blot analysis, using her stored frozen plasma sample, were obtained 15 days after her death.

As the result of this case, the authors strongly recommend that FXIII activity be analyzed whenever physicians encounter patients with unexplained bleeding disorders, and especially when the results of routine clotting time tests such as assessments of aPTT and PT are within the normal or subnormal ranges. In the future, a quick point-of-care test(s) for the measurement of FXIII activity must be developed to make an early diagnosis of life-threatening AH13 [15] and treat the condition immediately [4,14].

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H.S., H.U., S.S. and K.T. collected the clinical data, M.S. and A.I. performed the laboratory examinations and analyzed the results, and H.S. and A.I. drafted the article.

Conflicts of interest

None of the authors declared any conflict of interests.

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CASE REPORT

Hemorrhagic-acquired factor XIII deficiency associated with tocilizumab for treatment of rheumatoid arthritis

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Abstract Factor XIII (FXIII) is the final enzyme in the coagulation cascade. Acquired FXIII deficiency is caused by inhibitors of FXIII or decreased synthesis and/or increased consumption of FXIII, which leads to severe bleeding. Recently, we experienced a case of hemorrhagic-acquired factor XIII deficiency that occurred during treatment with the IL-6 inhibitor tocilizumab for rheumatoid arthritis. A 48-year-old man was referred because of right hip pain due to a hematoma. Laboratory findings showed that routine coagulation tests were normal, while FXIII activity was slightly low (52.4 %). The patient was successfully treated with plasma-derived factor XIII concentrates. The time course of recovery suggests that tocilizumab might have inhibited FXIII production. To our knowledge, this is the first report of acquired factor XIII deficiency associated with administering of tocilizumab. When recurrent bleeding is seen during administering of tocilizumab, acquired factor XIII deficiency may have been induced, thus attending physicians should consider this disease in a differential diagnosis.

Keywords Acquired factor XIII deficiency · Biologics · Bleeding disorder · Rheumatoid arthritis

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Introduction

Factor XIII (FXIII), a fibrin stabilizing factor, is composed of A subunit (FXIII-A) and B subunit (FXIII-B) dimers, and circulates in blood as a heterotetramer, where it is activated by thrombin and crosslinked fibrin monomers. Congenital FXIII deficiency is an extremely rare autosomal recessive disorder, with an estimated incidence of approximately 1 in 2 million [1, 2]. The clinical features of defective fibrin stabilization are highlighted by severe bleeding in congenital deficiency cases [3]. Acquired FXIII deficiency results from specific autoantibodies [4, 5], or its overconsumption and/or hypo-biosynthesis by disseminated intravascular coagulation, major surgery, liver diseases, and other disorders known to be rarely complicated with bleeding symptoms [6]. The disease is difficult to diagnose, since screening coagulation studies routinely ordered, such as prothrombin time (PT) and activated partial thromboplastin time (APTT), yield normal results. Here, we report a case of hemorrhagic-acquired FXIII deficiency that occurred in a patient during treatment with tocilizumab for rheumatoid arthritis (RA) (stage II, class II). To the best of our knowledge, this is the first report suggesting the

participation of tocilizumab in the pathogenesis of acquired FXIII deficiency.

Case report

A 48-year-old man came to the emergency department with progressive pain in the right thigh and weakness of the quadriceps muscle. He had been diagnosed with RA and Sjögren's syndrome (SS) 5 years earlier. The patient was unable to extend his right hip because of pain and a mass lesion in the right thigh was palpable. There was no prior episode of trauma or contusion. Laboratory examinations upon admission revealed that WBC and C-reactive protein (CRP) levels were increased, while hematocrit, platelet count, PT, and APTT were within normal ranges (Table 1). Computed tomography (CT) showed a mass lesion 60 mm long, 70 mm wide, and 60 mm in anterior–posterior length in the pelvis. Magnetic resonance imaging (MRI) revealed a hyper-intense lesion with a short TI inversion recovery (STIR) sequence, with no enhancement by a gadolinium-containing contrast agent (Fig. 1). After admission, we suspected femoral nerve palsy due to the mass lesion, thus an open biopsy was performed, which only identified a fibrin clot. Tissue culture findings revealed no infection. At the time of open biopsy, one of our differential diagnoses was bleeding. However, we were not aware of the bleeding tendency because the routine coagulation tests were normal. The patient recovered after 3 weeks of rest and was subsequently discharged.

Twenty days after discharge, the patient came to the hospital again with progressive pain, swelling in the thigh, and subcutaneous bleeding in the right inguinal area. Laboratory tests at the second admission showed slightly reduced hematocrit and fibrinogen levels, with normal white blood cell count, differential cell count, CRP, platelets, PT, APTT, and bleeding time (Duke method), von Willebrand factor activity, determined by a Ristocetin cofactor assay, was also within a normal range (Table 1). CT showed a diffuse high-density area from the inguinal region to the gluteus medius muscle (Fig. 2). A rare coagulation disorder was suspected, because of recurrent bleeding episodes despite the normal APTT and PT levels. Accordingly, FXIII activity in plasma was determined using a Berichrom FXIII kit (Dade Behring, Marburg, Germany), which revealed a mild decrease (52.4 %).

A review of past history of medical treatment revealed that right total hip arthroplasty (THA) had been performed 18 months before the present episode, without bleeding complications. At the time of that procedure, no abnormalities were identified in regard to platelet count, or PT and APTT levels. For control of severe RA, administering of methylprednisolone at 5 mg/day, methotrexate at 12.5 mg/week, were already initiated before THA. Tocilizumab at 600 mg/month were administered after the right THA. He had never administered additional drugs starting from the first bleeding episode. Since the patient had not experienced bleeding complications prior to the

Table 1 Laboratory tests

	First hospitalization (day 1)	Second hospitalization (day 20)
Hematology		
WBC	*10300/μL*	4700/μL
Neu	83.7 %	63.8 %
Lym	8.0 %	23.2 %
Mon	7.1 %	11.1 %
Eos		
Baso		
RBC	*384 × 10⁴/μL*	349 × 10⁴/μL
Hb	12.9 g/dL	11.2 g/dL
Ht	40.4 %	35.4 %
Plt	27.6 × 10⁴/μL	28.9 × 10⁴/μL
Biochemistry		
TP	6.0 g/dL	6.0 g/dL
Alb	3.9 g/dL	4.0 g/dL
AST	26 IU/L	*45 IU/L*
ALT	*57 IU/L*	*78 IU/L*
T-Bil	0.8 mg/dL	1.0 mg/dL
D-Bil	0.3 mg/dL	0.3 mg/dL
r-GTP	*65 IU/L*	*66 IU/L*
IDH	329 IU/L	248 IU/L
BUN	14 mg/dL	15 mg/dL
s-Cre	0.7 mg/dL	0.7 mg/dL
CRP	*1.2 mg/dL*	<0.02 mg/dL
Coagulation tests		
APTT	29.2 s	28.3 s
PT	112 %	114 %
Fibrinogen	ND	*179 mg/dL*
Antithrombin	ND	119.0 %
Plasminogen	ND	105.0 %
Protein C	ND	125.0 %
von Willebrand	ND	*306.0 %*
Coagulation factor		
II	ND	112.0 %
V	ND	>150 %
VII	ND	142.6 %
VIII	ND	>150 %
IX	ND	116.0 %
X	ND	81.0 %
XI	ND	95.3 %
XII	ND	95.5 %
XIII	ND	*52.4 %*

Italics indicate abnormal values

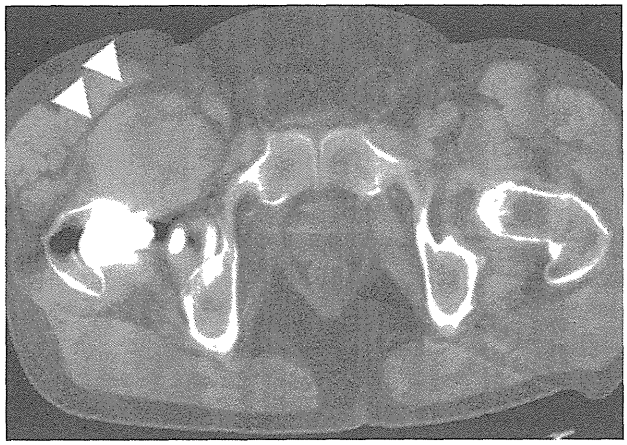

Fig. 1 Plain CT of pelvic cavity during first hospitalization. A mass lesion can be seen in front of the right femoral bone (*filled triangle*)

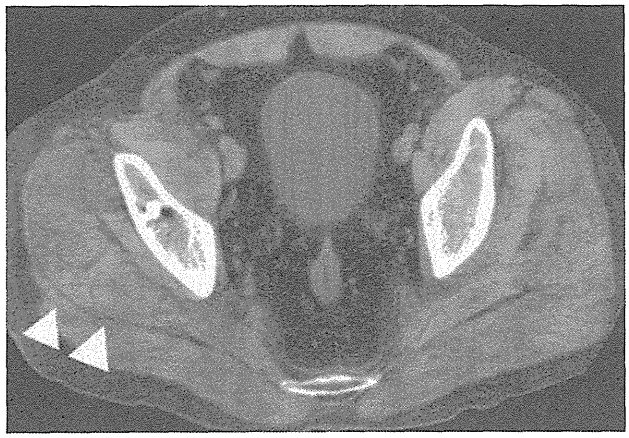

Fig. 2 Plain CT of pelvic cavity during the second hospitalization

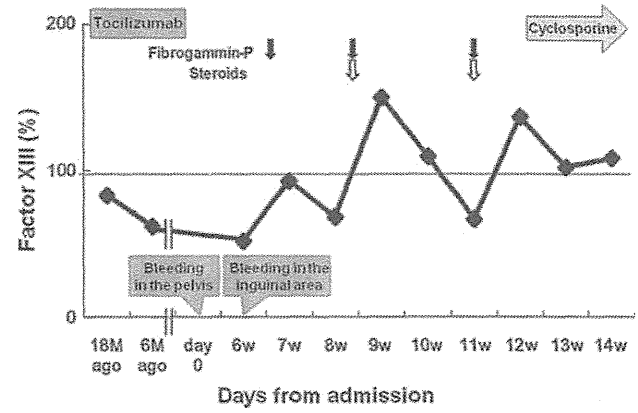

Fig. 3 Clinical course and FXIII activities. THA was performed 18 months prior. *Solid vertical arrows* with "Fibrogammin-P®" indicate 720 U/day of Fibrogammin-P® for 3 days, *open vertical arrows* with "steroids" show 2 courses of methylprednisolone at 1 g/day for 3 days, *arrow* with "cyclosporine" depicts 200 mg/day of cyclosporine, and *arrow* with "tocilizumab" indicates 600 mg/day of tocilizumab

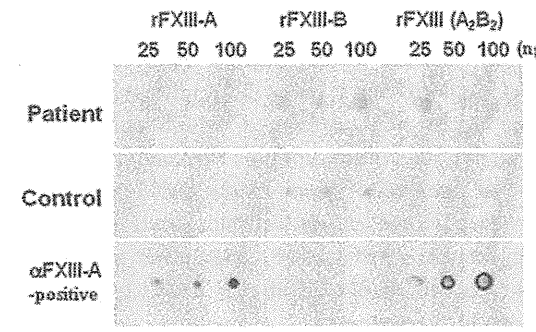

Fig. 4 Dot-blot test. No immunoglobulins that bind the recombinant A and B subunits of factor XIII (rFXIII-A and rFXIII-B) or rFXIII tetramer were detected. *Filter at the bottom* shows a representative sample obtained from a patient with anti-FXIII-A antibodies (Ref. [6])

present episode, the FXIII deficiency appeared most likely to have been acquired and not of hereditary origin. Furthermore, tocilizumab was considered to be causative of the FXIII deficiency, thus it was stopped 1 month before the first hospitalization. Following that stoppage, human plasma-derived FXIII (Fibrogammin-P®; CSL Behring GmbH, Marburg, Germany) was administered at 720 U/day for 3 days, which caused a rapid increase in FXIII, though it returned to a substantially low level after several days.

The clinical course led us consider the possibility of the existence of an inhibitor to FXIII. Steroid pulse therapy (2 courses of methylprednisolone at 1 g/day for 3 days) and immunosuppressive therapy (cyclosporine at 200 mg/day) were administered together with repeated injections of human plasma-derived FXIII (Fig. 3), after which FXIII activity returned to and remained at normal levels, and bleeding complications never reappeared. We conducted a sensitive dot-blot technique using the blood samples at second admission. But, we could not detect immunoglobulins G, M, and A, which bind the A and B subunits of FXIII (Fig. 4). Two months after second discharge, FXIII activity was gradually decreased (68.9 %) after resumption of tocilizumab. We conducted a cross-mixing test of patient plasma taken a half year after second admission with normal plasma. It showed a pattern of FXIII deficiency without existence of FXIII inhibitors (Fig. 5).

Discussion

Autoimmune diseases, such as systemic lupus erythematosus (SLE) and RA, are known to be accompanied by antibodies to coagulation factors, resulting in a mild to severe bleeding tendency. There are several reports of autoimmune diseases in patients possessing an FXIII

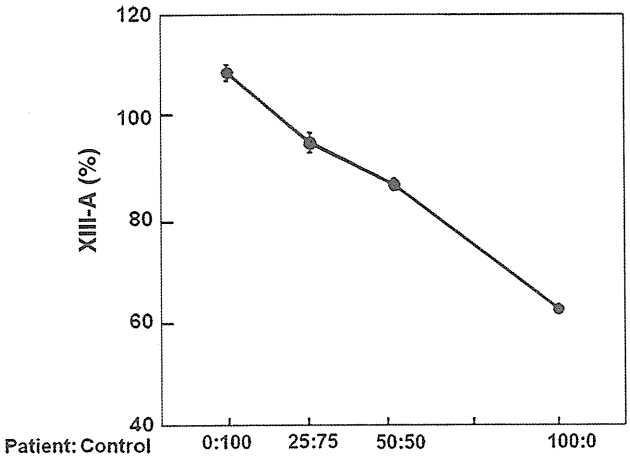

Fig. 5 Four-step dilution cross-mixing test using ammonia release assay for FXIII activity (XIII-A). A deficiency pattern is shown

inhibitor [4, 7]. Initially, the bleeding symptom in our patient was also considered to be due to the presence of an FXIII inhibitor, because of the underlying disease, RA/SS. However, at a later stage of the clinical course, results of a cross-mixing test and dot-blot technique did not reveal the existence of FXIII inhibitors (Figs. 4, 5), while they suggested an FXIII deficiency.

Hemorrhagic-acquired FXIII deficiency is generally characterized by an extreme decrease in FXIII activity, which leads to severe bleeding. However, in the present case, depression of FXIII activity was mild as compared with typical cases. Even in heterozygotes with congenital FXIII deficiency with 50 % FXIII activity, obvious bleeding following provocation, such as surgery, dental extraction, and trauma, has been reported [8]. A recent review also noted that the former description of 5 % plasma FXIII activity being sufficient for normal hemostasis, based on observations of congenital FXIII deficiency cases, is not adequate, because the FXIII level to delineate the boundary between hemostasis and hemorrhage might be much higher, and may also become more controversial in both congenital and acquired settings [6]. In the present case, tissue damage after THA and mild FXIII deficiency may have functioned together in an additive manner for the manifestation of bleeding symptoms.

Our patient developed RA 5 years before the onset of symptoms and THA was performed without any bleeding complications 1 year before onset. After that THA procedure, tocilizumab treatment for RA was started. This time course suggests that administering of tocilizumab inhibited the production of FXIII, thus we retrospectively examined FXIII activity in frozen blood samples obtained prior to beginning tocilizumab. FXIII activity was normal at the time of the THA operation (83.1 %), then FXIII activity was decreased to 62.3 % at 6 months after administering of tocilizumab. By the time of the inguinal hematoma episode, that activity had decreased further (52.4 %), as shown in Fig. 3. We considered that tocilizumab directly reduced FXIII under the pathological condition of RA/SS, as the FXIII activity returned to a normal range after stopping tocilizumab. RA and/or major surgery might decrease factor XIII activity; however, that activity in the perioperative period when the serious bleeding occurred was in a normal range (83.1 %). As for RA, the patient was treated for 4 years before coming to us and factor XIII activity before the first infusion of tocilizumab infusion was also in a normal range (83.1 %). These results indicate that tocilizumab-reduced FXIII production was not the direct cause of THA or RA.

In our case, several months had been needed to recover FXIII activity from stopping tocilizumab. We cannot clearly explain about the long-term adverse effect of tocilizumab to FXIII activity because the pathological mechanism of tocilizumab-induced FXIII deficiency is unknown. Iwasa et al. [9] recently reported the case of multiple ulcers in the small and large intestines as the adverse effect of 3 months administering of tocilizumab. In this case, the patient needed 6 weeks discontinuation of tocilizumab for recovery. In our case, the patient needed 4 months after discontinuation of tocilizumab to recover FXIII activity. It assumed that such a long recovery period was required because of a long term administration of tocilizumab (18 months). In the pharmacokinetics of the concentration for a dose of 8 mg/kg tocilizumab given every 4 weeks, steady-state was reached following the first administering for maximum concentration (C_{max}) and after 8 and 20 weeks for steady-state area under curve (AUC) and minimum concentration (C_{min}), respectively [10]. One of our speculations is that an accumulation effect of tocilizumab is due to longer administering. Another possibility is that the ability to recover FXIII requires long-term treatment even after decreasing the effect of tocilizumab. Additional clinical study is required to resolve the mechanisms in the future.

Tocilizumab is an interleukin (IL)-6 inhibitor that suppresses immune reactions. There are few reports concerning the relationship between IL-6 and FXIII. In a study of endotoxemia rats treated with lipopolysaccharide (LPS), administering of C1-esterase inhibitor and FXIII increased IL-6 levels, suggesting a positive relationship between FXIII concentration and IL-6 level [11]. On the other hand, results of an in vitro study showed that the expression of FXIII decreased while that of IL-6 increased when monocytes were cultured with LPS [12], which are consistent with the in vivo findings that plasma FXIII levels were decreased after LPS infusion [11]. Latter results also suggest that administering of an IL-6 inhibitor can prevent a decrease in FXIII expression. These conflicting results

cannot explain the phenomenon observed in our case. On the other hand, several kinds of drugs are known to induce acquired FXIII deficiency [7, 13–15]. However, no relationship between tocilizumab and acquired FXIII deficiency has been described, and other biologics such as TNF antagonists are not different from tocilizumab.

Laboratory test results obtained at the second admission showed a slight decrease in fibrinogen, indicating that fibrinogen and FXIII levels might have decreased to some degree because of bleeding (Table 1). Fibrinogen is an acute phase reactant and acceleration of IL-6 leads to its expression in the liver. Thus, an IL-6 inhibitor might inhibit the expression of fibrinogen. In a study of rats treated with the selective mineralocorticoid receptor antagonist eplerenone after coronary ligation, mineralocorticoid blockade led to a transient upregulation of monocyte chemoattractant protein-1, tumor necrosis factor-alpha, IL-1β, IL-6, IL-10, and IL-4, as well as an increase in FXIII-A protein expression in the healing myocardium [16], indicating that an IL-6 receptor inhibitor may inhibit the production of FXIII-A protein. Nevertheless, the mechanism by which tocilizumab suppresses plasma FXIII level remains unclear and should be elucidated in future studies.

In conclusion, when recurrent bleeding is seen despite normal results for APTT and PT during administering of tocilizumab for treatment of RA, hemorrhagic-acquired FXIII deficiency should be considered in a differential diagnosis.

Conflict of interest The authors declare that they have no conflict of interest.

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Regular Article

Molecular modeling predicts structural changes in the A subunit of factor XIII caused by two novel mutations identified in a neonate with severe congenital factor XIII deficiency☆

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ABSTRACT

Introduction: Coagulation factor XIII (FXIII) is a fibrin-stabilizing factor, which contributes to hemostasis, wound healing, and maintenance of pregnancy. Accordingly, patients with congenital FXIII deficiency manifest a life-long bleeding tendency, abnormal wound healing and recurrent miscarriage. In order to understand the molecular mechanisms of congenital FXIII deficiency, genetic analysis and molecular modeling were carried out in a Japanese male neonate with severe FXIII deficiency.

Methods and Results: Two novel mutations, Y204Stop (or Y204X, TAT to TAA) and S708R (AGC to AGG), were heterozygously identified by nucleotide sequencing analysis in exons V and XV of the gene for the A subunit of FXIII (FXIII-A). Y204X and S708R would lead to nonsense mediated mRNA decay and misfolding of the FXIII-A molecule, respectively. Using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, the presence of these mutations was confirmed both together in the proband and one each separately in either the maternal or paternal sides of his family. In addition, moderately decreased FXIII activity was associated with the presence of either mutation. Molecular modeling predicted that the mutant molecule of S708R would be structurally compromised by the substitution of the Ser with the larger extended bulky and positively charged Arg side-chain.

Conclusion: It is probable that the impaired tertiary structure of the mutant S708R molecule leads to its instability, which is at least in part responsible for the FXIII deficiency of this patient. This is consistent with the fact that the mutations and the reduced FXIII activities co-segregate among the patient's family members.

Introduction

In humans, transglutaminases (TGases) are made up of at least 9 homologous enzymes that cross-link a number of proteins. This kind of reaction not only enhances the original functions of substrate proteins, but also adds new functions to them [1]. Factor XIII (FXIII) is a plasma TGase circulating in blood as a heterotetramer, consisting of two catalytic A subunits (FXIII-A) and two non-catalytic B subunits (FXIII-B). It is a proenzyme activated by thrombin at the final stage of the blood coagulation cascade. Activated FXIII then crosslinks fibrin molecules among themselves as well as linking to α_2-plasmin inhibitor, fibronectin, etc. [2]; thus it plays essential roles in hemostasis, wound healing, and maintenance of pregnancy. Accordingly, lifelong bleeding tendencies as well as abnormal wound healing and recurrent miscarriage are common symptoms of congenital FXIII deficiency. Hemorrhagic acquired FXIII deficiency and autoimmune hemorrhaphilia XIII due to anti-FXIII autoantibodies also manifest severe subcutaneous, intramuscular, intrathoracic, -abdominal bleedings, and have been on the rise recently [3].

Congenital FXIII deficiency is relatively rare and its frequency is thought to be about one case among 2 million population [4]. Its genetic and molecular mechanisms have been analyzed extensively *in vitro* [5,6]. The mechanisms of such deficiencies have also been studied in detail by using FXIIIA (*F13A*) gene knock-out mice *in vivo* [7,8]. In the present study, we identified two novel mutations in the *F13A* gene of a neonate who developed recurrent umbilical bleeding typical of congenital FXIII deficiency; further, we made a theoretical examination of their effects on the structure of FXIII-A using molecular modeling *in silico*.

Methods

A male newborn having been delivered after 36 weeks of gestation started to repetitively manifest excessive umbilical bleedings on the

☆ This study and was presented in part at the 23rd ISTH meetings in Kyoto, Japan, in July 2011.

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5 day of birth, and was diagnosed to have severe FXIII deficiency. The clinical features of this patient, with special regard to a short half-life of injected FXIII, were reported elsewhere in detail [9].

This study was performed with approval of our Institutional Review Board. All works were conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from patient's parents and family members.

Amine Incorporation Assay for FXIII Activity

Ten microliters of plasma was incubated with 1 U bovine thrombin, 5 mM $CaCl_2$, 20 mM Tris–HCl (pH 7.5), 0.2% *N,N*-dimethylcasein, 2 mM monodansylcadaverine, and 2 mM dithiothreitol in a 0.1 mL mixture at 37 °C for 60 min. The reaction was terminated by adding 0.1 mL of 10% trichloroacetic acid. The precipitate was collected by centrifugation, washed three times with 0.5 mL of ethanol-diethyl ether mixture (1:1), and dissolved in 0.3 mL of 8 M urea, 1% sodium dodecyl sulfate (SDS) and 50 mM Tris–HCl (pH 8.0). The fluorescent intensity of emission at 520 nm with excitation at 360 nm was measured.

Enzyme-linked Immunosorbent Assay (ELISA) for FXIII-A Antigen

An anti-human FXIII-A monoclonal antibody, a kind gift from Dr. G. Reed of Massachusetts General Hospital (Harvard Medical School, Boston, MA), was coated in a microtiter plate for the measurement of FXIII-A. Plasma samples diluted 1/2,000 with 20 mM Tris–HCl (pH 7.5) and 150 mM NaCl were applied and incubated for 2 hours at room temperature. After washing, an anti-human FXIII-A polyclonal antibody (in-house) was added and incubated for 60 min. After washing, horseradish peroxidase-conjugated anti-rabbit IgG was added and incubated for 60 min. Absorbance at 450 nm was recorded by a microtiter plate reader Biolumin 960 (Molecular dynamics, San Diego, USA) and compared to standard curves, using purified FXIII-A.

Statistical Analysis

Results are presented as medians and interquartile ranges (IQR) as well as means and standard deviation (S.D.). Comparison between groups by the non-parametric (Mann–Whitney and Wilcoxin/Kruskal–Wallis) tests was carried out using the software program JMP ver. 6.0.3 and that ver. 10.0.0 (SAS Institute, Cary, NC), and differences were considered to be statistically significant at a *p*-value of less than 0.05.

Genomic Sequencing and PCR-RFLP Analysis

Genomic DNA (1 μg) was amplified using PCR (polymerase chain reaction) using a total of 17 pairs of gene-specific primers described previously [10,11]. The purified DNA was directly sequenced by the dideoxynucleotide method using a Dye Terminator Cycle Sequencing Kit with an ABI sequence analyzer, 310 Perkin Elmer, Norwalk, CT, USA).

Four oligonucleotides were synthesized to detect two newly identified mutations: a pair of primers for exon V including the Y204X codon; forward, 5′-CAATGAGAAAGAAAGAGAAGAGCA-3′ and reverse, 5′- TGTGACAAAAAATATGAAGTAAAAATG-3′. Genomic DNA obtained from each individual was amplified by PCR employing this pair of primers. PCR was also carried out employing a primer set of a sense primer (5′-GGCATCGGAAGCTGATAGCCA-3′) and an antisense primer (5′-GGGTTCATCTCAGCTTCCTGTG-3′) corresponding to the 5′-region of exon XV including a codon for S708 or R708. Each of these PCR products was digested using *Nla*III or *Bst*NI endonuclease at 37 °C for 2 h and then applied to a 2% agarose gel.

Molecular Modeling

The several crystal structures of human FXIII-A available in the Protein Data Bank all show the dimeric protein in the same overall three-dimensional conformation. The highest resolution wild-type FXIII-A crystal structure, accession code 1EVU [12], was used as a starting point for structural analysis and modeling. Computational mutagenesis and modeling of possible S708R conformations was carried out with the program Coot [13].

Results and Discussion

Novel Y204X and S708R Mutations Identified in the F13A Gene

The neonate showed considerably low FXIII activity (4% of normal), despite the fact that the test was carried out by a commercial laboratory using an ammonia release assay kit with a relatively low sensitivity between 3-5% [4]; this was consistent with the fact that the neonate's FXIII activity was found to be 1.7% of normal by an amine incorporation assay at our hands [14]. Thus, he was diagnosed as having severe FXIII deficiency. Because he had neither underlying pathological conditions nor disease states to induce secondary/acquired FXIII deficiency [3,4], he was suspected of being a case of severe congenital FXIII deficiency. Accordingly, we looked extensively for a possible genomic defect(s) in his *F13A* gene (Fig. 1a & b). Genetic analysis revealed two novel heterozygous mutations in the patient's genomic DNA, Y204X (TAT to TAA) and S708R (AGC to AGG) in exons V and XV, respectively (Suppl. Fig. 1a & b). The former nonsense mutation leads to truncation of the FXIII-A molecule at the N-terminal part of its core domain, and the latter missense substitution results in extension of the side-chain of residue 708 in the C-terminal part of barrel-2 domain, as discussed later in detail. The Y204X and S708R mutations were not found in 16 and 41 unrelated Japanese individuals, respectively.

At the time of submission of this manuscript, these mutations of the *F13A* gene had not been listed in the FXIII mutation database (http://www.f13-database.de/); where 34 missense, 5 nonsense, 21 insertion/deletion, and 9 splicing mutations are registered currently. These mutations spread all over *F13A*'s exons and the FXIII-A molecule. In addition, a computer-assisted literature search using PubMed revealed a similar S708N mutation reported by Castaman et al.; it was predicted that the substituted Asn residue would disrupt a hydrogen-bond with A291 in the core domain, and thus would result in the destabilization of the mutant molecule [15]. Interestingly, the Ser residue at position 708 is only relatively conserved among the TGase superfamily in humans (Fig. 2).

Several other nucleotide substitutions were also identified by DNA sequencing analysis; homozygously, L564 (CTG) to P564 (CCG) in exon XII and Q651 (CAG) to E651 (GAG) in exon XIV, both of which are well known genetic polymorphisms of the *F13A* gene [10];

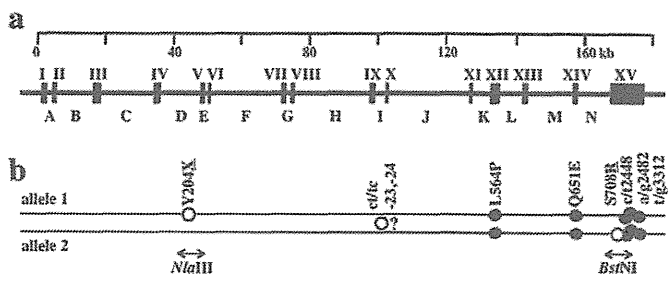


Fig. 1. Gene structure of *F13A* (a) and the positions of mutations (b).The human *F13A* gene spans about 177 kb. Exons are indicated by wide bars and Roman numerals, and introns by capital letters. Open circles stand for heterozygous mutations (underlined) or polymorphisms, while solid circles indicate homozygous polymorphisms. Positions of primer pairs for genetic diagnosis of two novel mutations are indicated by arrows and names of endonucleases.

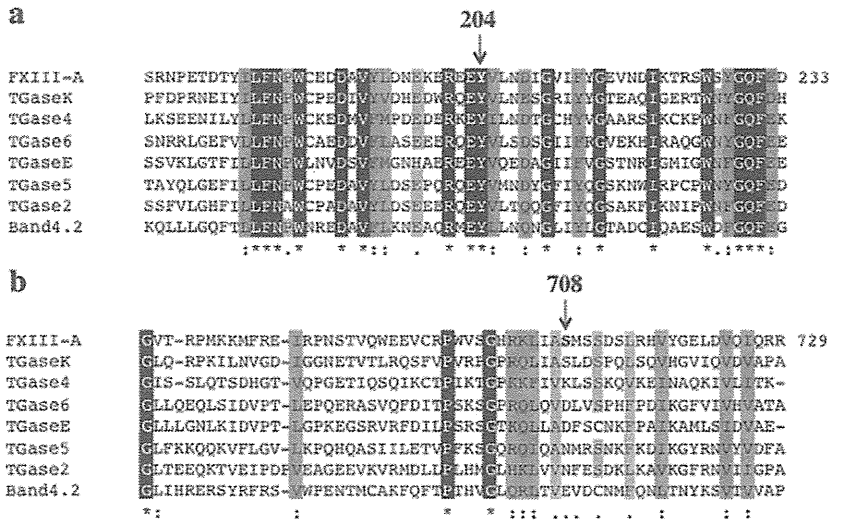


Fig. 2. Amino acid sequence alignment of exons V and XV around Y204X (a) and S708R mutations (b). Unexceptionally conserved residues are highlighted by black background and indicated by asterisks, and less conserved residues are gray-shadowed and shown by dots (colons mean more conservation than do periods), at the bottom.

homozygously, 2448 c to t, 2482 a to g, and 3317 t to g in the 3′-noncoding region in exon XV; heterozygously, ct to tc in intron I at 24, 23 nucleotides upstream exon X (Fig. 1b). These nucleotide substitutions also belong to known genetic polymorphisms and are not considered to be causative of FXIII deficiency [16,17].

Compound Heterozygous Mutations of Y204X and S708R Confirmed by Family Study

In order to investigate the segregation of these two mutations in the proband's family, genetic diagnosis was performed by the PCR-RFLP method using *Nla*III and *Bst*NI endonucleases (Figs. 1 & 3). The Y204X mutation was found in the proband, his father, an uncle and a grandfather, and the S708R mutation was found in the proband, his mother and a grandmother (Fig. 3a & b). Thus, it was confirmed that the proband was a compound heterozygote for the Y204X and S708R mutations, and that the former mutation on null allele 1 was inherited from the paternal side and the latter on allele 2 from the maternal side. This is consistent with the fact that all these carriers of either mutation themselves showed moderately reduced or nearly normal levels of FXIII activity and/or FXIII-A antigen (Fig. 3b) [9].

Medians (IQR) of FXIII activity for Y204X and S708R heterozygotes (n = 2 and 3, respectively) were 76.5 (70.0-77.3)% and 55.9 (53.9-57.9)%, respectively, while those of FXIII-A antigen were 67.8 (59.3-92.0)% and 41.8 (41.3-42.2)%, respectively. Thus, the FXIII activity of Y204X heterozygotes was higher than that of S708R heterozygotes (mean +/− S.D.; 74.6 +/− 3.1% versus 55.9 +/− 2.0% of the normal). The FXIII-A antigen level of the former was also higher than that of the latter (73.0 +/− 12.6% versus 41.8% +/− 0.5% of the normal). However, statistical analyses revealed no significant differences in FXIII activity nor FXIII-A antigen levels between these 2 mutation-genotypes. The reason(s) for these differences remains unknown.

The limitations of this study include the small number of heterozygotes evaluated. Moreover, further tests are not possible because the patient was a neonate and he moved out to the other area together with his family members.

Molecular Modeling of the Y204X and S708R Mutations

An analysis of the FXIII-A crystal structure gives some insight into the possible consequences of the two mutations on protein structure and thus on enzyme function. Each subunit in the FXIII-A dimer folds into four sequential domains: the N-terminal sandwich, a central core which contains the catalytic triad residues and calcium binding site, and two barrels at the C-terminus (Fig. 4a) [12,18]. The Y204X variant results in an N-terminal fragment that contains only 28% of the residues of the FXIII-A sequence. This fragment forms the N-terminal sandwich domain and is missing nearly all of the central core domain and its catalytic triad residues, and thus is not capable of enzymatic activity even if it is properly folded into a stable three-dimensional structure. However, it is highly likely that such an aberrant mRNA carrying a premature termination codon as the Y204X mutation will be rapidly eliminated by the nonsense-mediated mRNA decay pathway [19,20].

The possible structural consequences of the S708R substitution are more complex. In the FXIII-A crystal structure, the S708 side-chain is

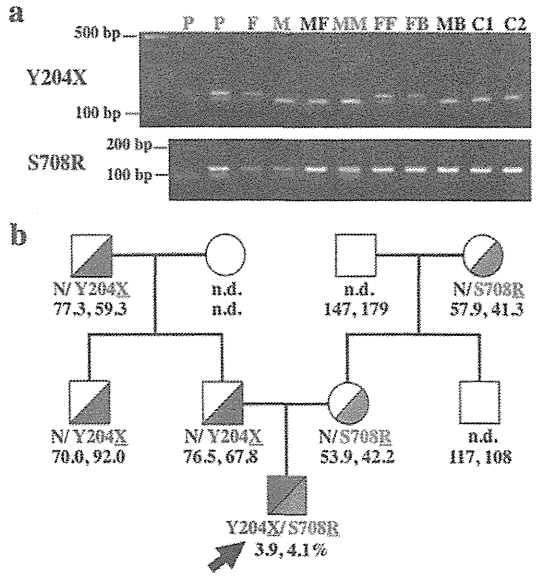
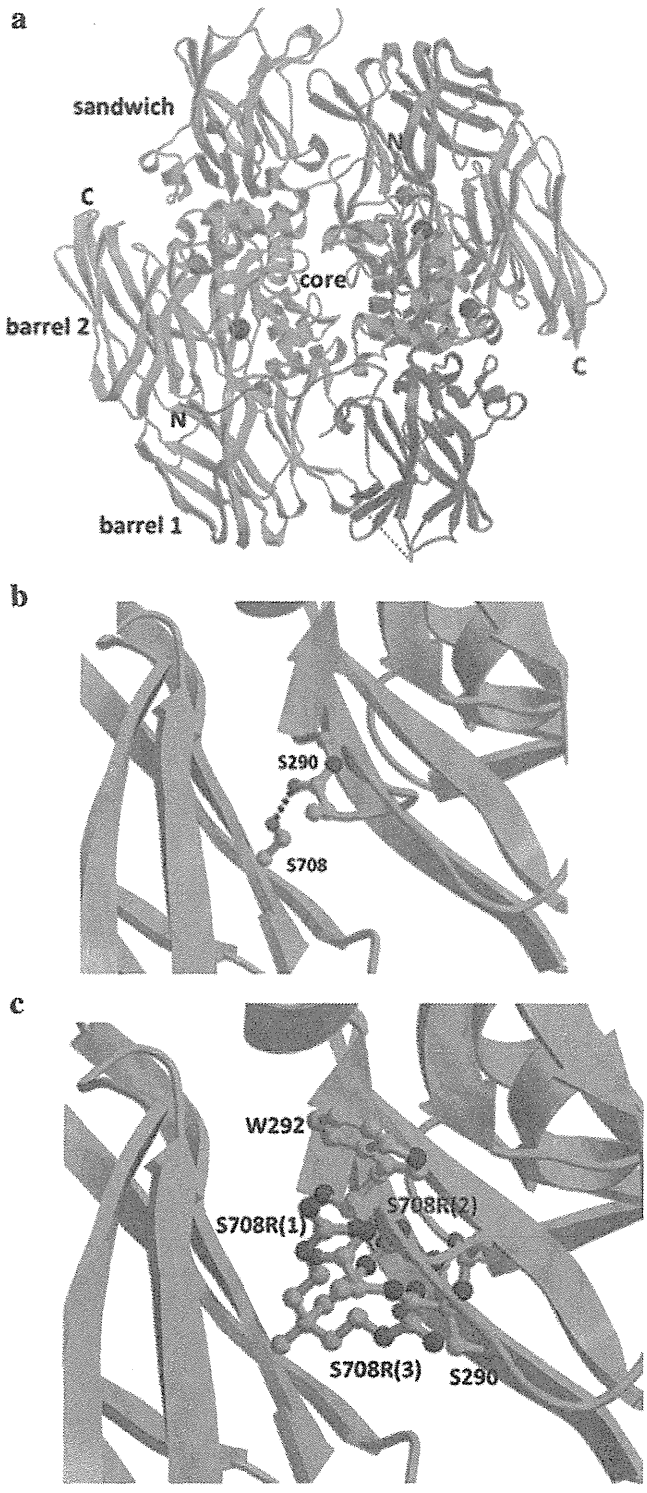


Fig. 3. Genetic diagnosis of the Y204X and S708R mutations (a) and family pedigree (b). **a**, For analyses of the Y204X substitution in exon V and the S708R mutation in exon XV, amplified DNA fragments were digested with *Nla*III and *Bst*NI endonucleases, respectively. Genetic diagnosis was carried out in the patient and his family members. MW stands for the molecular weight size marker of the 100 bp ladder. P; proband, F; father, M; mother, MF; mother's father; MM; mother's mother, FF; father's father, FB; father's brother, MB; mother's brother, C1 and C2; controls. **b**, Black and gray fill-ins indicate the Y204X and S708R mutations, respectively. An arrow indicates the proband. FXIII activities and FXIII-A antigen levels are determined by an amine incorporation assay and ELISA, respectively. FXIII activities (% of the normal) are followed by FXIII-A antigen levels (% of the normal). n.d.; not determined because a sample was not available.

located on the surface of the C-terminal barrel 2 domain, and is directed toward the adjacent catalytic core domain such that the S708 hydroxyl group forms a hydrogen bond with the main-chain carbonyl of A291 (Fig. 4b). Substitution of the small S708 side-chain in the S708R variant is predicted to create many steric clashes between the much larger R708 side-chain and several catalytic core residues. Several possible R708 side-chain conformations, with energetically favorable torsion angles, were modeled but all generated steric clashes, most severely with Arg223 and Ser290-Ala291-Trp292 in the catalytic core (Fig. 4c). Thus while the S708R substitution might allow proper folding of each individual domain, it would likely prevent the domains from packing against each other properly, especially the barrel 2 and catalytic core domains.



All published FXIII-A crystal structures represent an inactive conformation since the domains are arranged such that a barrel 1 loop prevents substrate access to the active site. It is speculated that both barrels 1 and 2 must shift dramatically to yield an active FXIII-A conformation [18]; such a shift is seen in the crystal structure of an activated form of the homologous enzyme TGase 2 [21]. That this active open conformation of TGase 2 has been successfully crystallized while even activated forms of FXIII-A (thrombin-cleaved or calcium-bound) crystallize in catalytically inactive closed conformations [12,22], suggests that the packing of barrel 2 domain and catalytic core domain is less important for TGase 2 stability than for FXIII-A. In this context it is interesting to note that while S708 is conserved in all 6 mammalian FXIII-A sequences including human, chimpanzee, dog, cow, mouse, and rat (data not shown), the homologous residue is an Asn in TGase 2, and this N667 side-chain does not interact with any catalytic core residues in the closed, inactive TGase 2 structure [23]. FXIII-A has a more complicated activation mechanism than TGase 2 and other homologous enzymes; it circulates in its inactive form as an A_2B_2 heterotetramer. Activation involves thrombin cleavage of FXIII-A, dissociation from the regulatory FXIII-B, and substrate binding. The immediate structural effect of the S708R substitution is predicted to be disruption of proper barrel 2-catalytic core interactions. This in turn may diminish mutant protein levels, either because the altered, open conformation of the S708R variant is not able to bind FXIII-B properly or because the open conformation is unstable in the absence of bound substrate.

In conclusion, we identified two novel mutations of the *F13A* gene in a Japanese neonate with severe congenital FXIII deficiency, and predicted structural changes in the mutant proteins by molecular modeling. This report will add new information to understand the significance of FXIII-A mutations [24].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.thromres.2012.05.003.

Conflict of Interest Statement

None of the authors has any conflict of interest.

Acknowledgements

The first and second authors equally contributed to this study. We thank Dr. Tsukasa Osaki for his assistance in statistical analyses and Ms. Leslie Boba for her assistance in preparation of the manuscript. This study was supported by a research grant from The Japanese Ministry of Health, Labor, and Welfare, and by a Grant-in-Aid for Scientific Research

Fig. 4. Modeling of FXIII-A mutants. a, Location of mutated residues in the dimeric FXIII-A crystal structure. The two FXIII-A molecules are shown as ribbon diagrams with labeled N- and C-termini (subunit 1 in cyan and yellow; subunit 2 in magenta and yellow, Suppl. Fig. 2). The Y204X mutant results in a truncated fragment which corresponds primarily to the non-catalytic N-terminal sandwich domain (dark cyan and dark magenta domains in subunits 1 and 2, respectively; the Y204 positions are marked with dark grey spheres). The S708R mutation alters a residue in the C-terminal barrel 2 domain (green spheres in yellow domains) which is adjacent to the catalytic core. Calcium ions bound to the core domain and the location of the catalytic C314 are shown as orange and blue spheres, respectively. **b**, S708 location and interactions. In the wild-type FXIII-A crystal structure, the small S708 side-chain (green carbon and red oxygen atoms, green bonds, Suppl. Fig. 2) is located on the surface of the barrel 2 domain (yellow) and buried in the interface with the core domain (cyan). The S708 side-chain hydroxyl forms a hydrogen bond (dashed line) with the carbonyl oxygen of A291 (cyan carbon atoms and bonds). **c**, S708R substitution. The S708R mutation replaces the wild-type serine with a much larger side-chain (three representative conformations of the R708 side-chain with energetically favorable torsion angles are shown with green carbon atoms and bonds) which creates unfavorable steric clashes with the neighboring core domain. The most severe clashes are with Arg223 (view partially obstructed by loop) and Ser290-Trp292 (shown with cyan carbon atoms and bonds). Figures prepared using Molscript [25] and Raster3D [26].

from Yamagata University. VCY is supported by U.S. National Institutes of Health grants (DK075897 and GM61388).

Authorship

MS performed all the laboratory experiments and analyses and VCY conducted structure modeling analyses and drafted the paper. NF worked on clinical studies and AI created the research project, and wrote the paper.

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ORIGINAL ARTICLE *Rare bleeding disorders*

A case of acquired FXIII deficiency with severe bleeding symptoms

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Summary. Acquired factor XIII (FXIII) deficiency due to an autoantibody against FXIII is a very rare, yet potentially life-threatening bleeding disorder. As the standard coagulation tests (prothrombin time and activated partial thromboplastin time) are normal, the specialized tests are required to make an accurate diagnosis. Here, we report a case of acquired FXIII deficiency with severe bleeding symptoms. A 75-year-old man was referred to our hospital because of severe bleeding tendency after a tooth extraction. Laboratory findings showed that routine coagulation studies were normal, but factor XIII (FXIII) activity was low (3%). The presence of FXIII inhibitor was detected with dot blotting studies. Although the bleeding tendency was very severe, it was successfully controlled by infusion of FXIII concentrates combined with immunosuppressive treatment (oral prednisolone). Fibrin cross-linking study showed the significant delay of the γ-chain dimer and α-chain polymer formation. Western blotting revealed the marked decrease in FXIII-A level. The mixing study of FXIII activity measured using amine-incorporation assay showed the incomplete inhibition pattern. There seems to be little agreement as to the treatment strategy of acquired FXIII deficiency. In this patient, the use of FXIII concentrates was very useful in the initial treatment of bleeding symptom. The use of steroids was also effective in increasing FXIII activity without any serious complications.

Keywords: acquired FXIII deficiency, FXIII inhibitor

Factor XIII (FXIII) is a final enzyme in the coagulation cascade and is responsible for fibrin stabilization. The clinical feature of defective fibrin stabilization is highlighted by the manifestation of severe bleeding in congenital FXIII deficiency [1].

Acquired FXIII deficiency caused by the autoantibody against FXIII, is a rare bleeding disorder [2]. Patients who develop such acquired FXIII inhibitors may present severe bleeding symptoms. However, the diagnosis using standard coagulation tests, prothrombin time (PT) and activated partial thromboplastin time (APTT) is quite difficult. Herein we present a case of acquired inhibitor against FXIII with severe bleeding tendency, successfully treated using FXIII concentrates and oral prednisolone as an immunosuppressive treatment.

A 75-year-old male suffering from severe bleeding tendency was referred to a rural hospital. There was no history of bleeding diathesis or familial bleeding. He had a subcutaneous haematoma from the right cervical region to the chest region after a tooth extraction. Laboratory tests showed low haemoglobin (Hb) and leucocyte levels but platelet level was normal. The fibrinogen level, PT and APTT were also normal. On the twentieth day of illness, the subcutaneous haematoma enlarged and extended to the right abdominal region, and the patient also presented with haematemesis. An upper gastrointestinal endoscopy could not identify the bleeding point. The Hb level decreased to 6 g/dL, for which the patient received a transfusion of packed red blood cells. He was transferred to our hospital for a more detailed examination and for keeping the bleeding under control. We found that the FXIII activity level had decreased (<3%). Thus, the patient was suspected of having acquired FXIII deficiency. After administration of FXIII concentrate, the enlarged haematoma (Fig. 1) gradually resolved and there was no progression of anaemia. The half-life of therapeutically administered FXIII concentrates (1,440 units) in circulation was shortened to 20 h in this patient (cf. 7–12 days) (Fig. 2). To confirm the diagnosis with acquired FXIII deficiency due to an FXIII inhibitor, dot blotting was performed. It revealed the presence of immunoglobulin which was bound to FXIII-A subunit (Fig. 3). We started immunosuppressive

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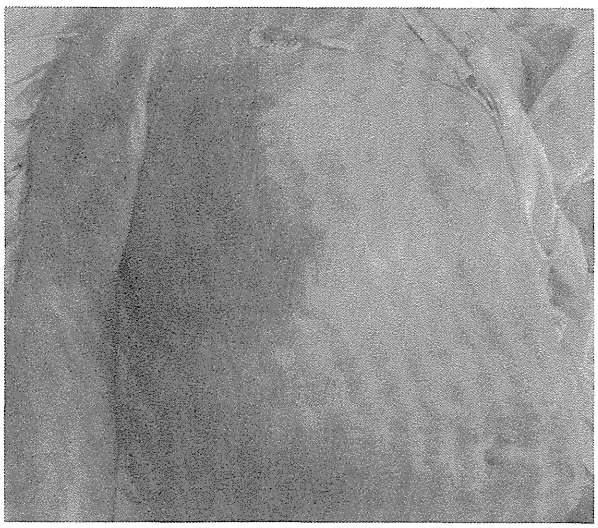

Fig. 1. Subcutaneous bleeding and haematoma on admission. Extensive subcutaneous haematomas were observed spanning the right axilla to the precordial region and the abdominal wall at the time of hospital admission.

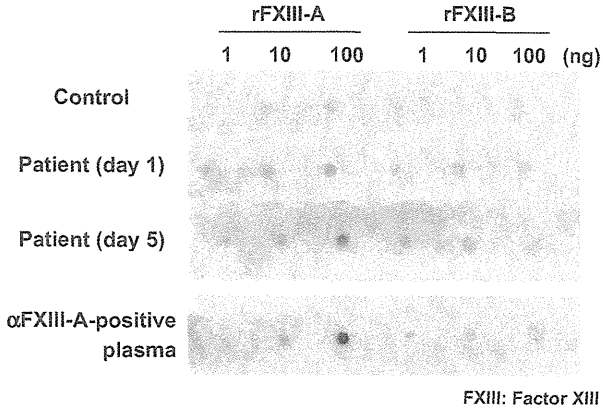

Fig. 3. Results of dot blot for FXIII inhibitor. Nitrocellulose membrane bound with recombinant FXIII-A and FXIII-B proteins was incubated with diluted control and patient plasma. Immunoglobulin combined with FXIII was detected using peroxidase-labelled antihuman immunoglobulin. Immunoglobulin binding to FXIII-A was detected. Day 1: First day of hospital admission. Day 5: Fifth day of hospital admission, after administration of FXIII concentrates.

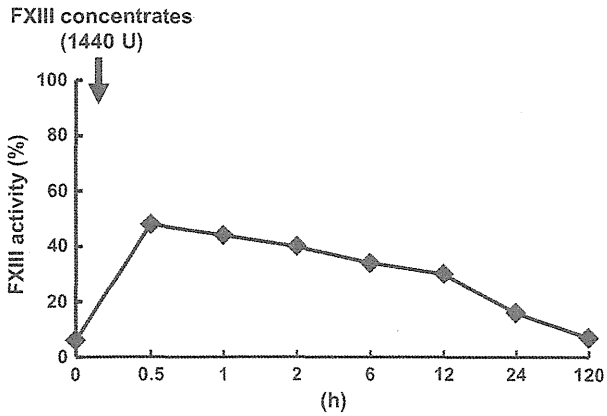

Fig. 2. Changes in FXIII activity after infusion of 1,440 U FXIII concentrates. Changes in FXIII activity (chromogenic-substrate method) after administration of FXIII concentrates. The half-life of FXIII concentrates was reduced to approximately 20 h.

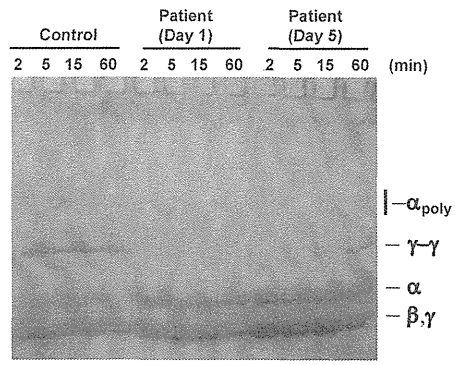

Fig. 4. Fibrin-cross-linking analysis. Formation of both γ-dimer and α-polymer was significantly delayed when calcium chloride and thrombin were added to the patient plasma. Day 1: First day of hospital admission. Day 5: Fifth day of hospital admission, after administration of FXIII concentrates. [Correction added after online publication 13 March 2012: The previously truncated image of Figure 4 has been replaced with a full version. There are no other data changes].

treatment with prednisolone (1mg kg^{-1} per day), and subsequently the dose was tapered. Thereafter, FXIII activity recovered and bleeding disappeared. The patient is well now.

Further examinations of the FXIII inhibitor were initiated. Fibrin cross-linking study indicated that the formation of fibrin γ-chain dimers and α-chain polymers was markedly delayed (Fig. 4). Western blotting revealed that the FXIII-A level was markedly decreased and FXIII-B level was slightly decreased (Fig. 5; day 1). After infusion of FXIII concentrate, levels of both subunits increased (Fig. 5; day 5). FXIII is a tetrameric protransglutaminase, and its activity was measured using amine-incorporation study. The mixing study of FXIII activity was performed after incubation of patient plasma and control in different dilutions for 2 h at 37 °C (Fig. 6). A pattern of incomplete inhibition was observed.

Acquired FXIII deficiency is a rare bleeding disorder caused by autoantibodies against FXIII. Bleeding can be acute to mild but when severe, carries a high risk of death. The patient in this study also showed serious bleeding symptoms, but routine coagulation studies revealed no abnormalities. If patients have bleeding symptoms with no abnormal findings in routine tests, the possibility of acquired FXIII deficiency should be considered. Acquired FXIII deficiency has been described in association with certain drugs and underlying disease, such as lymph proliferative disorders, malignant tumours and autoimmune disorders. In the case of our patient, we could not find the underlying disease.

Serious bleeding caused by acquired FXIII deficiency can be treated effectively by FXIII concentrates

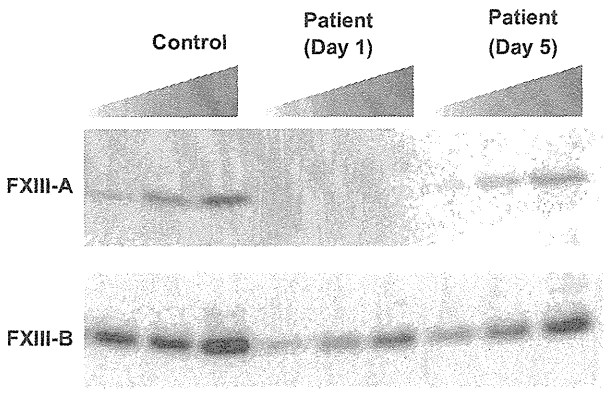

Fig. 5. Western blot analysis. Patient and control plasma were diluted in stages and western blot analysis was performed on each dilution using anti FXIII-A antibody (top) and anti FXIII-B antibody (bottom). FXIII-A was significantly reduced and FXIII-B was slightly reduced on the first day of admission. Recovery of both subunits was observed on the fifth day of hospital admission, after administration of FXIII concentrates. Day 1: First day of hospital admission. Day 5: Fifth day of hospital admission, after administration of FXIII concentrates.

[3]. In our patient, FXIII concentrates were administered soon after decreased FXIII activity was identified, and his bleeding symptoms improved drastically. Treatments such as corticosteroids, cyclophosphamide, rituximab and plasmapheresis have been reported to reduce and/or eliminate FXIII inhibitors [2–5]. However, the indications and long-term effects of these treatments have not been fully assessed. It is necessary to evaluate more cases and analyse data to learn more about its pathogenesis and treatment strategies.

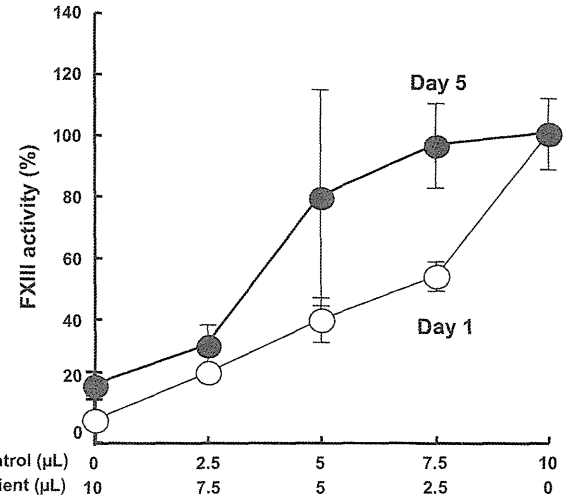

Fig. 6. Mixing test of plasma by amine-incorporation activity. Patient and control plasma were mixed in various proportions for 2 h at 37 °C. When the uptake of monodansylcadavarine into dimethylcasein was measured, an incomplete inhibition pattern was recognized on the first day of hospital admission, and a nonlinear aberrant pattern was recognized on the fifth day of hospital admission. The cause of the nonlinear aberrant pattern was not clear.

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Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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Factor XIII is a key molecule at the intersection of coagulation and fibrinolysis as well as inflammation and infection control

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Abstract Factor XIII (FXIII) is a transglutaminase consisting of two catalytic A subunits (FXIII-A) and two non-catalytic B subunits (FXIII-B) in plasma. FXIII-B protects FXIII-A from its clearance. FXIII-A is also present as a homodimer inside megakaryocytes/platelets and monocytes/macrophages. Although possible functions of intracellular FXIII-A have been proposed, these remain to be established. Intra- and extra-cellular FXIIIs support platelet adhesion and spreading as well as clot retraction, suggesting that FXIII is important for the stabilization of platelet–fibrin clots. Intra- and extra-cellular FXIIIs also support immobilization and killing of bacteria as well as phagocytosis by macrophages. Thus, FXIII may function in innate immunity. Congenital FXIII deficiency due to defective *F13-A* genes manifests as a life-long bleeding tendency, abnormal wound healing, and recurrent miscarriage. Although congenital FXIII-B deficiency used to be thought rare, reports of such cases have increased recently. As the bleeding tendency is often mild, patients with FXIII-B deficiency may be overlooked by physicians. Patients with acquired FXIII deficiency, in particular those with autoimmune hemorrhaphilia due to anti-FXIII antibodies, are on the increase, at least in Japan. It is important to diagnose such cases as early as possible, and to treat them with immunosuppression in combination with FXIII replacement therapy as their bleeding symptoms can be life-threatening.

Keywords Plasma transglutaminase · Fibrin-stabilizing factor · Anti-fibrinolytic factor · Innate immune factor · Autoimmune hemorrhaphilia XIII/13 · Hereditary factor XIII B subunit deficiency

Introduction

Coagulation factor XIII (FXIII, or FXIII/13 to avoid confusion with FVIII/8 and FXII/12[1]) is a pro-enzyme of plasma transglutaminase (TGase) consisting of two enzymatic A subunits (FXIII-A) and two non-catalytic B subunits (FXIII-B), and plays a critical role in the generation of a stable hemostatic plug [1, 2]. Thus, FXIII is also called a fibrin-stabilizing factor which cross-links fibrin monomers to other fibrin monomers, as well as to α_2-plasmin inhibitor (α_2-PI) and fibronectin, and thus contributes to hemostasis, wound healing, and maintenance of pregnancy.

Congenital FXIII deficiency is a rare hemorrhagic disorder. Umbilical bleeding in the neonatal period is a characteristic, and the most frequent, symptom. Intracranial hemorrhage is less frequent but the leading cause of death from this condition at all ages. In addition to a life-long bleeding tendency, abnormal wound healing is seen in about 20 % of cases, and recurrent miscarriage in female cases is also a common symptom of FXIII deficiency. More than 500 cases of congenital FXIII deficiency have been identified. Most of the reported cases with congenital FXIII deficiency are caused by defects in the *F13-A* gene. Only few cases with FXIII-B deficiency, caused by founder effect mutations in the *F13-B* gene, had previously been

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[1] Occurring frequently in clinical fields and less commonly in scientific fields, even in the official journal of the International Society of Thrombosis and Haemostasis as well as in PubMed.

identified in Japan and Italy [2, 3]. However, a German group recently reported as many as 20 cases (one homozygote and 19 heterozygotes) with FXIII-B deficiency [4]. It therefore seems highly likely that FXIII-B deficiency may have been overlooked by physicians, since its bleeding symptoms are relatively mild [2, 3], as discussed later.

In this article, only few basic and clinical topics on FXIII will be reviewed in detail as highlighted by this author (as the organizer/chairman) at the International FXIII Symposiums in Feb 2011 in Wiesbaden, Germany and in Feb 2012 in St. Gallen, Switzerland.

FXIII and platelets: FXIII–fibrin–GPIIb/IIIa axis (Fig. 1)

FXIII-A exists in both extracellular (in plasma) and intracellular (as a cytosolic protein in megakaryocytes/platelets and monocytes/macrophages) spaces, although the function(s) of intra-cellular FXIII-A remain to be established. In particular, platelets cause spreading and adhesion by extending filopodia and lamellipodia, and clot retraction (CR) by retracting extended filopodia along fibrin strands.

Prolonged bleeding times, a sign of defective/abnormal primary hemostasis, were commonly observed in two separate lines of FXIII-A knockout (KO) mice [5, 6], which prompted recent investigations of possible roles for FXIII in platelet-related function. While platelet aggregation induced by ADP or collagen was normal, CR was lost in the platelet-rich plasma (PRP) of FXIII-A KO mice [7]. In contrast, there was no CR impairment in the PRP of tissue TGase KO mice compared to that of wild-type mice. In addition, a TGase inhibitor abolished CR in the PRP of wild-type mice. These results indicate that the enzymatic activity of FXIII is necessary for CR, at least in mice.

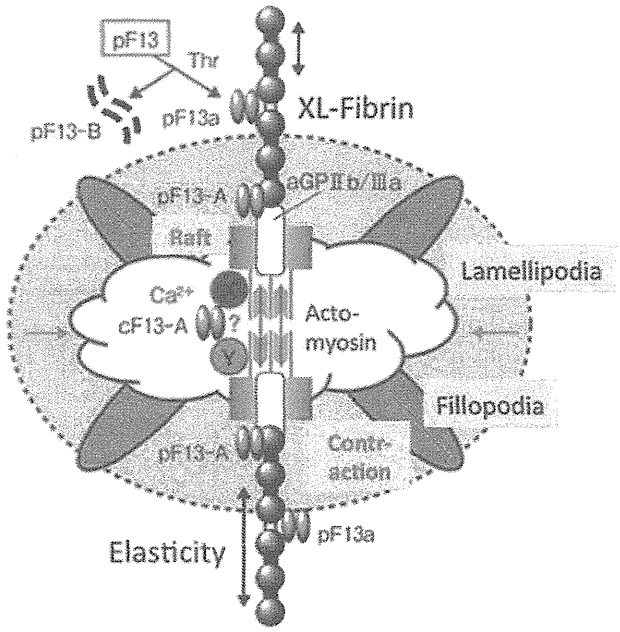

Fig. 1 FXIII–fibrin–GPIIb/IIIa axis for the CR reaction (hypothesis). Activated plasma FXIII (*pF13a*) by thrombin (*Thr*) cross-links fibrin monomers bound to activated GPIIb/IIIa (*aGPIIb/IIIa*) by the outside-in signal, and aGPIIb/IIIa clustered by the cross-linked (*XL-*) fibrin transduces signals to downstream effectors, and then to the terminal effectors, actin and myosin, to contract the whole body of a platelet. Intracellular FXIII-A (*cF13-A*) may cross-link cytoskeletal proteins (*actin*, *myosin*, *X* and *Y*) inside platelets. CR contributes to the stabilization of platelet–fibrin clots to form a hemostatic plug

The indispensable role of extracellular/plasma FXIII-A in the development of Ca^{2+}-dependent CR has previously been demonstrated in humans [8]. However, replacement of extrinsic FXIIIs, either plasma-derived FXIII or recombinant FXIII-A, only partially restored impaired CR in the PRP of FXIII-A KO mice in our system (Suppl. Fig. 2 of Ref. [7]), suggesting that both extracellular/plasma and intracellular/platelet FXIIIs are required for CR, at least in mice.

Activated FXIII (FXIIIa) is known to cross-link fibrin, glycoprotein (GP) IIb/IIIa, actin and myosin [1], which are involved in CR. Like cytosolic FXIII-A, both actin and myosin are located in the platelet cytosol and are cross-linked by Ca^{2+}-dependent TGase activity. Platelet FXIII-A can be activated by calpain, an endogenous intra-cellular protease, or by just Ca^{2+} ions [9]. Thus, it is possible that intracellular/platelet FXIII-A may function for CR via the cross-linking of cytosolic actin and myosin inside platelets. Recently, it has been reported that myosin IIA mediates platelet contraction [10]. This is consistent with the fact that in thrombin-stimulated platelets FXIII-A specifically binds to myosin IIA as well as four other platelet proteins, such as gelsolin, focal adhesion kinase (FAK), heat shock protein (HSP) 27, thrombospondin (TSP) I [11]. Gelsolin is a cytoskeletal protein that regulates actin assembly, while FAK is a tyrosine kinase that mediates signaling through GPIIb/IIIa and translocates to the cytoskeleton in platelets during the cytoskeletal rearrangement [12]. Accordingly, these cytoskeleton-related proteins must be involved not only in CR but also platelet adhesion and spreading as discussed below.

Although early platelet contraction has been reported to be fibrin-independent [10], fibrin cross-linked by FXIIIa must be indispensable for CR as afibrinogenemia (fibrinogen deficiency) also causes impaired CR [13].

The in vivo significance of CR for primary hemostasis is not fully understood. However, it has been shown to be essential for stable thrombus formation both ex vivo [14, 15] and in vivo [16, 17]. Thus, impaired CR may be, to some extent, responsible for the bleeding symptoms in patients with congenital FXIII deficiency.

With regard to other platelet reactions, it has been reported that the binding of fibrinogen in response to

thrombin receptor agonist peptide (TRAP) was significantly reduced in platelets from patients with FXIII-A deficiency [18]. Moreover, upon adhesion to fibrinogen, FXIII-A-deficient platelets showed a distinct extension pattern with increased filopodia and reduced lamellipodia formation, suggesting a delay in spreading. It was also shown that plasma FXIII binds to thrombin-receptor activated platelets via GPIIb/IIIa-bound fibrinogen [19]. More recently, Naseem [15] confirmed that FXIIIa supported platelet adhesion and spreading under static conditions that were dependent on GPIIb/IIIa and GPV/IIIa. FXIIIa also stimulated the formation of filopodia and lamellipodia in adherent platelets that was mediated exclusively by GPIIb/IIIa. Under conditions of arterial shear, FXIIIa accentuated platelet recruitment by von Willebrand factor and collagen.

Taken together, these findings indicate that FXIII supports platelet–fibrin clot formation by enhancing platelet adhesion and clot retraction via GPIIb/IIIa (FXIII–fibrin–GPIIb/IIIa axis, Fig. 1).

Novel function(s) of FXIII in innate immunity

As defense systems, both coagulation and complement cascades act locally; coagulation is initiated at a site of injury and complement at a site of infection. A site of open injury becomes a site of infection, in general. It has been shown that mannose-binding lectin-associated serine protease (MASP)-1 of the complement system cleaves fibrinogen and activates FXIII of the coagulation system [20], while MASP-2 activates prothrombin [21]. The MASP-catalyzed deposition and polymerization of fibrin on the surface of microorganisms may contribute to the defense system by limiting the dissemination of infection. Indeed, human FXIII sequesters bacteria, such as *Escherichia coli* and *Staphylococcus aureus*, in the blood clot [22]. Sequestration was strongly reduced in FXIII-deficient plasma. More recently, Loof et al. [23] have clearly shown that coagulation induced by bacteria leads to immobilization and killing of *Streptococcus pyogenes* bacteria inside the clot. The entrapment is mediated via cross-linking of bacterial surface proteins to fibrin fibers by FXIIIa. FXIII KO mice developed severe pathologic inflammation at the local site of streptococcal skin infection, and local FXIII administration reduced bacterial dissemination during early infection in wild-type animals. It is important to note that bacterial killing and cross-linking to fibrin networks have been confirmed in tissue biopsies from patients with streptococcal necrotizing fasciitis, supporting the concept that coagulation is part of the early innate immune system [23].

This scenario mimics horseshoe crab hemolymph coagulation system [24], which is extremely sensitive to bacterial lipopolysaccharides (LPS). Serine protease zymogen factor C is auto-catalytically activated in the presence of Gram-negative bacteria or LPS, and activated factor C activates coagulation factor B, which in turn converts the proclotting enzyme into the clotting enzyme. The clotting enzyme promotes the proteolytic conversion of coagulogen to coagulin, which spontaneously forms an insoluble polymer, a physical barrier at the site of microbial invasion.

Factor C contains five sushi domains, two of which are the core LPS-binding region [25]. Two sushi peptides (S1 and S3 derived from the first and third sushi domains) composed of 34 amino acids inhibit LPS-induced septic shock in mice by disrupting LPS aggregates, hence, neutralizing the LPS toxicity. Similarly, Muszbek most recently found that FXIII-B composed of 10 sushi domains also binds *Staphylococcus aureus* or Gram-positive bacteria (personal communication from Prof. L. Muszbek of Debrecen Univ. by E-mail in Mar. 2012). Thus, FXIII-B may contribute to innate immunity as well.

Macrophage is a key player in innate immunity. Adány [26] reported that monocytes activated by interleukin-4 were converted to M2 cells, increasing the expression of intracellular FXIII-A. In addition, monocytes obtained from healthy individuals are activated to macrophages, which had increased FXIII-A expression as well as enhanced phagocytic activity for sensitized red blood cells and complement-coated yeasts ex vivo [27]. On the other hand, monocytes obtained from cases with FXIII deficiency lacked phagocytic activity even when activated to macrophages.

Neutrophil is another major player of innate immunity. Nahrendorf [28] reported that recruitment of both macrophages and neutrophils into artificially infarcted loci were significantly reduced in the infarcted heart of FXIII-A KO mice. Phagocytic activity of macrophages was unchanged while that of neutrophils was reduced, indicating the role of intracellular FXIII-A in phagocytosis at least in neutrophils in vivo.

Increasing evidence suggests that platelets mediate inflammation and promote clearance of bacteria from the blood circulation. Toll-like receptor 2 (TLR2) is expressed on the platelet surface and recognizes bacterial lipopeptides, lipoproteins, and peptidoglycans from Gram-negative bacteria. Stimulation with a lipopeptide TLR2 agonist leads to activation of platelets, and platelet binding to monocytes [11]. Interestingly, TLR2 agonist-stimulation significantly increases the percent of platelet-positive monocytes over resting or thrombin-stimulation. Although FXIII-A specifically binds to myosin IIA, gelsolin, FAK, HSP27, TSPI in resting platelets [11], FXIII-A-binding to FAK, TSP1, and HSP27 decreased after TLR2 agonist-stimulation, while FXIII-A-binding to gelsolin increased. The FXIII-A interactions with these platelet proteins may

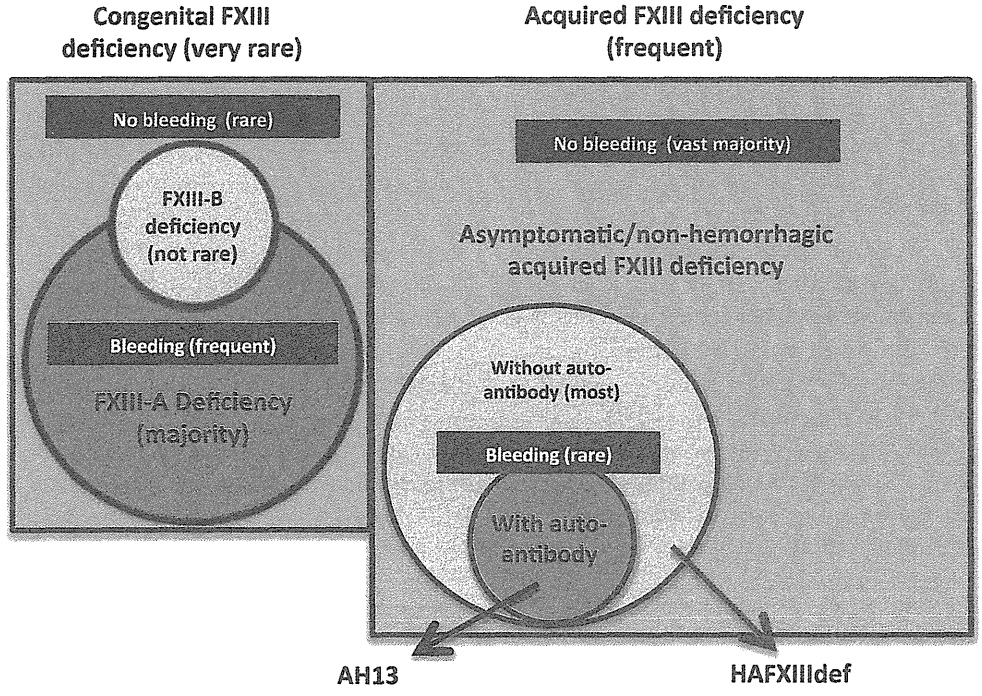

Fig. 2 HAFXIIIdef and AH13 (concept). There must be vast numbers of patients with acquired FXIII deficiency secondary to various disease states, due to hypo-synthesis and/or hyper-consumption of FXIII. Only limited numbers of cases with severe deficiency manifest bleeding symptoms (HAFXIIIdef). Among them, there are few cases of autoimmune hemorrhaphilia due to antibodies against either FXIII-A or FXIII-B (*AH13*). Most such cases show severe bleeding symptoms and require both FXIII replacement and immunosuppressive therapy, simultaneously. In contrast, bleeding symptoms of HAFXIIIdef may be generally arrested by FXIII replacement therapy alone

be related to platelet binding to monocytes to localize the immune response to the site of injury.

Acquired hemorrhaphilia XIII/13 (Fig. 2)

Acquired FXIII deficiency is a relatively common disorder, which is frequently caused by secondary FXIII reduction due to hypo-synthesis and/or hyper-consumption through a primary disease(s), such as leukemia, myelo-dysplastic syndrome and liver diseases, and disseminated intravascular coagulation, major surgery, Henoch–Schonlein purpura, chronic inflammatory bowel disease, etc. FXIII reduction in these disease states is caused in the absence of anti-FXIII inhibitors (Fig. 2).

There is an autoimmune disease, tentatively designated as "acquired hemophilia[2]", which results from the presence of autoantibodies directed against coagulation factors, most commonly factor VIII/8 (FVIII) [30]. It is also termed acquired FVIII inhibitors [31]. Recently, recognition of this bleeding disorder is increasing; for example, the incidence of acquired hemophilia-A due to anti-FVIII inhibitors (AHA) has been estimated at 1.5 cases per one million population per year [32]. The literature of AHA has also been significantly expanded by data on as many as 501 patients reported to European Acquired Hemophilia Registry [33].

In contrast, information on only a small number of cases of another autoimmune bleeding disorder, designated as "autoimmune/acquired hemorrhaphilia[2] due to anti-FXIII/13 inhibitors (AH13)", has been collected [34, 35]. However, AH13 has also recently been increasing at least in Japan (28 cases as of Mar. 2012; unpublished data). AH13 must be distinguished from regular "hemorrhagic acquired FXIII deficiency (HAFXIIIdef)" [34] (Fig. 2), as AH13 tends to be more severe than regular HAFXIIIdef, and requires immunosuppressive therapy to eradicate autoantibodies, together with FXIII replacement therapy to stop bleeding.

The number of Japanese AH13 cases is larger than that summarized after reviewing publications throughout the world between the 25-year period 1967–1992 [36, 37].

[2] Acquired h(a)emophilia is a tentative, working name for this category of diseases, but remains unofficial as it is not included in the current version of the WHO ICD (2007). "Acquired h(a)emorrhaphilia" seems to be a more logical and proper appellation, because the term hemorrhaphilia stands for "love of bleeding/hemorrhage" while the word hemophilia literally means "love of blood" [29]. Thus, the author uses the term hemorrhaphilia for a bleeding disorder caused by anti-FXIII/13 inhibitors.

Fewer than 10 papers on the topic have been published from other countries in the twenty-first century, as reports of isolated single cases may not be readily accepted by major medical journals. Further, it is very likely that many additional patients are systematically overlooked by physicians, as decreased FXIII activity cannot be detected by routine coagulation tests such as activated partial thromboplastin time (aPTT) and prothrombin time (PT), and FXIII assays are usually not performed. It must be more frequent than ever thought, in other countries, too.

The Japanese AH13 cases were mostly in the elderly, with a mean age of 66 years [35]. In contrast to congenital FXIII deficiency, no intracranial bleeding was seen among these cases, except for one AH13 case complicated with brain hemorrhage (a 68-year-old man has been diagnosed in Mar. 2012, unpublished data). In about half, no underlying condition was observed, while the remaining half had other autoimmune diseases, cancers, hepatitis, etc. Interestingly, several Japanese AH13 cases were complicated by aortic aneurysm (Ref. [35] and unpublished data). Most of the cases were treated with FXIII concentrates because of the lack of bypassing agents for this purpose. Most of these patients were also successfully treated with immunosuppressive therapy, generally by employing prednisolone, cyclophosphamide, and rarely by rituximab, with one exception of spontaneous remission [38]. In the patients who received immunosuppressive therapy, their anti-FXIII antibodies disappeared and/or their FXIII activities were normalized after an average of 4 months.

A 66-year-old female with severe AH13 recently died as a result of long delay in reporting the results of her FXIII activity assay, which was outsourced to a commercial laboratory (Sugiyama and Ichinose, in preparation). It is very important to diagnose early and treat early to save patients' lives.

Patients with suspected diagnoses of AH13 are examined for the presence of anti-FXIII inhibitors by mixing tests for FXIII activity of mixed plasma between a patient and a healthy individual as well as by a dot blot test for antibodies against recombinant FXIII-A and recombinant FXIII-B. We have diagnosed a total of 19 AH13 cases. Anti-FXIII inhibitors reacted with either FXIII-A or FXIII-B antigens (17 and two cases for FXIII-A and FXIII-B, respectively, as of Mar. 2012). Such an antigen–antibody complex may be cleared rapidly from the circulation, such that the patients' FXIII levels and FXIII activities decrease significantly.

During the 10 months of our nationwide campaign, which was supported by the Japanese Ministry of Health, Labor and Welfare in 2009, we diagnosed five new patients with AH13, among 20 newly suspected cases of HAFXIIIdef. When FXIII activity was reduced to less than 50 % of normal, it was proportional to the difference in α_2-PI levels between plasma and serum (plasma − serum α_2-PI), very likely due to its cross-linking to fibrin by FXIIIa [39]. Therefore, decreased amounts of the plasma − serum α_2-PI ex vivo may reflect reduced FXIII activity in vivo. This may, in turn, lead to decreased resistance to fibrinolysis in a hemostatic clot, and its premature lysis [40]. It has been reported that model thrombi from an FXIII-deficient patient lysed more quickly than normal thrombi; replacement therapy with FXIII concentrate normalized lysis at about 50 % FXIII activity in plasma [41]. Complete stabilization of thrombi was also achieved in vitro at 0.5 U/mL FXIII (50 % of normal) [42]. FXIII levels at more than 50 % of normal may be sufficient to achieve the plateau amount of plasma − serum α_2-PI, i.e. approximately 20 % of plasma α_2-PI which coincides with the plateau level of cross-linked α_2-PI by FXIIIa [43].

In addition, cross-linked α_2-PI may play a significant role in the inhibition of spontaneous lysis of a retracted clot more than in that of a non-retracted clot [40]. Consistently, we found that CR of platelet–fibrin was absent in FXIII-A KO mice [7] manifesting severe bleeding symptoms [44, 45] as described above.

Further, the plasma − serum α_2-PI level and its ratio to the adjusted plasma α_2-PI were confirmed to be useful diagnostic markers for severe FXIII deficiency, by examination of a total of 61 suspected HAFXIIIdef cases (as of Mar. 2012, unpublished data).

Mild bleeding symptoms in congenital FXIII-B deficiency (Table 1; Suppl. case review)

Through the recent nationwide survey on AH13 [35, 39], we identified a new case of severe FXIII-B deficiency in a 74-year-old Japanese man (paper in preparation by Wada and Ichinose, Case 11 in Suppl. case review). To the best of the author's knowledge, he is the oldest case of complete congenital FXIII-B deficiency ever first diagnosed (Table 1). This is consistent with the idea that congenital FXIII-B deficiency is a mild bleeding tendency and tends to be overlooked by physicians [2, 3], even up to the age of his mid-seventies as in this case. Hence, this author strongly recommends that FXIII activity be analyzed whenever physicians come across patients with unexplained bleeding disorders, especially when the results of routine clotting time tests, such as aPTT and PT, are within the normal or subnormal ranges. This holds true for the early diagnosis and treatment of HAFXIIIdef and AH13 [34, 35] as well.

Recently, a German group also reported a case of severe congenital FXIII-B deficiency (Case 10 in Suppl. case review) [4]. To the author's best knowledge, only 11 cases

Table 1 Bleeding symptoms and treatments in patients with severe FXIII-B deficiency

Case no.	Ethnic origin	Gender	Age (years)	FXIII-B (%)	FXIII-A (%)	Miscarriage (times)	Postpartum bleed. (times)	Gynecol./ obstetr. hemorrhage(s)	Postop bleeding	Other bleeding	Treatment	Author
1	Japanese	Female	32	<2	10–24[a]		1			Subcutaneous	Red cells concentrate, fresh frozen plasma	Saito [50]; Hashiquchi [52]
2	Japanese	Female	35[a]	ND	<10		2					Saito [50]
4	Italian	Female	30	<5	1	2	2	Menorrhagia	Yes	Epistasis, subcutaneous	Whole blood, transfused (not specified)	Girolami [48]; Izumi [49]
5	Italian	Female	33	<5	3		1		Yes	Epistasis	Whole blood	Girolami [48]; Izumi [49]
6	Italian	Female	34	<10	<10		2	Menorrhagia	Yes	Subcutaneous		Capellato [51]; Souri [53]
7	Japanese	Female	22	ND	<10				Yes			Koseki [3]
9	Hispanic	Female	19–76	UD	<10			Menorrhagia	Yes			Lovejoy [46]; Alvarado [54]
3	Japanese	Male	30[a]	<2	10	–	–	–	No			Saito [50]
8	Japanese	Male	1	ND	5	–	–	–		Umbilical		Koseki [3]
10	Indian	Male	32	<0.1	<2	–	–	–	Yes			Ivaskevicius [4]
11	Japanese	Male	74	<5	5	–	–	–	No	Intramuscular, subcutaneous, gingival, lower gastrointestinal	FXIII concentrates	Unpublished

ND not determined, *UD* undetectable

[a] Personal communication from Dr. M. Saito of Kanazawa University by E-mail in Feb 2012