Type Aa (case 17), type Ab (case 15) or type B (case 7) plasma was mixed with plasma from healthy controls. Amine incorporation (AI) (closed circle) and ammonia release (AR) activities (open circles) were measured with the five-step dilution test. (B) The inhibition ratios of AI and AR activities were calculated with the following equation: 100 × ([control + patient]/2 − [1 : 1 mixture])/([control + patient]/2), for each AH13 type. Individual AH13 cases were plotted. (C) Recombinant activated FXIII A subunit (rFXIII-A*) was incubated with plasma of AH13 cases, and the AI activity was assayed. The inhibition value was calculated with the following equation: 100 × (activity of rFXIII-A* incubated with AH13 plasma)/(activity of rFXIII-A* incubated with FXIII-A deficient plasma). ***$P < 0.001$; **$P < 0.01$. Act, activity.

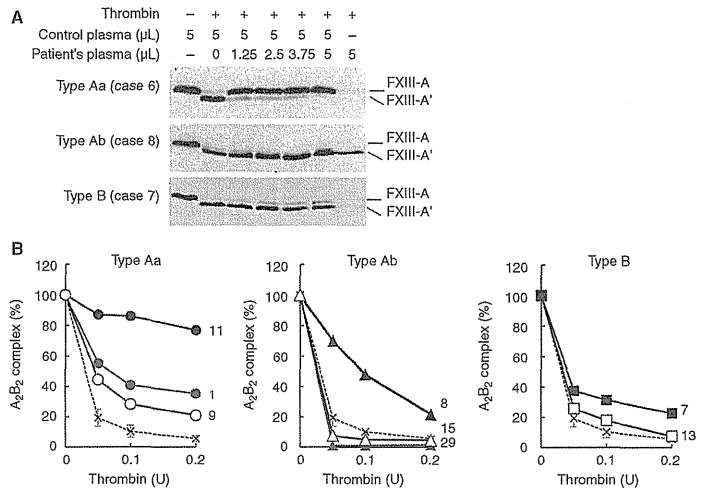

Fig. 4. Influence of anti-FXIII autoantibodies on activation of FXIII. (A) To test whether anti-FXIII autoantibodies inhibit activation peptide (AP) cleavage, healthy control plasma was incubated with AH13 plasma and used in a reaction with thrombin. Cleavage of AP was determined by western blotting with an anti-FXIII A subunit (FXIII-A) polyclonal antibody. (B) Thrombin-dependent and Ca^{2+}-dependent dissociation of the A_2B_2 complex. Recombinant A_2B_2 was incubated with type Aa, Ab or B plasma in a reaction with 0.05, 0.1 or 0.2 U of thrombin for 20 min in the presence of calcium ions. The amounts of A_2B_2 complex in the reaction mixture were measured by ELISA. The numbers indicate individual AH13 cases, and a broken line represents the result for FXIII-A-deficient plasma.

from a type Aa case, FXIII-B and FXIII-A appeared in different fractions, and formed two separate peaks (Fig. 6A). Anti-FXIII-A IgG was also detected in the same fractions as FXIII-A. In addition, immunoprecipitation studies revealed much less coimmunoprecipitated FXIII-B and FXIII-A as well as high recovery of FXIII-A with protein A–Sepharose from type Aa plasma (Fig. S5). These results confirmed that, in type Aa cases, FXIII-A is exclusively complexed with anti-FXIII-A IgG and not with FXIII-B.

A mixing study revealed that when rFXIII-A was preincubated with type Aa plasma, serum, or IgG, subsequently added rFXIII-B could not form A_2B_2 heterotetramers (Fig. 6B; Table 1; Fig. S6). However, A_2B_2 heterotetramers were readily formed when type Ab plasma was premixed with rFXIII-A and rFXIII-B was added subsequently. This was also true when plasma from patients with either congenital FXIII-A or FXIII-B deficiency was employed.

FXIII-B exchange by type Aa autoantibodies

When FXIII concentrates were infused into a type Aa patient (case 23) for hemostatic therapy, levels of the A_2B_2 heterotetramer increased immediately, but then returned to the basal level, i.e. 0% of normal, as early as 24 h after FXIII dosing (Fig. 7A). However, both FXIII-A and FXIII-B antigens showed sustained increases for > 48 h. It is important to note that the level of FXIII-A-bound IgG also increased, and it remained in the patient's plasma in concert with the FXIII-A antigen (Fig. 7A). These results strongly suggest that the FXIII-A of the A_2B_2 heterotetramer contained in infused FXIII concentrates was transferred to the FXIII-A–IgG complex *in vivo*. Essentially the same result was obtained in cases 24 and 25 (Fig. S7A).

To test this, we incubated the A_2B_2 heterotetramer with plasma from the type Aa patient for 12 h *in vitro*. The level of the A_2B_2 heterotetramer rapidly decreased with time, and the level of the FXIII-A–IgG complex increased in a symmetric manner (Fig. 7B; Fig. S7B).

A decrease in the level of the A_2B_2 heterotetramer coupled with an increase in the level of the FXIII-A–IgG complex was also confirmed in serum obtained from type Aa cases (Fig. S7C). Gel-filtration analyses of the reaction mixtures revealed that most FXIII-A did not exist in a complex with FXIII-B, but was bound to anti-FXIII-A IgG instead (Fig. S7D). In contrast, the level of the A_2B_2 heterotetramer did not decrease and the level of the FXIII-A–IgG complex did not increase when the A_2B_2 heterotetramer was incubated with plasma obtained from patients with congenital FXIII deficiencies.

Table 1 Properties of autoantibodies in AH13 cases

Case	Major type	Inhibition of activity (%) AI**	AR**	FXIII-A* AI†	Activation AP cleavage	*Dissociation*‡ (% inhibition)	Fibrin crosslinking in *mixed plasma* (min§)	Complex formation
1	Aa	46	74	35	Inhibited	41	5–15	Inhibited
2	Aa	23	52	4	Inhibited	40	5–15	Inhibited
3	Aa	29	NE	NE	Inhibited	NE	NE	Inhibited
4	Aa	7	69	15	Inhibited	38	2–5	Inhibited
5	Aa	57	80	29	Inhibited	38	15–60	Inhibited
6	Aa	2	65	< 1	Inhibited	21	15–60	Inhibited
7	B	< 1	4	< 1	Inhibited (slightly)	31	2–5	Not inhibited
8	Ab	24	29	62	Not inhibited	48	< 2 (α-polymerization delay)	Not inhibited
9	Aa	27	80	< 1	Inhibited	23	< 2	Inhibited
10	Aa	< 1	79	< 1	Inhibited	54	5–15	Inhibited
11	Aa	71	90	6	Inhibited (completely)	86	> 60	Inhibited
12	Aa	11	91	18	Inhibited	35	5–15	Inhibited
13	B	< 1	6	18	Not inhibited	18	< 2	Not inhibited
14	Aa	58	88	4	Inhibited	35	5–15	Inhibited
15	Ab	57	1	85	Not inhibited	< 1	< 2 (α-polymerization delay)	Not inhibited
16	Aa	40	NE	21	Inhibited	41	5–15	Inhibited
17	Aa	47	86	41	Inhibited	34	15–60	Inhibited
18	Aa	12	71	< 1	Inhibited	16	5–15	Inhibited
19	Aa	23	85	6	Inhibited	20	5–15	Inhibited
20	Aa	20	82	< 1	Inhibited	26	5–15	Inhibited
21	Aa	18	76	< 1	Inhibited	20	5–15	Inhibited
22	Ax	1	< 1	< 1	Not inhibited	< 1	< 2	Not inhibited
23¶	Aa	35	66	< 1	Inhibited	21	15–60	Inhibited
24	Aa	38	85	19	Inhibited	25	15–60	Inhibited
25	Aa	61	72	< 1	Inhibited	32	> 60	Inhibited
26	Aa	65	NE	< 1	Inhibited	33	5–15	Inhibited
27	Aa	66	76	< 1	Inhibited	14	5–15	Inhibited
28	Aa	62	88	17	Inhibited	15	5–15	Inhibited
29	Ab	75	30	41	Not inhibited	< 1	> 60	Not inhibited
30	Aa	44	89	47	Inhibited	36	5–15	Inhibited
31	Aa	13	23	2	Inhibited	21	2–5	Inhibited
32	Aa	50	85	33	Inhibited	32	15–60	Inhibited
33	Aa	31	74	< 1	Inhibited	24	5–15	Inhibited

AI, amine incorporation; AP, activation peptide; AR, ammonia release; Ax, not determined because of extremely low reactivity in ELISA; FXIII-A*, activated factor XIII; NE, not examined. †The inhibition value of AI activity of FXIII-A* was calculated as follows: 100 × (activity of rFXIII-A* incubated with AH13 case's plasma)/(activity of rFXIII-A* incubated with FXIII-A deficient plasma). ‡The inhibition value of *FXIII dissociation with 0.1 U of thrombin was calculated as follows:* 100 × (*remaining* A_2B_2 in the reaction with case plasma – remaining A_2B_2 in the reaction with FXIII-A-deficient plasma)/(100 – remaining A_2B_2 in the reaction with FXIII-A-deficient plasma). §Completion of γ-γ dimer formation was judged by the disappearance of γ-chain monomer. ¶Case 23 was female and 63 years of age. She had intramuscular bleeding but did not receive FXIII concentrates at the time of examination. **The inhibition ratio of AI or AR was calculated as follows: 100 × ([activity of case's plasma + activity of control plasma]/2 – activity of 1 : 1-mixed plasma)/([activity of case's plasma + activity of control plasma]/2).

AH13 cases (cases 6, 10, 12, 15, 18, 21, 25, 26, and 28) who received FXIII concentrates within 10 days before blood collection are underlined.

A_2B_2 heterotetramer levels did not change in plasma from type Ab and type B cases, and FXIII–IgG complex levels did not increase, even after 24 h (Fig. 7C).

Discussion

Hemorrhagic disorders of fibrin stabilization were previously classified as follows [19,20]: class I (hereditary disorders), class II (acquired inhibitors), and class III (acquired lack of FSF). Classes I and II were also divided into five subtypes: class I type I (lack of FSF activity; corresponds to congenital FSF deficiency), class I type II (dysfibrinogenemia with crosslinking defects), class II type I (inhibitors of FSF generation), class II type II (inhibitors of FSF activity) [21–25], and class II type III (blocking fibrin reactivity to FSF).

Type Aa and Ab autoantibodies obviously correspond to the former class II type II and type I, respectively [19,20]. However, we unexpectedly found that the A_2B_2 heterotetramer was almost completely absent in plasma

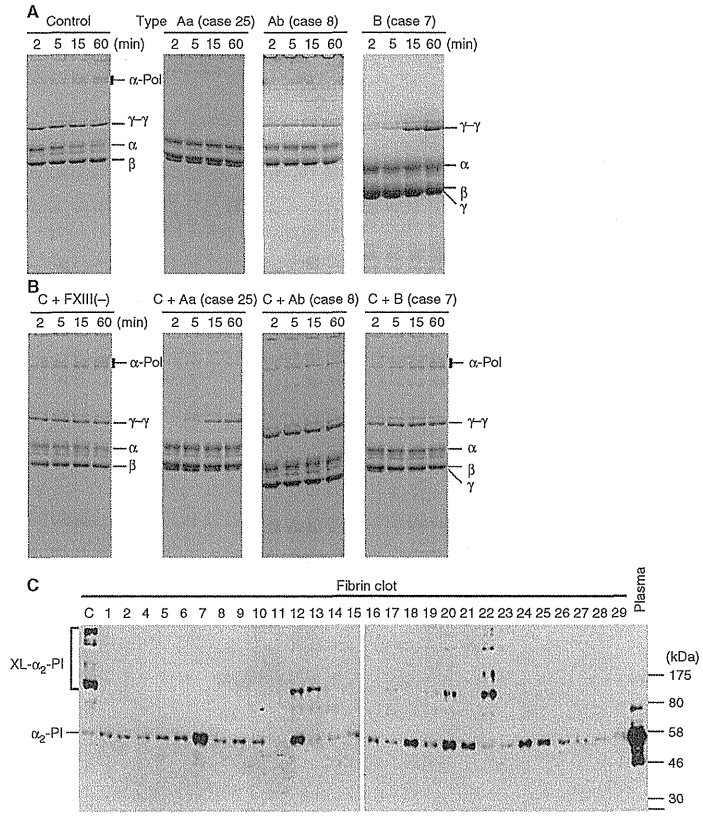

Fig. 5. Inhibition of fibrin-crosslinking reactions by anti-FXIII autoantibodies. (A) Fibrin-crosslinking reactions were examined in the plasma from a healthy control, a type Aa patient (case 25), a type Ab patient (case 8), or a type B patient (case 7). (B) Healthy control plasma was incubated with an equal volume of plasma from patients with FXIII A subunit deficiency: type Aa (case 25), type Ab (case 8), or type B (case 7). Fibrin-crosslinking reactions were then performed. (C) AH13 plasma was treated with thrombin and $CaCl_2$ for 1 h, and the α_2-plasmin inhibitor (α_2-PI) incorporated into the fibrin clots was then detected by western blotting with an anti-α_2-PI antibody. Control plasma was loaded in the lane at the right end. C, control; XL-α_2-PI; crosslinked α_2-PI. α-Pol, α-Polymer.

from type Aa cases. The existing FXIII-A in patients' plasma was not complexed with FXIII-B, but was instead exclusively bound to anti-FXIII-A IgG. This is because type Aa autoantibodies not only prevent the assembly of the A_2B_2 heterotetramer, but also replace FXIII-B in the A_2B_2 heterotetramer with type Aa anti-FXIII-A autoantibodies themselves as the counterpart of FXIII-A in the A2B2 heterotetramer, which results in the formation of FXIII-A–IgG complexes. In an antibody-abundant condition in the circulation in AH13 cases, FXIII-A may be captured by its autoantibodies immediately after it is released from its synthesizing cells [26–31], before it can form a complex with FXIII-B [32], in plasma of type Aa AH13 cases *in vivo*.

The type Aa autoantibodies bound to FXIII-A efficiently prevent its activation [13–16], probably by blocking the access of thrombin to its AP. In addition, the formation of complexes between FXIII-A and its antibodies may accelerate their clearance; the rates of disappearance of both FXIII-A antigen and anti-FXIII-A IgG were relatively rapid, and their half-lives must therefore be < 10 days [33,34], as shown in case 23 *in vivo*. Accordingly, the presence of at least some type Aa autoantibodies simultaneously inhibits FXIII activation and enhances the clearance of FXIII-A–IgG complexes, which is consistent with the significantly reduced FXIII-A antigen levels observed in plasma from type Aa cases. The dual mode of action of type Aa autoantibodies suggests that large amounts of FXIII concentrates might be required to achieve hemostatic levels of the functional FXIII protein in bleeding AH13 patients, in whom the recovery rate of FXIII activity is decreased and its half-life is shortened.

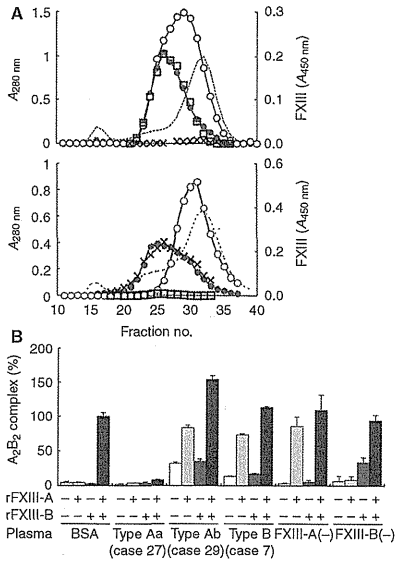

Fig. 6. Inhibition of heterotetramer assembly by type Aa anti-FXIII autoantibodies. (A) Plasma obtained from a healthy control or case 10 (type Aa) was fractionated on a Sepharose CL6B column. FXIII A subunit (FXIII-A) (closed gray circles and bold gray line), FXIII B subunit (FXIII-B) (open circles and bold line), and A_2B_2 (open squares) antigens, as well as FXIII-A-bound IgG (x) in each fraction, were measured by ELISA. A thin broken line indicates absorbance at 280 nm. (B) Recombinant FXIII-A (rFXIII-A) (or recombinant FXIII-B (rFXIII-B) preincubated in AH13 plasma was mixed with rFXIII-B (or rFXIII-A). The amounts of resulting A_2B_2 complexes (black bar) were then measured by ELISA (a result in bovine serum albumin [BSA] was assigned as 100%). Open bars: no rFXIII-A and rFXIII-B (indicates endogenous FXIII complex). Light gray bars: rFXIII-A alone. Dark gray bars: rFXIII-B alone.

Because type Aa autoantibodies have relatively low reactivity to FXIII-A*, cleavage of AP and dissociation of FXIII-B may induce drastic conformational change(s) in the epitope region(s) of FXIII-A molecules for type Aa autoantibodies. As type Aa autoantibodies preferentially react with native FXIII-A, preactivated FXIII-A* products may be more effective as hemostatic reagents in the treatment of actively bleeding type Aa AH13 cases, because they can escape from type Aa autoantibodies.

Only three of the AH13 cases examined possessed type Ab autoantibodies. Type Ab autoantibodies react only weakly with native FXIII-A, and thus do not interfere with the assembly of A_2B_2 heterotetramers. Thus, a normal A_2B_2 molecular form and a normal level of A_2B_2 antigen are present in plasma of type Ab AH13 cases. Accordingly, type Ab can readily be differentiated from type Aa by the absence of A_2B_2 antigen in the latter. In other words, types Aa and Ab can be differentially diagnosed with immunologic assays alone.

It is important to note that type Ab autoantibodies, only slightly, if at all, inhibit γ-dimerization of fibrin. This is probably because γ-dimerization by FXIIIa* proceeds and is completed so quickly that type Ab autoantibodies cannot prevent this reaction. In addition, type Ab autoantibodies start to function as FXIII inhibitors only after the generation of activity. In contrast, type Aa autoantibodies can efficiently block such rapid γ-dimerization, because they inhibit FXIII activation before the generation of activity.

α-Polymerization by FXIIIa* proceeds slowly, and even type Ab autoantibodies can severely inhibit it. Not only α-polymerization but also crosslinking of α_2-PI with the α-chain is inhibited by type Ab autoantibodies, suggesting that the crosslinking reaction between α_2-PI and the α-chain also occurs more slowly than γ-dimerization. An absence or a reduced amount of XL-α_2-PI may lead to the severe bleeding symptoms of AH13 [10,35,36].

A Hungarian type B AH13 patient showed extremely severe decreases in all FXIII parameters; FXIII activity and FXIII-A, FXIII-B and FXIII-A_2B_2 antigens were almost completely absent [37]. One of the two type B patients (case 13) showed moderately reduced FXIII parameters, whereas the other patient (case 7) had a normal level of FXIII-B antigens but considerably decreased FXIII activity and levels of FXIII-A and FXIII-A_2B_2 antigens. The reason(s) for this variation in FXIII-B antigen levels remain unknown. The type B autoantibodies may bind to the FXIII-B molecule in A_2B_2 heterotetramers, and may rapidly eliminate A_2B_2 heterotetramers from the circulation in type B patients. This assumption is consistent with the shortened half-life of the activity of the infused A_2B_2 heterotetramer (fibrogammin-P) observed in the Hungarian case.

Type B cases represent ~ 10% of AH13 cases, probably because type B autoantibodies are not neutralizing FXIII inhibitors, and thus can only be detected with immuno-

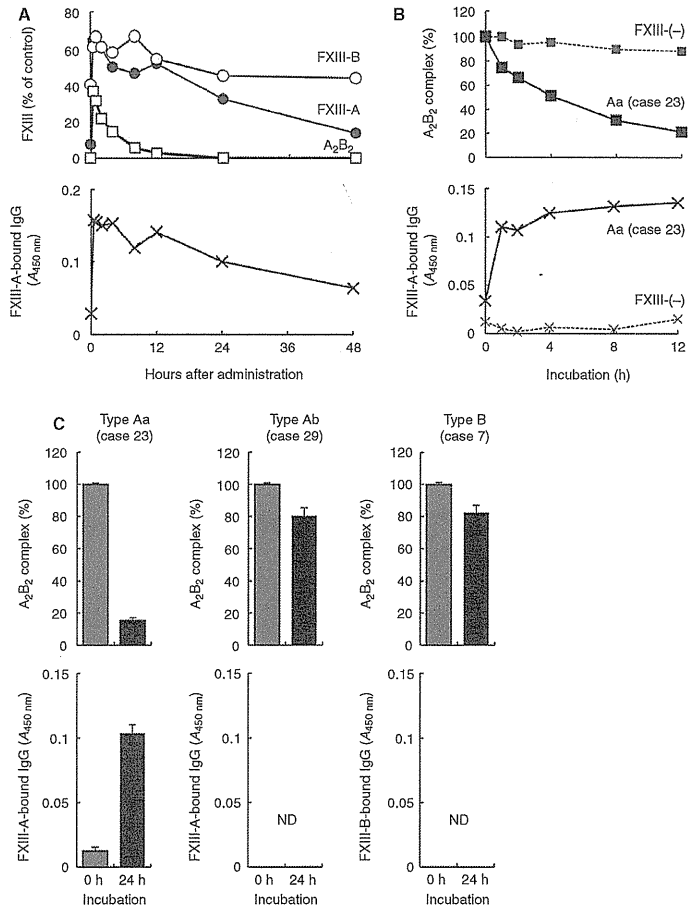

Fig. 7. Removal of FXIII A subunit (FXIII-A) from the FXIII heterotetramer by type Aa autoantibody. (A) Plasma-derived FXIII concentrates (A_2B_2) were infused into a type Aa AH13 patient (case 23), and plasma levels of FXIII-A (gray circles), FXIII B subunit (FXIII-B) (open circles), A_2B_2 (open squares) antigens and FXIII-A-bound IgG (X) were measured by ELISA at the indicated time intervals. (B) Recombinant A_2B_2 (rA_2B_2) was mixed with plasma from case 23 *in vitro*, and the levels of A_2B_2 complexes and FXIII-A-bound IgG were examined. The broken line shows the result of the incubation of rA_2B_2 with FXIII-A-deficient plasma. (C) rA_2B_2 was also incubated with the three types of AH13 plasma for 24 h *in vitro*, and the amounts of A_2B_2 complex and FXIII-A-bound IgG (cases 23 and 29) or FXIII-B-bound IgG (case 7) were measured by ELISA. ND, not detected.

logic methods, rather than with the more common functional assays. The hemorrhagic symptoms of our type B cases seemed to be somewhat mild, and similar to those of congenital FXIII-B deficiency [38].

Finally, it is important to note that the AH13 cases examined often showed mixed characteristics of the three types of anti-FXIII autoantibody. This is very likely because their autoantibodies are oligoclonal rather than

monoclonal [15,39,40], as suggested by the fact that most of our patients had anti-FXIII autoantibodies of more than one IgG subclass (Table S2). In addition, multiple clones may share the same subclass. Thoroughly distinguishing and characterizing each type of anti-FXIII autoantibody would be possible if each clone of antibody-synthesizing B cells could be separated; this work remains for the future.

In conclusion, there are three major types of anti-FXIII autoantibody, with distinct targets and mechanisms, that cause AH13; type Aa blocks A_2B_2 tetramer assembly and steals A_2 from the tetramer, and inhibits proteolytic FXIII activation by thrombin; type Ab inhibits activated FXIII exclusively; and type B accelerates the clearance of FXIII–antibody complexes from the circulation.

Addendum

M. Souri and T. Osaki performed experiments and wrote the paper. A. Ichinose created the research project and wrote the paper.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

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

Table S1. Classification of AH13 cases and FXIII antigens (Agn) and activities (Act) in patients' plasma.
Table S2. IgG subclasses and reactivity of anti-FXIII autoantibodies.
Fig. S1. Correlation between FXIII-A and FXIII-B (A) or A_2B_2 (B) antigen or amine incorporation activity (C) in AH13 plasma.
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Table S1. Classification of AH13 cases and FXIII antigens (Agn) and activities (Act) in patients plasma

Case	Antibody*	Major Type	FXIII-A (U/mL)	AgnFXIII-B (U/mL)	AgnA_2B_2 (U/mL)	Amine-incorporation Act (U/mL)	Amine-incorporation Specific	Fibrin-crosslinking Act(min of γ–γ-completion)	
1	Anti-FXIII-A	IgG	Aa	0.14	1.00	<0.02	0.06	0.44	>60
2	Anti-FXIII-A	IgG	Aa	0.04	1.47	<0.02	0.04	1.13	15-60
3	Anti-FXIII-A	IgG	Aa	0.62	0.72	<0.02	0.18	0.29	>60
4	Anti-FXIII-A	IgG	Aa	1.56	1.07	<0.02	0.27	0.17	15-60
5	Anti-FXIII-A	IgG	Aa	0.83	1.08	<0.02	0.16	0.19	>60
6	Anti-FXIII-A	IgG	Aa	<0.02	0.67	<0.02	<0.02	–	>60
7	Anti-FXIII-B	IgG	B	<0.02	1.23	0.14	<0.02	–	15-60
8	Anti-FXIII-A	IgG	Ab	0.781.1	1	0.87	0.26	0.34	<2 (less α_{poly})
9	Anti-FXIII-A	IgG	Aa	<0.02	0.85	<0.02	<0.02	–	>60
10	Anti-FXIII-A	IgG	Aa	0.42	1.08	<0.02	0.06	0.15	>60
1	1Anti-FXIII-A	IgG	Aa	1.83	1.04	<0.02	0.09	0.05	>60 (less clot)
12	Anti-FXIII-A	IgG	Aa	1.59	1.71	<0.02	0.61	0.38	15-60
13	Anti-FXIII-B	IgG	B	0.34	0.37	0.41	0.28	1.14	<2
14	Anti-FXIII-A	IgG	Aa	0.50	1.05	<0.02	0.07	0.14	>60
15	Anti-FXIII-A	IgG	Ab	2.64	1.53	2.52	0.15	0.05	<2 (less α_{poly})
16	Anti-FXIII-A	IgG	Aa	0.52	0.82	<0.02	0.06	0.12	>60
17	Anti-FXIII-A	IgG	Aa	0.59	0.57	<0.02	0.26	0.45	>60
18	Anti-FXIII-A	IgG	Aa	0.30	0.59	<0.02	0.31	1.04	>60
19	Anti-FXIII-A	IgG	Aa	0.98	0.61	<0.02	0.60	0.62	>60
20	Anti-FXIII-A	IgG	Aa	0.71	0.65	0.07	0.37	0.52	15-60
21	Anti-FXIII-A	IgG	Aa	<0.02	0.89	<0.02	<0.02	–	>60
22	Anti-FXIII-A	IgG	Ax	0.87	0.89	0.80	0.52	0.59	<2
23	Anti-FXIII-A	IgG	Aa	0.15	0.62	<0.02	0.04	0.29	>60
24	Anti-FXIII-A	IgG	Aa	0.68	0.66	0.05	0.23	0.34	>60
25	Anti-FXIII-A	IgG	Aa	0.64	0.50	<0.02	0.16	0.26	>60
26	Anti-FXIII-A	IgG	Aa	0.25	0.94	<0.02	0.03	0.12	>60
27	Anti-FXIII-A	IgG	Aa	<0.02	0.34	<0.02	0.02	–	>60
28	Anti-FXIII-A	IgG	Aa	<0.02	0.68	<0.02	<0.02	–	>60
29	Anti-FXIII-A	IgG	Ab	0.28	0.76	0.37	0.04	0.15	>60
30	Anti-FXIII-A	IgG	Aa	0.82	0.53	<0.02	0.1	1 0.14	>60
31	Anti-FXIII-A	IgG	Aa	1.16	0.52	<0.02	0.70	0.60	5-15
32	Anti-FXIII-A	IgG	Aa	<0.02	0.59	<0.02	0.01	–	>60
33	Anti-FXIII-A	IgG	Aa	0.21	0.76	0.03	0.21	0.98	>60
Mean ± SD	AH13 (n = 33)			0.58 ± 0.61	0.85 ± 0.32	0.18 ± 0.47	0.19 ± 0.20	0.42 ± 0.33	
	Control (n = 80)			1.06 ± 0.27	0.94 ± 0.23	1.04 ± 0.30	1.00 ± 0.28	0.96 ± 0.26	<2

Ax; not determined because of extremely low reactivity in ELISA.
*; determined by dot-blot assay
AH13 cases received FXIII concentrates within 10 days before blood collection were underlined and colored in blue.

1

Table S2. IgG subclasses and reactivity of anti-FXIII autoantibodies

Case	Major Type	IgG Subclass IgG1	IgG2	IgG3	IgG4	Reactivity (relative to case 1 or 7) FXIII-A	FXIII-B	(% of FXIII-A or -B) FXIII-A*	A_2B_2
1	Aa	±	–	+	–	1.00	–	39	86
2	Aa	+	–	+	+	1.28	–	37	72
3	Aa	n.e.†	n.e.†	n.e.†	n.e.†	n.e.†	–	n.e.†	n.e.†
4	Aa	±	–	–	+	0.76	–	40	77
5	Aa	+	–	±	+	2.24	–	61	79
6	Aa	+	–	+	+	1.53	–	3	62
7	B	±	+	–	–	<0.01	1.00	n.e.†	89
8	Ab	±	–	–	–	0.30	–	205	67
9	Aa	±	–	–	+	0.64	–	11	71
10	Aa	+	–	+	+	1.70	–	18	79
11	Aa	+	–	–	+	1.56	–	41	63
12	Aa	+	–	+	+	2.44	–	28	63
13	B	–	+	±	–	<0.01	1.08	n.e.†	92
14	Aa	+	–	+	±	1.41	–	19	64
15	Ab	–	–	–	+	0.04	–	234	81
16	Aa	+	–	–	–	2.87	–	23	61
17	Aa	+	–	–	+	2.95	–	27	64
18	Aa	±	–	+	+	0.78	–	2	52
19	Aa	+	–	–	+	1.46	–	37	63
20	Aa	±	–	–	+	0.70	–	30	83
21	Aa	±	–	+	+	0.62	–	17	74
22	Ax	–	–	–	–	<0.01	–	n.d.	n.d.
23	Aa	+	–	±	+	1.86	–	1	73
24	Aa	+	–	+	+	1.38	–	15	75
25	Aa	+	–	±	+	2.19	–	14	78
26	Aa	+	–	+	±	1.95	–	7	71
27	Aa	+	–	±	±	1.80	–	9	70
28	Aa	+	–	+	+	1.75	–	21	71
29	Ab	–	–	–	±	0.01	–	128	77
30	Aa	±	–	–	±	0.71	–	68	95
31	Aa	–	–	+	–	0.36	–	51	98
32	Aa	+	–	+	–	2.56	–	19	89
33	Aa	–	–	+	–	0.96	–	24	84

†; not examined, *; not determined because of extremely low reactivity in ELISA, n.d.; not determined.
AH13 cases received FXIII concentrates within 10 days before blood collection were underlined and colored in blue.

2

Fig. S2. Inhibition of thrombin- and Ca^{2+}-dependent dissociation by anti-FXIII autoantibodies.

The results are presented as box-and-whisker plots denoting the median, interquartile range, and the minimum and maximum data points. The results of individual AH13 cases were shown for AH13 cases with type Aa (black dots), type Ab (closed triangles), and type B (closed squares).

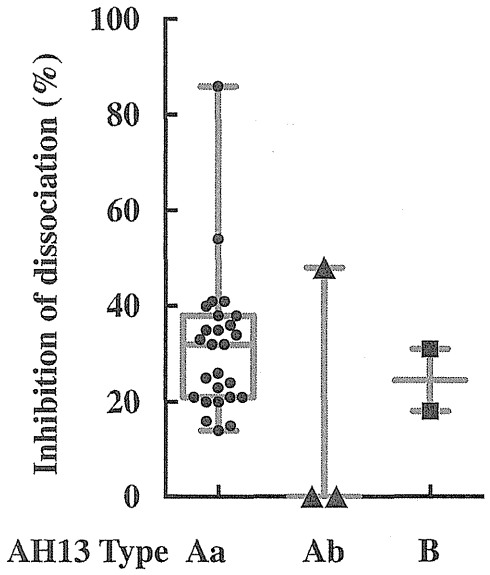


Fig. S1. Correlation between FXIII-A and FXIII-B (A) or A_2B_2 (B) antigen or amine-incorporation activity (C) in AH13 plasma.

(A) A significant correlation between FXIII-A and FXIII-B antigens was observed in healthy controls (n = 80, open circles; slope = 0.30, $R^2 = 0.15$, $P = 0.0005$), but not in AH13 cases with type Aa (n = 27, red dots; slope = 0.17, $R^2 = 0.089$, $P = 0.13$), type Ab (n = 3, orange dots; slope = 0.299) and type B (n = 2, blue dots; slope = -2.53). **(B)** A strong correlation between FXIII-A and A_2B_2 antigens was observed in healthy controls (slope = 0.87, $R^2 = 0.65$, $P < 0.0001$). Although the values of AH13 cases with types Ab (slope = 0.91) and B (slope = 0.79) followed a linear regression model in the same way as those of healthy controls, whereas the values of type Aa cases (slope = 0.0057, $R^2 = 0.029$, $P = 0.39$) did not. **(C)** Amine-incorporation (AI) activity (Act) significantly correlated to FXIII-A antigen (Agn) in healthy controls (slope = 0.60, $R^2 = 0.37$, $P < 0.0001$). Values of AH13 cases with type B and type Aa varied widely.

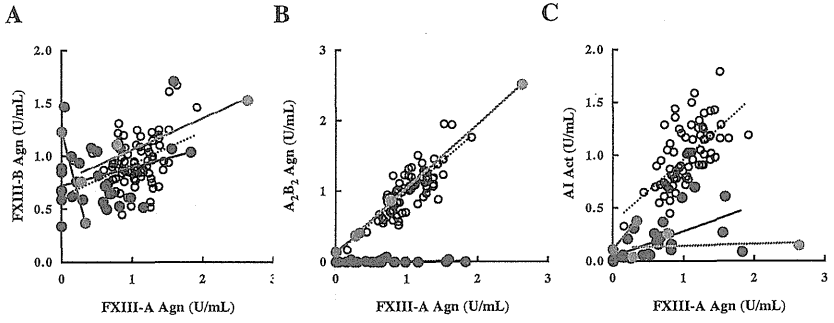


Fig. S4. Gel-filtration analysis of FXIII antigens in plasma of types Ab and B cases.
Plasma obtained from case 15 (type Ab) or case 13 (type B) was fractionated by Sepharose CL6B column. FXIII-A (closed gray circles and a bold gray line), FXIII-B (open circles and a bold line), and A_2B_2 (open squares) antigens present in each fraction were measured by ELISA (A450). A thin broken line indicates absorbance at 280 nm (A280).

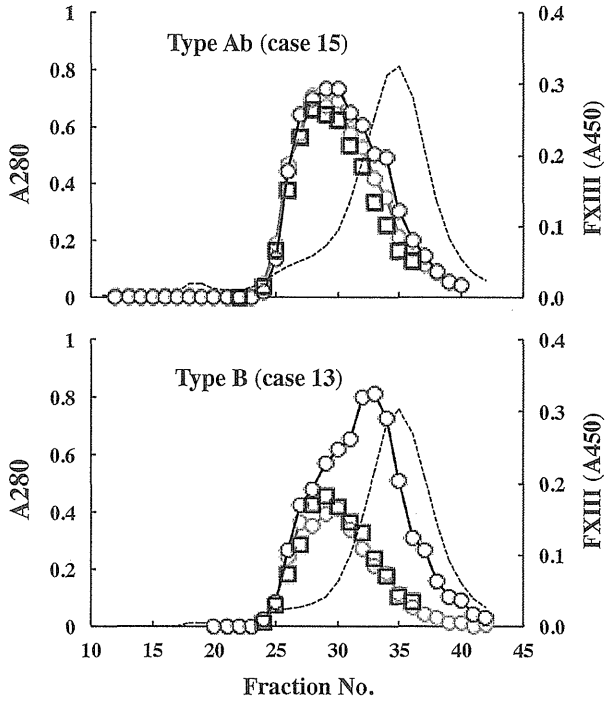


Fig. S3. Inhibition of fibrin-crosslinking reactions by anti-FXIII autoantibodies.
(A) SDS-PAGE separated fibrin clots stained with CBB. Plasma of two type Ab AH13 patients (cases 15 and 29) were examined. **(B)** Fibrin crosslinking reactions were examined using normal plasma mixed with plasma of case 15, case 29 (type Ab), or case 17 (type Aa).
(C–D) Plasma-derived fibrin clots obtained from the control, two type Ab cases, and a single type Aa case were analyzed by western blotting using an (**C**) anti-fibrinogen or (**D**) anti-a_2-PI antibody.

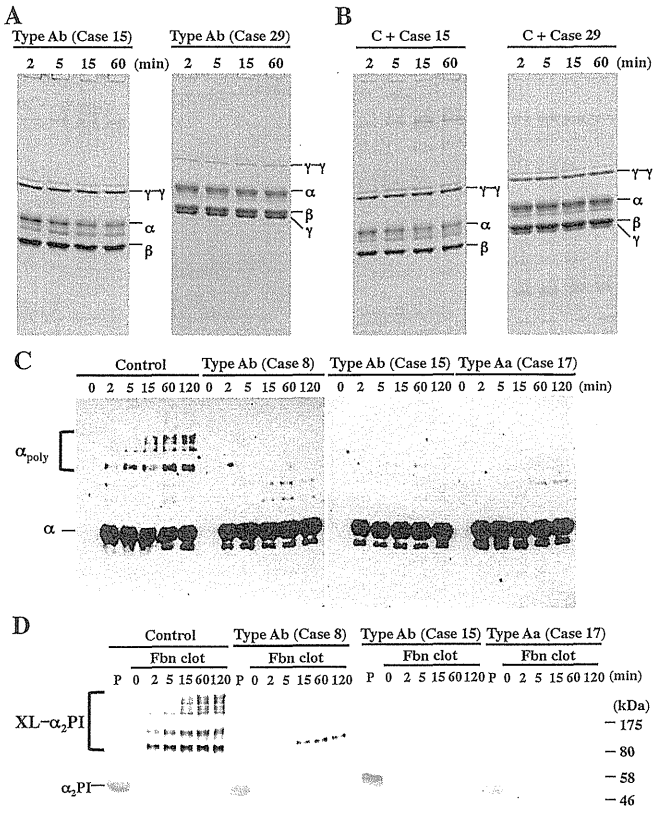


Fig. S6. Inhibition of heterotetramer assembly by type Aa serum and IgG.

(A) One hundred ng of rFXIII-A were incubated with 10 mL of serum obtained from a type Aa case (case 10) or healthy control (C) at 37°C for 1 hour, and reacted with 200 ng of rFXIII-B at 37°C for 30 min. rFXIII-A was immunoprecipitated with an anti-FXIII-A mAb, and co-precipitated FXIII-B was visualized by Western blotting using an anti-FXIII-B antibody. **(B)** Twenty ng of rFXIII-A were incubated with 2 mL of serum (cases 12, 20, 21, 24, 26, 27, 28, or healthy control) at 37°C for 2 hours, and the A_2B_2 heterotetramer assembly was estimated by ELISA. **(C)** Inhibition of heterotetramer assembly by type Aa IgG. One mg of IgG purified from serum (type Aa case 10 or healthy control) using protein A-Sepharose was reacted with 100 ng of rFXIII-A at 37°C for 1 hour. Two hundred ng of rFXIII-B were added and further incubated at 37°C for 30 min. rFXIII-A was recovered using an anti-FXIII-A mAb and protein A-Sepharose, and co-precipitated FXIII-B was visualized by Western blotting using an anti-FXIII-B antibody.

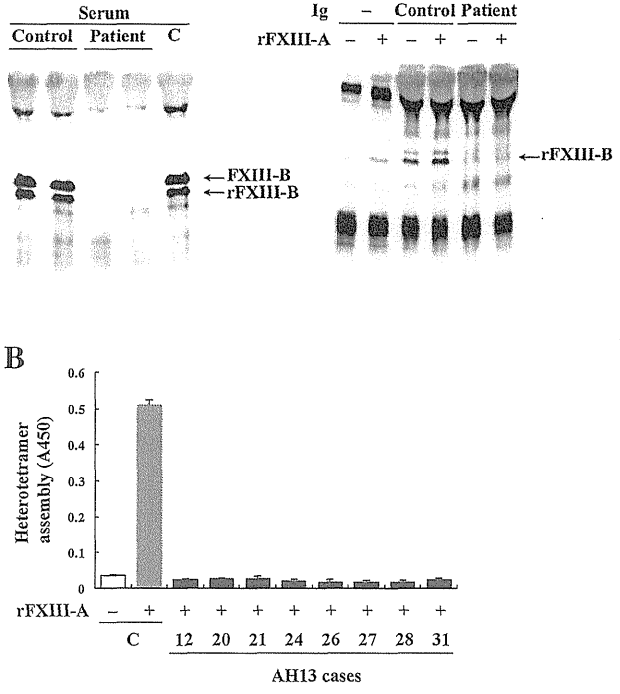
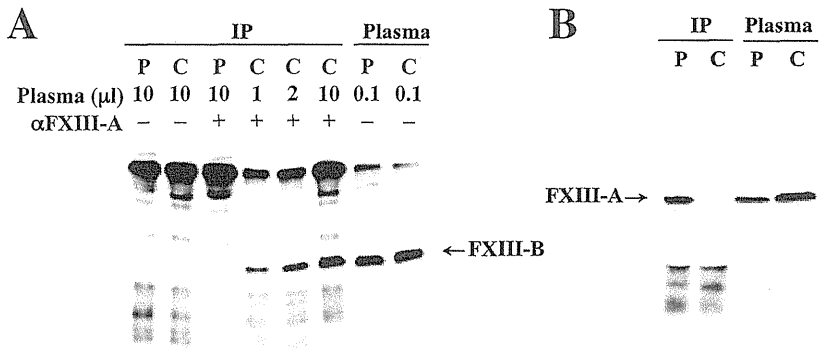


Fig. S5. Immunoprecipitation of FXIII from plasma of type Aa patient.

(A) Ten mL of type Aa (case 10, P) or 1, 2, or 10 mL of healthy control plasma (C) were reacted with anti-FXIII-A mAb at room temperature for 1 hr. Antibodies were collected using Protein A-Sepharose, and the resin was washed three times with TBS-T and boiled with 50 mL of SDS-sample buffer without 2-mercaptoethanol. Fifteen mL of the supernatant (IP) were subjected to SDS-PAGE followed by Western blotting using an anti-FXIII-B antibody. **(B)** Ten-mL plasma from a type Aa case (case 10, P) or healthy control (C) were reacted with Protein A-Sepharose (10 mL) at room temperature for 1 hour. The resin was collected by centrifugation, washed three times with 0.1% Tween 20, 20 mM Tris (pH 7.5) and 150 mM NaCl (TBS-T), and boiled with 30 mL of SDS-reducing buffer. Ten μL of the supernatant (IP) and 1/50-dilution of plasma were subjected to SDS-PAGE, followed by Western blotting using an anti-FXIII-A antibody.



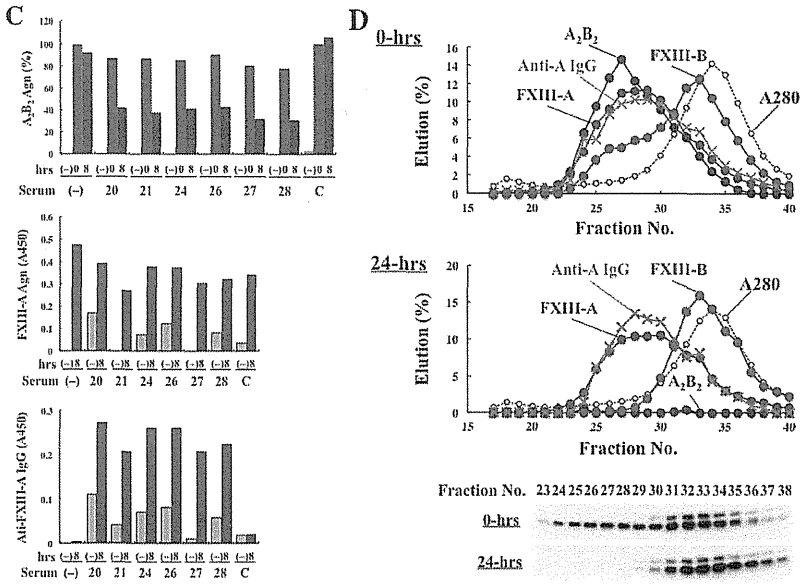
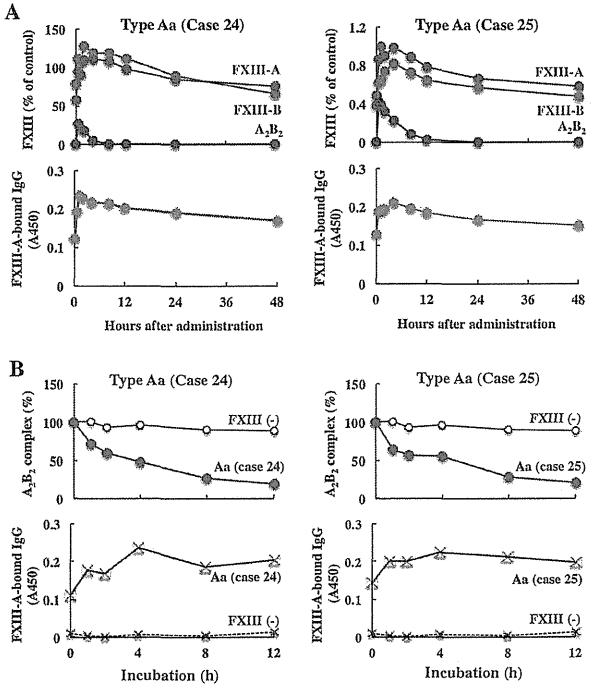


Fig. S7. Removal of FXIII-A from FXIII heterotetramer by type Aa anti-FXIII-A autoantibodies.

(A) Plasma-derived FXIII concentrates (A_2B_2) were infused into two type Aa patients (case 24 or case 25), and FXIII-A (red circles), FXIII-B (blue circles), and A_2B_2 (black circles) antigens, as well as FXIII-A-bound IgG (orange circles) were measured by ELISA at the indicated time intervals. **(B)** rA_2B_2 was mixed with plasma of case 24 or case 25 *in vitro*, and the plasma levels of A_2B_2 antigen (red circles) and FXIII-A-bound IgG (purple x) were measured by ELISA. rA_2B_2 was also incubated with FXIII-A-deficient plasma, and A_2B_2 antigen (open circles) and FXIII-A-bound IgG (black x) were measured. **(C)** Forty ng of rA_2B_2 were incubated with 4 mL of serum (cases 20, 21, 24, 26, 27, 28, or healthy control) at 37°C for 0 or 8 hours, and FXIII-A-bound FXIII-B, FXIII-A and FXIII-A-bound IgG were determined by ELISA. **(D)** Two mg of rA_2B_2 were incubated with 0.1 mL of case 24 serum at 37°C for 0 or 24 hours, and applied to a Sepharose CL6B column. FXIII-A, FXIII-B, FXIII-A-bound FXIII-B, and FXIII-A-bound IgG in the fractions were determined by ELISA. FXIII-B in the fractions was also detected by Western blotting using an anti-FXIII-B antibody.



Rapid immunochromatographic test for detection of anti-factor XIII A subunit antibodies can diagnose 90 % of cases with autoimmune haemorrhaphilia XIII/13

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Summary

Autoimmune haemorrhaphilia XIII/13 (AH13) is an acquired life-threatening bleeding disorder due to anti-factor XIII (FXIII) autoantibodies (auto-Abs). AH13 patients may die of haemorrhage without correct diagnosis and proper treatment because of lack of awareness and the absence of rapid easy-to-use tests specific for this disease. Currently, the definitive diagnosis is established by cumbersome and time-consuming laboratory tests such as dot-blot assays and enzyme-linked immunosorbent assays (ELISA), and therefore these tests are generally not carried out. To save AH13 patients' lives, there is an urgent necessity for developing a rapid test for FXIII auto-Abs. We first generated and characterised mouse monoclonal antibodies (mAb) against human FXIII A subunit (FXIII-A), and then developed a rapid immunochromatographic test (ICT) for detection of anti-FXIII-A auto-Abs using one mAb with a dissociation constant of 9.3×10^{-11} M. The auto-Ab-FXIII-A complex was captured by the mAb on a nitrocellulose membrane and visualised by Au-conjugated anti-human IgG Ab. Mixing with healthy control plasma improved the detection of auto-Abs in patients having extremely low levels of FXIII-A. The specificity and sensitivity of the ICT were 87 % and 94 %, respectively. We also detected auto-Abs against activated FXIII (FXIIIa) in three patients by pre-converting FXIII to FXIIIa by thrombin treatment. ICT values were significantly inversely correlated with FXIII activity levels, indicating an association between the quantity of anti-FXIII autoantibodies and AH13. This reliable rapid ICT assay can be applied to a point-of-care test to detect anti-FXIII-A auto-Abs, and will contribute to early diagnosis and treatment of AH13.

Keywords

Acquired coagulation disorders, autoantibodies, autoimmune diseases, factor XIII / transglutaminases, diagnosis management

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Introduction

Factor XIII (FXIII) is a plasma pro-transglutaminase consisting of a catalytic A subunit (FXIII-A) dimer and a carrier B subunit (FXIII-B) dimer that circulates in the blood as a heterotetramer. During the final stage of blood coagulation, FXIII is activated by thrombin and Ca^{2+}, and the resultant activated FXIII (FXIIIa) crosslinks the γ-glutamyl-ε-lysine residues in fibrin and the α_2-plasmin inhibitor as well as fibrin monomers to form stable fibrin clots with increased resistance to mechanical stresses and fibrinolysis (1–4).

Acquired FXIII deficiency is mainly caused by a secondary FXIII reduction via hypo-synthesis and/or hyper-consumption due to a primary disease(s) (4, 5). However, anti-FXIII autoantibodies (auto-Abs) cause acquired haemophilia-like disease (or haemorrha-philia; termed AH13 in this manuscript), which manifests more severe bleeding symptoms than non-autoimmune haemorrhagic acquired FXIII deficiency (AFD) (5, 6).

Acquired FXIII inhibitors, especially auto-Abs, are classified into three major types: type I inhibitors that prevent the activation of FXIII (7–10); type II inhibitors that interfere with the transaminase activity of FXIIIa; and type III inhibitors that are directed against the fibrin itself, blocking the crosslinking sites for access to FXIIIa. This classification applies to anti-fibrin auto-Abs but not to anti-FXIII-B auto-Abs (11, 12). Therefore, we recently proposed classifying anti-FXIII auto-Abs into types Aa, Ab, and B by their immunological properties, i.e. directed against FXIII-A, FXIIIa, and FXIII-B, respectively (unpublished data). As of April 2014, approximately 80 %, 10 %, and 10 % of our 32 AH13 patients belonged to types Aa, Ab, and B, respectively.

AH13 is thought to be rare (13, 14). However, the number of patients has recently been increasing in Japan (15), probably because Japan has become a so-called "super-aging" society. In fact, we have diagnosed 44 Japanese AH13 cases during the last 11 years (as of October 2014; unpublished data), while only eight Japanese AH13 cases were reported by other researchers before 2000, to the authors' best kenowlege. In addition, 17 non-Japanese AH13 cases were documented in the last century (4, 13). AH13, however, is not well known even among Japanese physicians and some patients never receive the correct diagnosis and proper treatment.

Many patients with AH13 are considered to have an unexplained bleeding disorder because decreased FXIII activity cannot be detected by routine coagulation tests such as the prothrombin time and activated partial thromboplastin time tests.

The definitive diagnosis of AH13 can currently be established only through time-consuming and expensive laboratory tests, such as the dot-blot assay and enzyme-linked immunosorbent assay (ELISA) that are carried out in a limited number of medical facilities. Therefore, a rapid point-of-care test (POCT) (16–19) for AH13 is necessary because a prompt differential diagnosis between AH13 and AFD is essential for proper treatment.

In this study, we generated and characterized mouse monoclonal antibodies (mAbs) against human FXIII-A, and developed an immunochromatographic test (ICT) that rapidly detects anti-FXIII auto-Abs to diagnose AH13.

Materials and methods

Materials

Recombinant FXIII-A (rFXIII-A) was kindly provided by Zymogenetics (Seattle, WA, USA). Recombinant FXIII-B (rFXIII-B) was expressed by the baculovirus system and purified as previously described (20). Anti-FXIII-A and anti-FXIII-B polyclonal antibodies (pAbs) were purchased from Calbiochem (San Diego, CA, USA) and each immunoglobulin G (IgG) was purified and biotinylated as previously described (21). A horseradish peroxidase (HRP)-conjugated anti-mouse IgG, HRP-streptavidin, Protein G-Sepharose, and CNBr-activated Sepharose 4B were obtained from GE Healthcare Bioscience AB (Uppsala, Sweden). A Tetramethylbenzidine (TMB) Peroxidase Substrate Kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Bovine chymotrypsin, thrombin, and Gly-Pro-Arg-Pro(GPRP)-NH_2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was purchased from Wako Pure Chemical Ind. (Osaka, Japan). FXIII-A peptides were synthesised by Sigma-Genosys (Hokkaido, Japan).

Production of in-house mouse mAbs against human FXIII-A

Female six-week-old BALB/c mice were subcutaneously immunised every two weeks three times with purified human plasma-FXIII emulsified in complete Freund's adjuvant. Four days after intraperitoneal booster injections of FXIII, the mouse splenic cells were fused with NS-1 myeloma cells. Hybridomas producing a large quantity of anti-FXIII mAbs were screened for their ability to bind to purified FXIII by a sandwich ELISA using a rabbit anti-mouse γ-globulin antibody. After cloning by limiting dilution several times, the selected cell lines were cultured, and monoclonal antibodies (mAbs) were purified from culture supernatants by 40 % ammonium sulfate precipitation and gel-filtration using a Sephacryl S-200 column after digestion with pepsin.

ELISA

ELISA was performed as described previously (21) with several modifications. IgGs of anti-FXIII-A and anti-FXIII-B pAbs were coated for the measurement of FXIII-A and FXIII-B, respectively. Three doses (1, 5, and 25 ng) of rFXIII-A or rFXIII-B were then applied and incubated, followed by incubation with in-house mAbs (1TH2–8C4C, 1TH6–2H7F, and 1TH6–10E; 3.3 nM each).

To determine the dissociation constant (K_d) of in-house mAbs and rFXIII-A, ELISA was performed with increasing concentrations (0.07–4.3 nM) of the mAbs and a fixed concentration (5 ng, 0.6 nM) of rFXIII-A. Double reciprocal plots of the ELISA signal versus the concentration of the mAbs were evaluated. A K_d value for each mAb was determined as previously described (22).

Dot-blot analyses

Denatured rFXIII-A was prepared by boiling rFXIII-A in 125 mM Tris-buffer (pH 6.8) containing 0.1 % SDS. Native and denatured rFXIII-A (1, 10, and 100 ng) was spotted on nitrocellulose membranes. A dot-blot analysis was performed as previously described (23). One of the mAbs against FXIII-A and HRP-conjugated anti-mouse IgG was used for the primary and secondary antibodies, respectively.

The effect of mAbs on the FXIII activation or FXIIIa activity

To detect the inhibitory effects of mAbs against FXIII activation or FXIIIa activity, rFXIII-A (100 ng) was pre-incubated with mAbs (5 μg) at 37 °C for 1 hour (h) or thrombin (1 U) at 37 °C for 15 minutes (min), and then incubated with thrombin (1 U) at 37 °C for 15 min or mAbs (5 μg) at 37 °C for 1 h.

A standard amine-incorporation (AI) assay was performed using 5 mM $CaCl_2$, 0.2 % N,N-dimethylcasein, 2 mM monodansylcadaverine and 1 U bovine thrombin as previously described (8, 10, 21).

The effect of mAbs on the formation of the FXIII-A_2B_2 heterotetramer

Four doses (0.2, 1, 5, and 25 μg) of mAbs were incubated with rFXIII-A (1 μg) in 2 % bovine serum albumin overnight at 4 °C and subsequently incubated with rFXIII-B (1 μg) in 20 μl for 20 min on ice. The FXIII-A_2B_2 heterotetramer was detected by ELISA as previously described (21).

Proteolytic digestion and western blot

Proteolysis of rFXIII-A (2 mg/ml) with trypsin (using an enzyme-to-substrate ratio E/S 1/500, w/w) was carried out at 37 °C for 30 min in 20 mM Tris-buffered saline (TBS), pH 7.5 containing 10 mM $CaCl_2$. A western blot of the digested product was performed as previously described (23) with several modifications. One of the mAbs against FXIII-A and HRP-conjugated

anti-mouse IgG was again used for the primary and secondary antibodies, respectively.

Epitope analysis using FXIII-A peptide-coated plates by ELISA

Microtitre plates were coated with FXIII-A peptides (2.5 nmol; Suppl. Table 1, available online at www.thrombosis-online.com) in 100 µl of 50 mM carbonate buffer (pH 9.6) overnight at 4 °C. The plates were incubated with in-house mAbs (500 ng), and subsequently incubated with HRP-conjugated anti-mouse IgG. The reaction with the TMB substrate and the termination of the reaction were performed as described (21).

Epitope analysis using mass spectrometry-based identification of digested fragments bound to each mAb

Each anti-FXIII-A mAb-Sepharose (1.2 mg mAb/ml) column was prepared by coupling CNBr-activated Sepharose 4B with the in-house mAb according to the manufacturer's protocol. The rFXIII-A was then incubated with mAb-Sepharose at 4 °C for 2 h, followed by digestion using chymotrypsin (E/S = 1/100) at 37 °C for 6 h. After washing with TBS containing 0.1 % Tween 20 and deionised water, bound peptides were eluted by 0.1 M glycine buffer (pH 2.5), neutralised by 1.5 M Tris-buffer (pH 8.8), and subjected to carbamidomethylation with iodoacetamide. For mass spectrometry (MS) identification, the samples were desalted using a C-Tip (Nikkyo Technos, Tokyo, Japan) according to the manufacturer's protocol. To determine the ionisation efficiency of each peptide, rFXIII-A without mAbs was also digested using chymotrypsin, and carbamidomethylation and desalting were performed as described above.

Nanoflow liquid chromatography-tandem MS (nanoLC-MS/MS)

The desalted peptide solution was analysed by nanoLC-MS/MS as described previously (24) with several modifications, using the EASY-nLC 1000 system (Thermo Scientific, Hudson, NH, USA) on a nano-capillary column (NTTC-360/75-3, Nikkyo Technos). The nanoflow system was connected to a Quadrupole Orbitrap (Q-Exactive, Thermo Scientific) mass spectrometer equipped with a nanoelectrospray emitter. The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition. The full-scan spectra (m/z range 350–1800) were acquired. The 10 most intense ions were sequentially isolated and fragmented by higher-energy C-trap dissociation (25) at a normalised collision energy of 28 %.

Peptide identification

Raw file reads were searched against the Swiss-Prot human database (542,503 sequences) using Proteome Discoverer (version 1.4, Thermo Scientific) with the Sequest HT and Mascot (version 2.3, Matrix Science, Tokyo, Japan) search engines. Precursor and fragment mass tolerances were set to 5 ppm and 0.02 Da, respectively. Cysteine carbamidomethylation was set as a static modification. The results were filtered using Percolator with a false discovery rate of 1 %. The peak area of each identified peptide was estimated using Proteome Discoverer.

Clinical samples

The authors consulted physicians in charge of unexplained haemorrhagic patients (14, 15). From June 2009 to July 2014, patients with severe bleeding who did not have a personal or family history of bleeding or abnormal clotting times were recruited for this study. Control plasma samples were obtained from healthy volunteers. This study was approved by the Institutional Review Board of the Yamagata University School of Medicine. All procedures were conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all individuals.

ICT for the detection of anti-FXIII-A auto-Abs

The entire ICT procedure is summarised and an example is shown in Suppl. Figure 1 (available online at www.thrombosis-online.com). The in-house mAb, 1TH2–8C4C, was dispended onto a nitrocellulose strip (test line). Patient or healthy control plasma samples (1:10 dilution) were applied to a 96-well microtitre plate. The strip was then inserted into a well for 5 min at 37 °C, and after three washes, the strip was immersed in a solution containing the anti-human IgG Ab-gold conjugate for 15 min at 37 °C. Reactions were read using a reader device (FactScan, Denken Co., Ltd., Oita, Japan) and expressed as an absorbance unit relative to that of a plasma sample from an AH13 patient (AH13-Aa-1) (assigned as 1 arbitrary unit, AU).

Spiked ICTs

Equal volumes of patient and commercially available pooled normal plasma (Sysmex, Kobe, Japan) were mixed and incubated at 37 °C for 2 h prior to ICT (termed spiked ICT). To this reaction mixture, an equal volume of thrombin (0.01 U) in TBS containing 10 mM $CaCl_2$ with or without 1 mM GPRP-NH_2 was added and incubated at 37 °C for 2 h. Samples were then diluted 5-times with a dilution buffer for ICT.

Comparison of ICT and conventional dot-blot analysis

Conventional dot-blot analyses of clinical samples for the detection of anti-FXIII-A auto-Abs were performed as previously described (23). The sensitivity of the dot-blot analysis and direct and spiked ICTs was calculated from the results of 16, 15, and 16 samples from patients with AH13, respectively. The specificity of these tests was calculated from results of 23 patients with AFD. To assess the agreement between direct or spiked ICT and the dot-blot analysis, the kappa coefficients were calculated as previously described (26).

Statistical analysis

For statistical analysis, values were expressed as mean ± standard deviation. Comparisons between groups were performed using Kruskal-Wallis tests of SAS Enterprise Guide 6.1 (SAS Institute, Cary, NC, USA). Differences were considered significant at p-value of <0.05. Receiver operating characteristic (ROC) curve was used to determine the optimal cutoff values and the areas under curve (AUC) for ICT data.

Results

Characterisation of mouse mAbs against human FXIII-A

We first characterised three in-house mAbs for use in development of an ICT for detection of anti-FXIII-A auto-Abs; all mAbs readily detected native rFXIII-A at concentrations of 10 and 100 ng by dot-blot analyses (data not shown). Denatured rFXIII-A, however, was detected unambiguously only at 100 ng. Therefore, these mAbs recognized native rFXIII-A approximately 10 times more efficiently than denatured rFXIII-A.

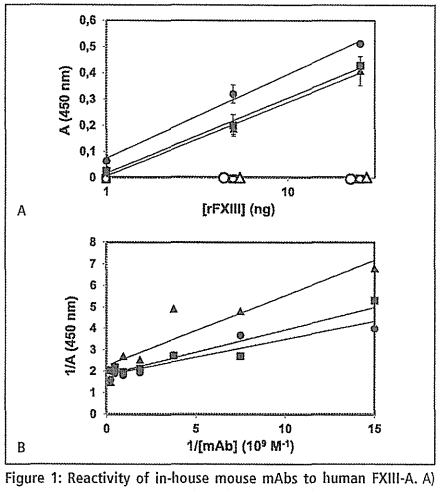

Figure 1: Reactivity of in-house mouse mAbs to human FXIII-A. A) Anti-FXIII-A mouse monoclonal antibodies (mAbs) (50 ng; 3.3 nM), 1TH2–8C4C (closed triangles, open triangles), 1TH6–2H7F (closed triangles, open triangles), or 1TH6–10E (closed squares, open squares) recognised rFXIII-A. Three doses (1, 5, 25 ng) of rFXIII-A (closed circles, closed triangles, closed squares) or rFXIII-B (open circles, open triangles, open squares) were captured by anti-FXIII-A or anti-FXIII-B pAbs (1 µg each), respectively. Data are presented as the means of two replicates with standard deviations (SD). A, absorbance. B) A double reciprocal plot of the ELISA signal versus the concentration of anti-FXIII-A mAbs. A fixed concentration of rFXIII-A (5 ng; 0.6 nM) was captured by anti-FXIII-A pAb (1 µg), and detected by increasing concentrations (0.07–4.3 nM) of mAbs, 1TH2–8C4C (closed circles), 1TH6–2H7F (closed triangles), or 1TH6–10E (closed squares). Data are presented as the means of two replicates.

All of the three mAbs bound to rFXIII-A in a dose-dependent manner in ELISA (▶ Figure 1A). We also determined a K_d value for each mAb against FXIII-A using double reciprocal plots of the ELISA signal versus the concentration of mAbs (▶ Figure 1B). The plots were linear and the K_d values of 1TH2–8C4C, 1TH6–2H7F, and 1TH6–10E were 9.3×10^{-11}, 1.4×10^{-10}, and 1.2×10^{-10} M, respectively.

When these mAbs were pre-incubated with rFXIII-A prior to thrombin activation, AI activities in the presence of 1TH2–8C4C, 1TH6–2H7F, and 1TH6–10E were 116 ± 5, 117 ± 9, and 122 ± 14 % (n = 3), respectively. These values were slightly higher compared with that obtained in the absence of mAbs (assigned as 100 %). Because we did not inactivate thrombin, mAbs may have protected rFXIII-A from extensive digestion of rFXIII-A by thrombin (incubated for a total of 45 min) and its consequent degradation.

We also tested whether mAbs inhibit FXIIIa pre-activated by thrombin. AI activities of FXIIIa in the presence of 1TH2–8C4C, 1TH6–2H7F, and 1TH6–10E were 174 ± 3, 160 ± 3, and 183 ± 11 % (n = 3), respectively. These values were considerably higher compared with the values obtained in the absence of mAbs (100 %) (incubation for a total of 1 h and 45 min). These results indicate that the mAbs do not inhibit either FXIIIa activity or FXIII activation. In addition, all three mAbs may bind near the FXIIIa inactivation site between the core and barrel-1 domains.

Finally, the heterotetramer formation was, in the presence of 0.2, 1, 5, and 25 µg of 1TH2–8C4C, 82, 71, 134, and 101 %, respectively, compared with that in the absence of mAbs (100 %). The heterotetramer formation was 91, 116, 137, and 87 % in the presence of 1TH6–2H7F, and 121, 117, 83, and 107 % in the presence of 1TH6–10E, respectively. These results indicate that the three mAbs do not inhibit FXIII-A_2B_2 heterotetramer assembly.

Epitope analysis of each mouse mAb

Western blot analysis of trypsin-digested rFXIII-A was performed to determine the approximate regions recognised by mAbs. There were three major bands, 24, 54, and 76 kDa visualised by Coomassie staining, corresponding to the barrel-1 and -2, β-sandwich and core, and β-sandwich and core and barrel-1 and -2 domains, respectively. However, the 24 and 76 kDa bands, but not the 54-kDa band, were detected by all three mAbs, indicating that all three mAbs recognise the barrel-1 or barrel-2 domain.

We used 35 synthesised peptides with sequences covering 83 % of the FXIII-A molecule to localise the epitope(s) of each mAb (Suppl. Table 1, available online at www.thrombosis-online.com). All mAbs bound to the FXIII-A-peptides (P) 3, 5, 26, 27, 28, and 29 coated on plates and generated strong ELISA signals over 0.05 (absorbance at 450 nm, Suppl. Figure 2A, available online at www.thrombosis-online.com). There were some differences in the binding intensity to the peptides between these mAbs, e. g. 1TH2–8C4C bound to P5

and P28, 1TH6–2H7F to P27 and P29, and 1TH6–10E to P3 and P26 more strongly than the other two mAbs, respectively. These results suggested that the epitopes of the mAbs were organised as epitopic mosaics distributed around the β-barrel-1 and/or β-sandwich domains of the FXIII-A molecule, and thus these might be discontinuous 'structural/conformational' epitopes.

Epitope mapping of each mouse mAb by MS of enzyme-digested fragments

To further localise the mAbs epitopes rFXIII was digested with chymotrypsin, and the rFXIII-A-derived fragments bound to each mAb were identified using MS. Chymotrypsin digestion produced 79, 58, and 56 fragments of rFXIII-A bound to 1TH2–8C, 1T6–2H7F, and 1TH610E, respectively, and of these nine, seven, and six major fragments were detected, respectively, with peak areas of over 10^9 (1.000E9), that originated from the β-sandwich and barrel-1 domains of FXIII-A (Suppl. Figure 2B and Suppl. Table 2, columns 11–13 highlighted, available online at www.thrombosis-online.com). In contrast, among 80 chymotrypsin-digested fragments derived from the same amount of rFXIII-A alone (Total), 54 fragments had peak areas of more than 10^9 and originated from domains throughout the entire FXIII-A molecule (Suppl. Figure 2B and Suppl. Table 2, total area in column 14 highlighted, available online at www.thrombosis-online.com).

The relative peak areas of the major mAb-bound fragments to the Total areas (Suppl. Table 2, column 14, available online at www.thrombosis-online.com) were estimated (in parentheses). The estimated relative area of FXIII-A-(amino acid numbers145–157), -(581–588), -(581–594), and -(607–619) bound to 1TH2–8C4C, those of FXIII-A-(581–588) and -(607–619) bound to 1TH6–2H7F and 1TH6–10E, and that of FXIII-A-(581–594) bound to 1TH6–2H7F were more than 0.5.

These results clearly indicated that the epitopes of all three mAbs were located primarily in the β-barrel-1 domain.

Development of a direct ICT using the in-house mAb

We developed an ICT for detection of anti-FXIII-A auto-Abs using 1TH2–8C4C because it demonstrated the highest affinity to rFXIII-A among the three mAbs. We first carried out 20 trial runs of a healthy control plasma sample on different days to assess the reproducibility of the ICT. The mean and standard deviation (SD) of the test line signal intensity were 0.09 and 0.03 AU, respectively.

We then determined a mean and SD using the plasma of 24 healthy controls to establish a cut-off value of the test (▶Figure 2A). The mean and SD of the test line intensity were 0.08 and 0.05 AU, respectively. We therefore established the cut-off value of 0.18 AU for the present ICT using the mean plus 2 SDs.

Evaluation of the direct ICT for detection of anti-FXIII-A auto-Abs in clinical samples

We performed the ICT on plasma samples from 38 AH13-suspected patients to determine whether anti-FXIII-A auto-Abs were present. As a result, 12 samples yielded a positive reaction in the ICT, while 26 samples yielded a negative reaction (▶Figure 2B and C). The conventional dot-blot assay used for definitive diagnosis classified 14 samples as positive for anti-FXIII-A auto-Abs and 24 samples negative (Suppl. Table 3, available online at www.thrombosis-online.com).

One (AFD-10) of the 12 positive samples assessed using ICT was considered to be a false positive because of the negative results obtained with the dot-blot assay and cross-mixing tests (data not shown). However, four of the 26 negative samples determined by ICT were false negatives. Therefore, the specificity of the ICT to detect anti-FXIII-A auto-Abs was 96% (22/23), while the sensitivity was 73% (11/15). A kappa coefficient of 0.77 was in the "good to fair" agreement range between the ICT and dot-blot assays.

Evaluation of spiked ICT for detection of anti-FXIII-A auto-Abs

Several patients with AH13 have extremely low levels of FXIII-A, and therefore the quantity of a quadripartite complex consisting of mAb (1TH2–8C4C), FXIII-A, auto-Abs, and anti-human IgG Ab-gold conjugate is not sufficient to be detected by the ICT. This is the most probable reason why the sensitivity of the direct ICT was not high. We therefore spiked an equal volume of healthy control plasma, 2 h prior to ICT, to provide sufficient FXIII-A for detection by ICT (Suppl. Figure 1C, available online at www.thrombosis-online.com).

We applied this spiked ICT to plasma samples from 39 AH13-suspected patients. One patient was examined by spiked ICT alone, when we did not have enough assay reagents. As a result, 18 cases yielded a positive reaction in the spiked ICT, while 21 cases yielded a negative reaction. Three (AFD-9, AFD-10 and AFD-19, ▶Figure 3A) of the 18 positive samples assessed using spiked ICT were considered to be false-positives, as described above, while one (type B AH13 patients) of the 22 negative samples were considered to be a false-negative. Therefore, the specificity of the spiked ICT was 87% (20/23), while the sensitivity was 94% (15/16). The kappa coefficient of 0.79 was in the "good to fair" agreement range between the spiked ICT and the dot-blot analyses.

Performance of spiked ICT for detection of auto-Abs in patients with various types of AH13

The ICT spiked with healthy control plasma could detect auto-Abs in all patients with AH13 type Aa (from AH13-Aa-1 to -Aa-14) and one type Ab (AH13-Ab-3) (▶Figure 3B), but not in a type B case (AH13-B-3). We therefore applied the spiked ICT to various AH13 cases including types Aa, Ab, and B to evaluate its performance. All samples from AH13 type Aa cases, as well as two type Ab and one B cases (AH13-Ab-1 and -3, and AH13-B-1) yielded positive reactions, while the remaining three cases (AH13-Ab-2 and AH13-B-2 and 3) yielded negative reactions (▶Figure 3B).

Modification of spiked ICT with FXIIIa for detection of AH13 type Ab

In order to improve the detection of AH13 type Ab, we spiked ICT with rFXIIIa pre-activated by thrombin because AH13 type Ab preferentially reacts with FXIIIa. As a result, we successfully detected auto-Abs in samples from all patients with type Ab (▶Figure 4A), but not in a healthy control plasma. This was also true for when the ICT spiked with healthy control plasma was performed after thrombin-treatment in the presence of calcium and the resultant serum was used (▶Figure 4B).

Correlation between ICT values and FXIII levels

Both direct and spiked ICTs (▶Figure 5) were significantly inversely correlated with FXIII activity levels but not with those of FXIII antigen in AH13 cases (Suppl. Figure 3, available online at www.thrombosis-online.com). Therefore, high ICT values may be implicated in pathogenic and pathologic conditions, i.e. a severe FXIII deficiency in AH13 patients.

Evaluation of direct and spiked ICTs by ROC curve analysis

We performed ROC curve analysis of our direct ICT by which patients with or without AH13 (24 healthy control, 23 AFD and 19 AH13) were diagnosed. As a result, the best cut off value of direct ICT was turned out to be 0.14 AU (Suppl. Figure 4, available online at www.thrombosis-online.com); the specificity and sensitivity of direct ICT were 89% (42/47) and 68% (13/19), respectively. The area under curve (AUC) was 0.74, which could be judged as moderately accurate (0.7<AUC≤0.9) according to an arbitrary guideline (27). The specificity and sensitivity of direct ICT with a cut-off value of 0.18 AU (average + 2SD) were 94% (44/47) and 58% (11/19), respectively.

We also performed ROC curve analysis of spiked ICT by which patients with or without AH13 (24 healthy controls, 23 AFD and 20 AH13) were diagnosed. As a result, the optimal cut-off value for our spiked ICT was 0.23 AU; the specificity and sensitivity of spiked ICT were 98% (46/47) and 80% (16/20), respectively. The AUC was 0.91, which could be judged as highly accurate (0.9<AUC<1). The specificity and sensitivity of spiked ICT with a cut-off value of 0.18 AU (average + 2SD) were 91% (43/47) and 80% (16/20), respectively. These results indicated that the cut-off

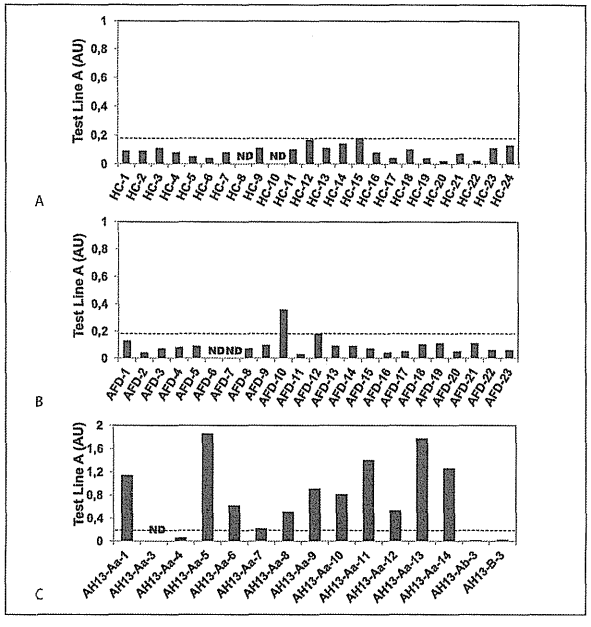

Figure 2: Development of a direct ICT to detect anti-FXIII-A auto-Abs. The absorbance is shown for 24 healthy control plasma samples (HC-1 to HC-24) (A), 23 samples from patients with AFD (AFD-1 through AFD-23) (B), and 15 samples from patients with AH13 (AH13-Aa-1, -Aa-3 through -Aa-14, -Ab-3, and B-3) (C). The cut-off value is indicated by a dashed line. ND, not detectable.