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 HAFD. 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.

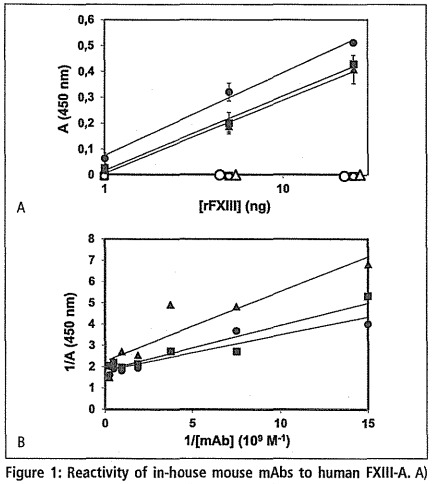

Figure 1: Reactivity of in-house mouse mAbs to human FXIII-A. A) Anti-FXIII-A mouse monoclonal antibodies (mAbs) (50 ng; 3.3 nM), 1TH2–8C4C (closed triangles, open triangles), 1TH6–2H7F (closed triangles, open triangles), or 1TH6–10E (closed squares, open squares) recognised rFXIII-A. Three doses (1, 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).

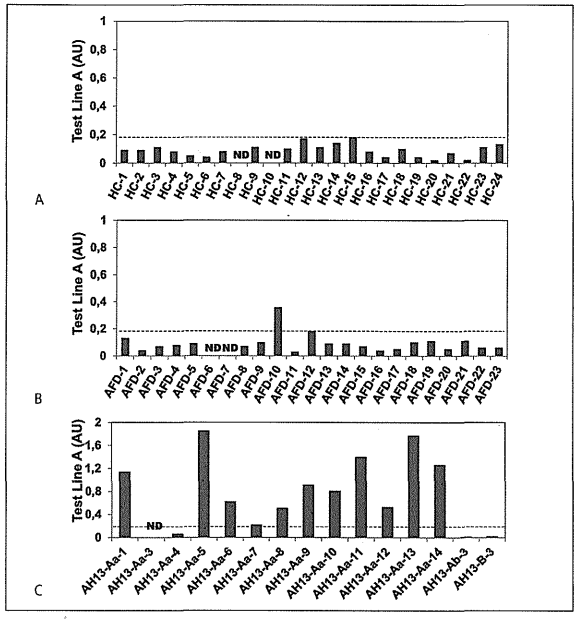

Figure 2: Development of a direct ICT to detect anti-FXIII-A auto-Abs. The absorbance is shown for 24 healthy control plasma samples (HC-1 to HC-24) (A), 23 samples from patients with AFD (AFD-1 through AFD-23) (B), and 15 samples from patients with AH13 (AH13-Aa-1, -Aa-3 through -Aa-14, -Ab-3, and B-3)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

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 ($0.7<AUC\leq0.9$) 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

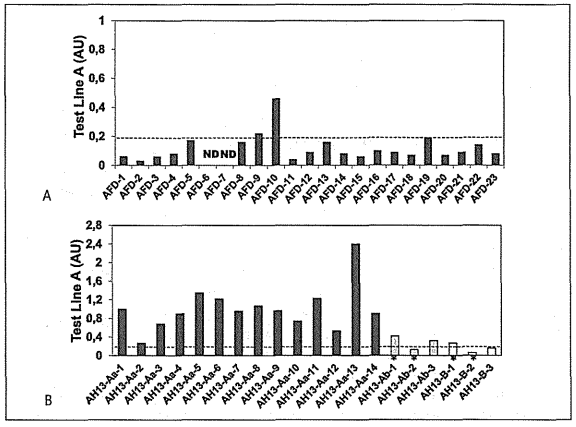

Figure 3: Modification of ICT after spiking with normal plasma samples. The absorbance is shown for 23 plasma samples from patients with AFD (AFD-1 through AFD-23) (A) and 20 samples from patients with AH13 (AH13-Aa-1 through -Aa-11, and -Ab-3) (B). The results of 13 AH13 cases of type Aa (AH13-Aa-1 through Aa-13), 3 of type Ab (AH13-Ab-1 through Ab-3), and 3 of type B (AH13-B-1 through B-3) are represented as a gray, closed, and open bars, respectively. * indicates samples (AH13-Ab-1, Ab-2, B-1, and B-2) that have already been diagnosed as AH13. The cut-off value is indicated by a dashed line. ND, not detectable.

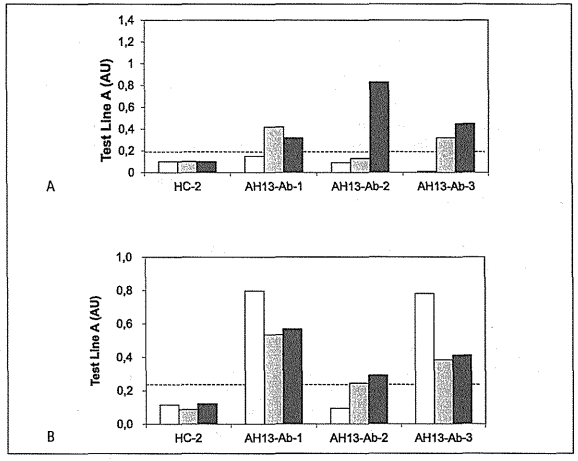

Figure 4: Application of ICTs to various types of AH13. A) The absorbance is shown for one healthy control plasma sample (HC-2) and three samples from patients with AH13 type Ab (AH13-Ab-1 to Ab-3). "Mere ICT", "ICT spiked with healthy control plasma", and "ICT spiked with FXIIIa" are represented as open, gray, and closed bars, respectively. A cut-off value is indicated by a dashed line. B) The absorbance is shown for one healthy control plasma sample (HC-2) and three samples from patients with AH13 type Ab (AH13-Ab-1 through -Ab-3). The results of ICTs spiked with healthy control plasma in the absence (open bars) or the presence of thrombin and $CaCl_2$ without (gray bars) or with GPRP -NH2 (closed bars) are shown. The cut-off value is indicated by a dashed line.

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 values obtained by ROC curve analysis and by average + 2 SD made comparable performance in ICTs. We therefore kept cut-off values obtained by average + 2 SD.

Discussion

This is the first report of a rapid test for the detection of anti-FXIII-A auto-Abs, which cause a life-threatening bleeding disease termed AH13. In this study, we first characterised three in-house mAbs in detail to confirm the rational basis for their use in an important life-saving diagnostic method. We then developed an ICT

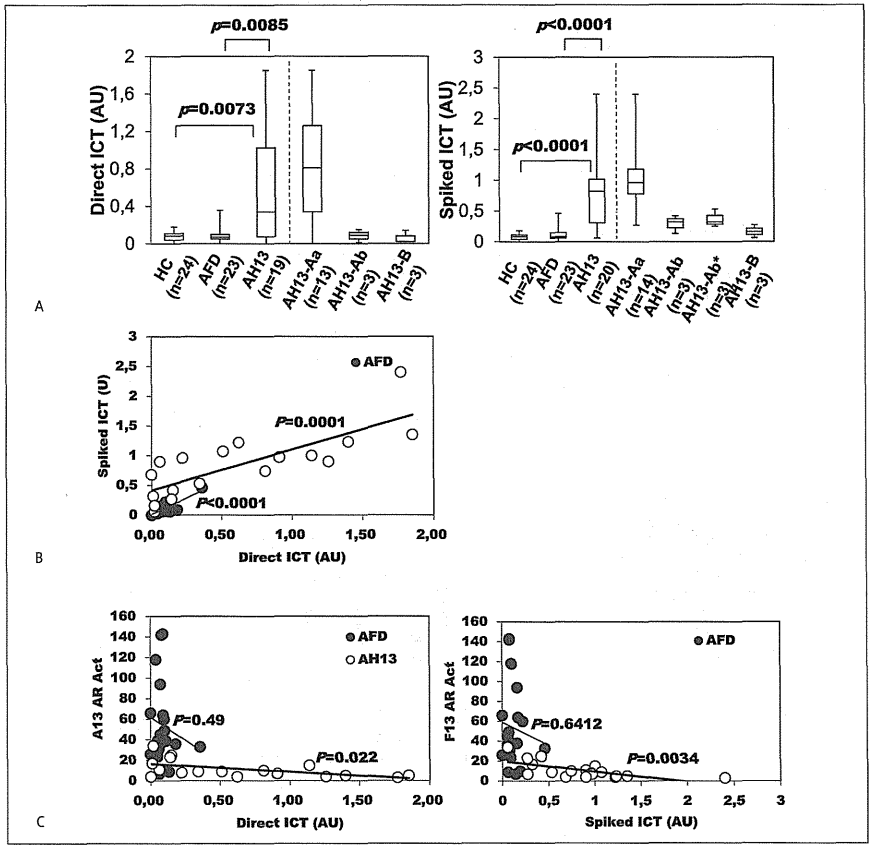

Figure 5: The correlation between ICT values and FXIII levels. A) Values of direct (left) and spiked (right) ICTs are shown. The results are presented as box-and-whisker plots denoting the median, interquartile range, and the minimum and maximum data points. The values of both direct and spiked ICTs were significantly higher in AH13 cases than in the healthy control (HC) group (0.60 ± 0.61 vs 0.08 ± 0.05, P = 0.0073 and 0.78 ± 0.54 vs 0.08 ± 0.05, P <0.0001, respectively) and HAFD patients (vs 0.09 ± 0.07, P = 0.0085 and vs 0.11 ± 0.09, P <0.0001, respectively). *; activated by thrombin in the presence of calcium. B) The correlation between direct and spiked ICTs are shown. There was a strong correlation between direct and spiked ICTs for both the AH13 and AFD groups (R^2 = 0.62 and P = 0.0001; R^2 = 0.67 and P <0.0001, respectively). C) Direct (left) and spiked (right) ICT values vs FXIII ammonia release (AR) activity (Act) levels are shown. For AH13 cases (open circles), a significant inverse correlation between both direct and spiked ICT values and FXIII-AR Act was observed (slope = −7.7, R^2 = 0.30, P = 0.022 and slope = −10.4, R^2 = 0.43, P = 0.0034, respectively).

to detect anti-FXIII-A auto-Abs and to differentiate AH13 cases from other HAF13D patients. The latter is essential for the proper treatment of AH13 because administration of immunosuppressive agents is indispensable for its Ab eradication therapy.

Among our three in-house mAbs against human FXIII-A, 1TH2–8C4C, had the highest affinity toward FXIII-A, and therefore it was selected as a capture antibody to be coated onto the surface of the assay membrane. This mAb was also confirmed to bind to the β-barrell-1 domain, a region where no function of FXIII-A had been assigned, to the best of our knowledge. This is consistent with the findings that none of our mAbs inhibited either the proteolytic activation of FXIII by thrombin or the catalytic activity of activated FXIIIa. Furthermore, the three mAbs did not interfere with the heterotetramer assembly between FXIII-A_2 and FXIII-B_2 dimers.

Taken together, 1TH2–8C4C is the most suitable Ab for the present ICT because it would not compete with patients' inhibitory anti-FXIII-A auto-Abs that target the plasma transglutaminase regions of FXIII-A. Thus, 1TH2–8C4C can detect not only non-neutralising auto-Abs but also neutralising auto-Abs against FXIII-A.

There are at least two major difficulties to overcome in developing an ICT to detect anti-FXIII-A auto-Abs in AH13 cases. The first is the low amounts of FXIII-A antigen that is frequently observed in patients' plasma. Our "direct ICT" can detect only an immune complex between FXIII-A antigen and its auto-Abs, but not free anti-FXIII-A Abs. Therefore, our direct ICT is made for the detection of only complexed anti-FXIII-A auto-Abs. This problem was overcome by employing the "spiked ICT," in which a normal amount of FXIII-A in control plasma was added to a patient's sample. Subsequently, the exogenous FXIII-A antigen supplied by the control plasma would be bound by free anti-FXIII-A auto-Abs in a patient's plasma, resulting in the formation of an additional amount of FXIII-A antigen-anti-FXIII-A auto-Ab complex. Accordingly, our spiked ICT can measure the total anti-FXIII-A auto-Ab level, if the quantity of a patient's anti-FXIII-A auto-Abs does not exceed the amount of FXIII-A antigen in the control plasma. In fact, this spiked ICT diagnosed all type Aa cases, without exception.

The second problem is harder to overcome, because it is based on the fundamental mechanisms of action of anti-FXIII auto-Abs. As described previously, there are at least three types of anti-FXIII auto-Abs, type Aa, Ab, and B, directed exclusively toward native FXIII-A, activated FXIII-A, and FXIII-B, respectively. Actually, some AH13 samples of either type Ab or B auto-Abs were classified as "false-negative". We therefore modified our ICT by adding pre-activated rFXIIIa into the original ICT reaction mixture, and successfully detected auto-Abs in all type Ab cases. A serum sample made from a mixture of type Ab and normal control plasma was used for ICT of AH13-suspected patients "in the field," because endogenously generated thrombin can activate FXIII-A in normal control plasma to form an immune complex between activated FXIIIa antigen and anti-FXIIIa auto-Abs.

Conversely, it is impossible to detect the type B auto-Abs by our current ICTs, because this type of auto-Abs is directed to the FXIII-B. We are developing another ICT to detect the type B auto-Abs using in-house mouse mAbs generated against FXIII-B, in the near future.

Our ultimate goal is to make our ICT suitable as a POCT (16–19), because a prompt detection of anti-FXIIII auto-Abs can lead to an immediate decision to start immunosuppressive treatment for patients with otherwise unexplained bleeding. To adapt our ICT system to a fully assembled POCT devise (17, 19), the current methods may need to be optimised further. In addition, incorporating a positive control of anti-FXIII Abs into a test strip as a control line will improve the usefulness of the test because it will reduce the number of strips as well as reagents needed to perform the assay. Although the present ICT study was carried out using a bench top absorbance reader, it was possible to read results visually (by three independent examiners in our laboratory, data not shown).

In conclusion, we have developed a new, rapid, and easy-to-use ICT for anti-FXIII-A auto-Abs that diagnoses 90% of AH13 patients. This novel ICT method may be applicable to a POCT, and contribute to saving more AH13 patients' lives.

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Author contributions

TO performed experiments and wrote the paper; SD, NK, and MY prepared in-house mAbs and developed ICT; SM assisted the experiments; AI created the research project and wrote the paper.

Conflicts of interest

None declared.

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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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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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In this article, a case of AH13 is defined as a patient who has both anti-FXIII autoantibodies and hemorrhagic symptoms.

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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 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 (Ontario, 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 recombinant FXIII-A (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 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.

LOW RESOLUTION FIG

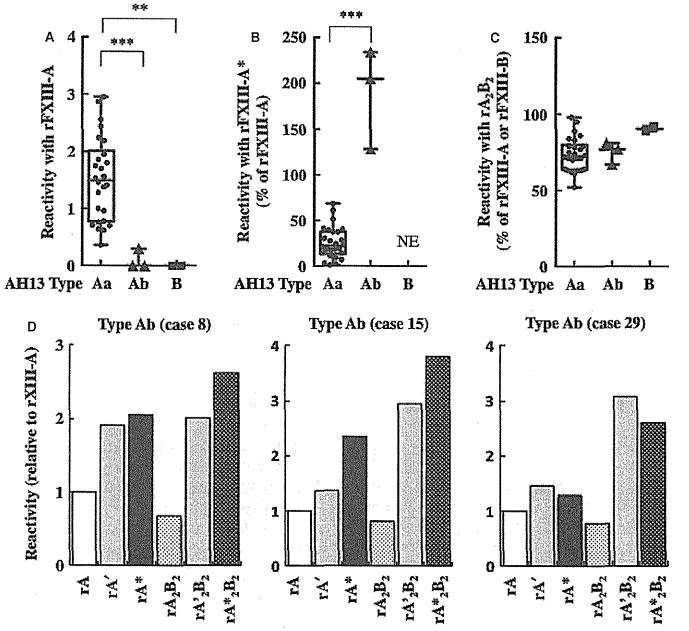

Fig. 1. Reactivity of anti-FXIII A subunit (FXIII-A) autoantibodies. (A–C) Reactivity of anti-FXIII-A autoantibodies with (A) recombinant FXIII-A (rFXIII-A), (B) activated rFXIII-A (rFXIII-A*) or (C) recombinant A_2B_2 (rA_2B_2) was determined for the three AH13 types. rFXIII-A reactivity is shown as 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. ***$P < 0.001$; **$P < 0.01$. (D) The reactivity of type Ab autoantibodies in cases 8, 15 and 29 with activation intermediates (rFXIII-A′, recombinant rA'_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). 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), 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).

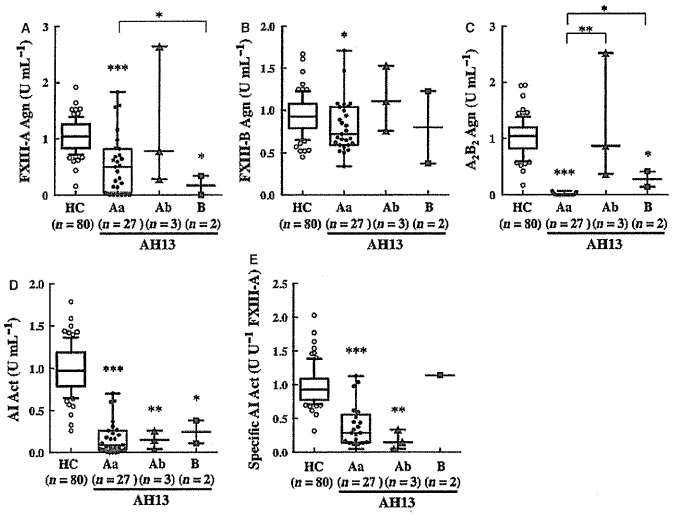

Fig. 2. FXIII parameters in plasma from healthy control and AH13 cases. (A–C) Levels of (A) FXIII A subunit (FXIII-A), (B) FXIII B subunit (FXIII-B) and (C) A_2B_2 heterotetramer antigens in plasma were measured by ELISA. (D) Amine incorporation (AI) activity was assayed with monodansylcadaverine and *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) and HAF13D. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. 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′ 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-polymeriza-

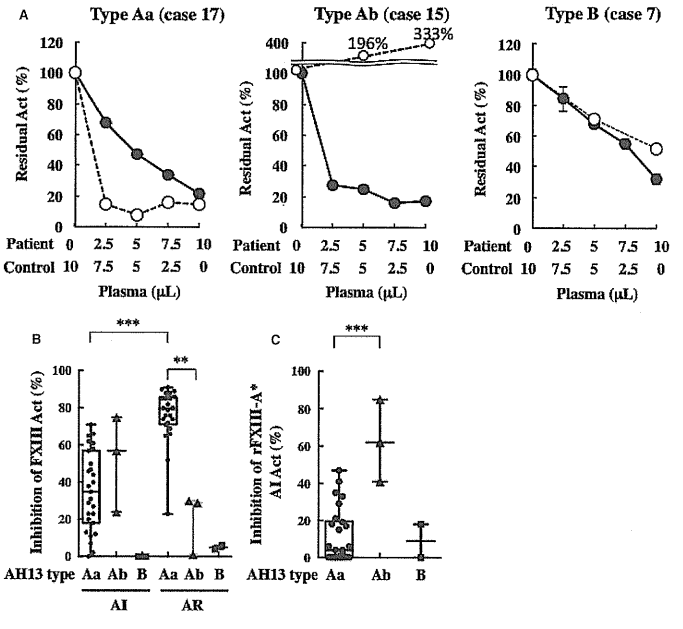

Fig. 3. Inhibition of FXIII activity by anti-FXIII autoantibodies. (A) Type Aa (case 17), type Ab (case 15) or type B (case 7) plasma was mixed with plasma from healthy controls. Amine incorporation (AI) (closed circle) and ammonia release (AR) activities (open circles) were measured with the five-step 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.

tion 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

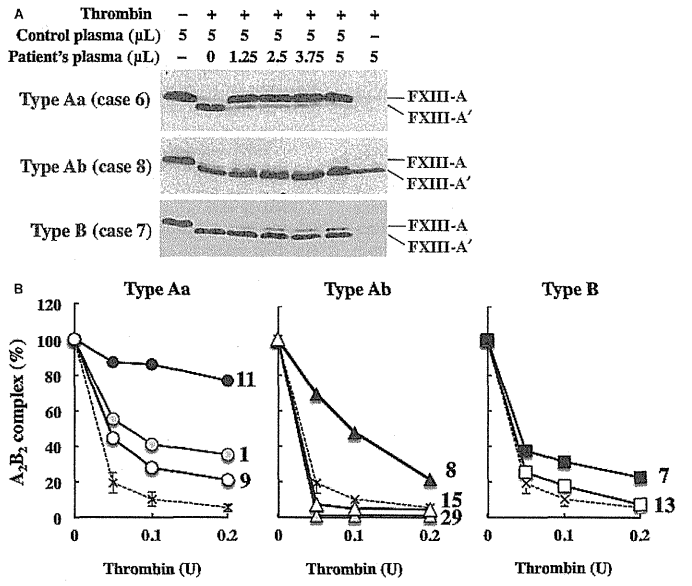

Fig. 4. Influence of anti-FXIII autoantibodies on activation of FXIII. (A) To test whether anti-FXIII autoantibodies inhibit activation peptide (AP) cleavage, healthy control plasma was incubated with AH13 plasma and used in a reaction with thrombin. Cleavage of AP was determined by western blotting 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.

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 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; 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).

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 γ-completion)	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	BE	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 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). †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.
AH13 cases who received FXIII concentrates within 10 days before blood collection are underlined.

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.

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).

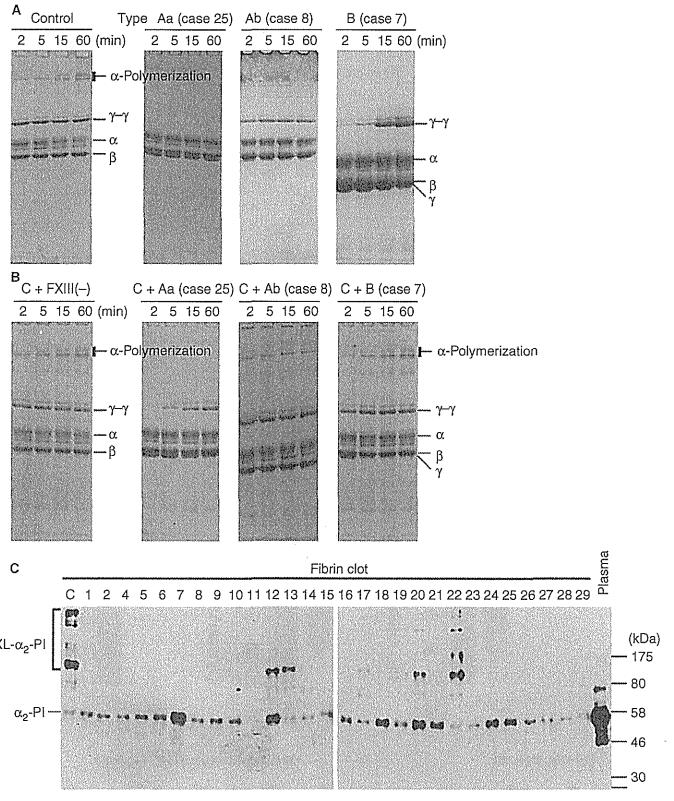

Fig. 5. Inhibition of fibrin-crosslinking reactions by anti-FXIII autoantibodies. (A) Fibrin-crosslinking reactions were examined in the plasma from a healthy control, a type Aa patient (case 25), a type Ab patient (case 8), or a type B patient (case 7). (B) Healthy control plasma was incubated with 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.

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 from type Aa cases. The existing FXIII-A in patients' plasma was not complexed with FXIII-B, but was instead

LOW RESOLUTION FIG

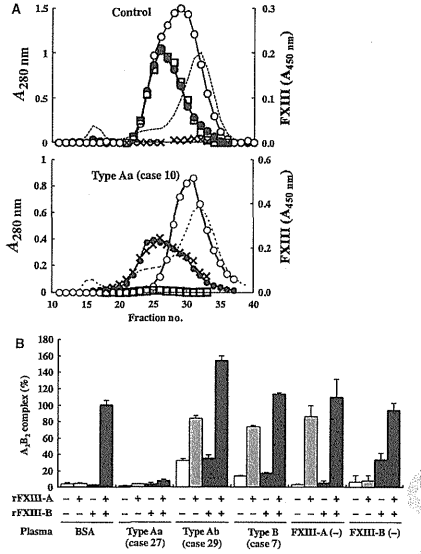

Fig. 6. Inhibition of heterotetramer assembly by type Aa anti-FXIII autoantibodies. (A) Plasma obtained from a healthy control or case 10 (type Aa) was fractionated on a Sepharose CL6B column. FXIII A subunit (FXIII-A) (closed gray circles and bold gray line), FXIII B subunit (FXIII-B) (open circles 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.

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 themselves with FXIII-B in the A_2B_2 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.

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

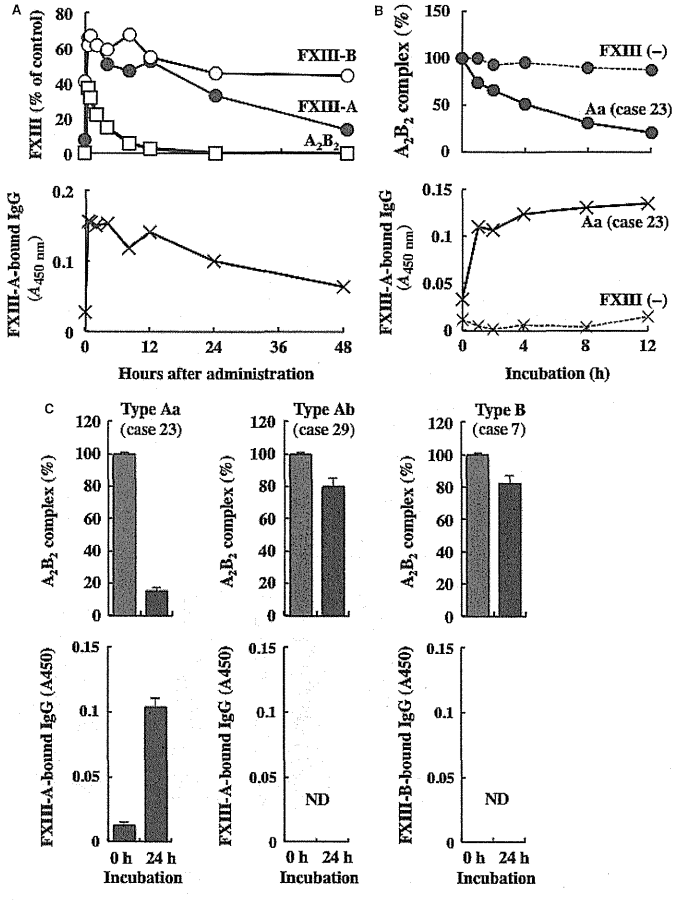

Fig. 7. Removal of FXIII A subunit (FXIII-A) from the FXIII heterotetramer by type Aa autoantibody. (A) Plasma-derived FXIII concentrates (A_2B_2) were infused into a type Aa AH13 patient (case 23), and plasma levels of FXIII-A (gray circles), FXIII B subunit (FXIII-B) (open circles), A_2B_2 (open squares) 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.

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 immunologic 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 A2 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.
Fig. S2. Inhibition of thrombin-dependent and Ca^{2+}-dependent dissociation by anti-FXIII autoantibodies.
Fig. S3. Inhibition of fibrin-crosslinking reactions by anti-FXIII autoantibodies.
Fig. S4. Gel-filtration analysis of FXIII antigens in plasma of type Ab and B cases.
Fig. S5. Immunoprecipitation of FXIII from plasma of a type Aa patient.
Fig. S6. Inhibition of heterotetramer assembly by type Aa serum and IgG.
Fig. S7. Removal of FXIII-A from FXIII heterotetramer by type Aa autoantibodies.

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CASE REPORT

Report of a patient with chronic intractable autoimmune hemorrhaphilia due to anti-factor XIII/13 antibodies who died of hemorrhage after sustained clinical remission for 3 years

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Abstract Although the incidence of autoimmune hemorrhaphilia due to anti-Factor XIII (FXIII, not FVIII or FXII to avoid confusion) antibodies (AH13) or hemorrhagic "acquired FXIII deficiency due to anti-FXIII autoantibodies" was previously considered rare, it has been on the increase in the twenty-first century, at least in Japan. An 83-year-old woman with an unexplained hemorrhage was admitted to our hospital for intramuscular hematoma and severe anemia. Her FXIII activity was reduced to 10 % of normal; since FXIII inhibitors and anti-FXIII-A subunit autoantibodies were detected, she was definitively diagnosed with AH13. Despite developing cardiac tamponade due to pericardial hemorrhage, she clinically recovered from AH13 after hemostatic therapy with FXIII-concentrates and immunosuppressive treatment with rituximab and cyclophosphamide. However, her FXIII activity remained low and she died of hemorrhage 3.5 years after admission. AH13 patients should be monitored for a prolonged period, as this disease is very likely a chronic intractable hemorrhagic disorder.

Keywords Autoimmune disease · Hemorrhagic disorder · Chronic disease · Hemostatic therapy · Immunosuppressive treatment

Acquired h(a)emophilia is a tentative name for this category of diseases, but unofficial because it is not included in the current version of the WHO ICD-10. "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" [Brinkhous, K.M. A short history of hemophilia, with some comments on the word "Hemophilia". In: KM Brinkhous and HC Hemker (eds). Handbook of Hemophilia, part 1, Amsterdam: Excerpta Medica, American Elsevier. 1975, p. 3–20]. Thus, the authors use the term hemorrhaphilia for a bleeding disorder caused by anti-FXIII autoantibodies, throughout this manuscript. H(a)emophilia must be used for the inherited hemorrhagic disease due to Factor VIII deficiency as listed in WHO ICD-10.

'Clinical' remission is defined as the disappearance of all bleeding symptoms, in this manuscript.

Masayoshi Souri and Akitada Ichinose belong to Japanese Collaborative Research Group on AH13.

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Introduction

Factor XIII (FXIII not FVIII or FXII) is a fibrin-stabilizing factor in the plasma consisting of A and B subunits (FXIII-A and FXIII-B, respectively). Because thrombin-activated FXIII cross-links fibrin monomers themselves and fibrin and α_2-plasmin inhibitor, congenital FXIII deficiency results in a lifelong severe bleeding tendency [1, 2]. Although congenital FXIII deficiency is rare [3], acquired FXIII deficiency is rather common, and mostly secondary to hyper-consumption or hypo-synthesis of FXIII (disseminated intravascular coagulation, surgery, trauma, leukemia, liver diseases, chronic inflammatory bowel diseases, artificial dialysis, etc.) [4]. However, patients with acquired FXIII deficiency rarely bleed because their FXIII levels remain only moderately reduced [5, 6]. In contrast, patients with autoimmune hemophilia-like disease (i.e., hemorrhaphilia) due to anti-FXIII antibodies (AH13) or hemorrhagic "acquired FXIII deficiency due to anti-FXIII autoantibodies" experience severe bleeding due to drastically decreased FXIII levels and the consequently reduced cross-linked α_2-plasmin inhibitor to fibrin [7].

Although AH13 used to be rare [8, 9], its incidence has been on the rise in the twenty-first century in Japan [10]. It is very likely because Japan has become a super-aging society first in the world, and because we have conducted a nation-wide survey supported by the Japanese Ministry of Health, Labor and Welfare. In this study, we report an 83-year-old woman with chronic intractable AH13.

Methods

This study was approved by the institutional review board of Yamagata University School of Medicine. All procedures were conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from this patient.

All experimental procedures have been described previously [11, 12] except for the enzyme-linked immunosorbent assay (ELISA) to detect anti-FXIII-A autoantibodies: briefly, 1 μL of plasma was incubated with 200 ng of recombinant (r)FXIII-A in a 10 μL mixture at 37 °C for 2 h and then diluted tenfold with a buffer. Ten microliters of the diluted plasma was added to a 96-well plate coated with anti-FXIII-A monoclonal antibody and reaction with peroxidase-conjugated anti-human Immunoglobulin G (IgG) was allowed for 60 min. ELISA was also carried out without adding rFXIII-A into patient's plasma to detect pre-existing anti-FXIII-A autoantibodies bound to own FXIII-A.

For dot blot assay [11], 50 and 100 ng of either rFXIII-A or rFXIII-B, or 100 and 200 ng of rFXIII complex (A_2B_2 tetramer) were blotted onto a nitrocellulose membrane, and reacted with the patient's plasma at a dilution of 1:2,000. Immunoglobulin bound to either one of these FXIII antigens was detected using peroxidase-conjugated anti-human immunoglobulins (G+M+A) and a chemiluminescent substrate.

Patient

The initial part of this patient's history was previously reported as acquired FXIII deficiency "in Japanese" by orthopedic surgeons [13]; in brief, our patient with unexplained hemorrhage was admitted to the Department of Orthopedic Surgery of our hospital on Oct 17, 2009 for large intramuscular hematoma in her left thigh and severe anemia. She experienced repeated life-threatening bleeding episodes at multiple sites, including retroperitoneal, intrathoracic, and mediastinal, for approximately 3 weeks. Her FXIII activity was reduced to 10 % of normal despite normal bleeding time, prothrombin time (PT), activated partial thromboplastin time (APTT), and platelet count (Table S1). She received repeated transfusions of red blood cell concentrates (Fig. 1, top), and the arteries flowing the bleeding sites were topically treated using transcatheter arterial embolization on Nov 1 and 7, 2009.

Plasma-derived FXIII-concentrates (1,200 U in 5 vials of Fibrogammin; CSL Behring, Tokyo, Japan) were administered daily starting on Nov 7 (Fig. 1, top) because of her isolated severe FXIII deficiency. On Nov 10, because of her severe hemorrhagic symptoms, she was transferred to the intensive care unit of our hospital, where she suddenly developed shock. She was then intubated and mechanically ventilated. Computed tomography (CT) revealed the presence of cardiac tamponade due to pericardial hemorrhage and an intrathoracic hematoma (Fig. S1 A, B).

To explore the pathogenesis of the patient's isolated acquired FXIII deficiency and determine its ideal treatment modality, the patient was referred to the Japanese Collaborative Research Group (JCRG) for detailed FXIII analyses. After being definitely diagnosed with AH13, she was started on rituximab, an anti-CD 20 monoclonal antibody, 375 mg/m^2/week for 4 weeks from Nov 13 (Fig. 1, top). Hemostatic therapy with FXIII-concentrates was also continued every other day. Despite our extensive search, we could not find any evidence for the underlying diseases of AH13, such as cancer, autoimmune disease, and medication history of isoniazid, antibiotics, and anticonvulsants [8, 9].

Although "clinical remission" was achieved, i.e., the hemorrhage was arrested, the patient's FXIII activity remained low (around 10 % of normal) despite her FXIII antigen level being much higher than normal (>200 %) because of the continued administration of the FXIII-concentrates (Fig. 1, top). These findings suggested the persistence of an FXIII inhibitor. Therefore, she was intravenously given cyclophosphamide 10 mg/kg every other week three times starting in Jan 2010. Since no improvement in FXIII activity was seen, rituximab was added in Feb 2010. However, the combination of these immunosuppressants did not have any immediate effect on FXIII activity (Fig. 1, bottom).

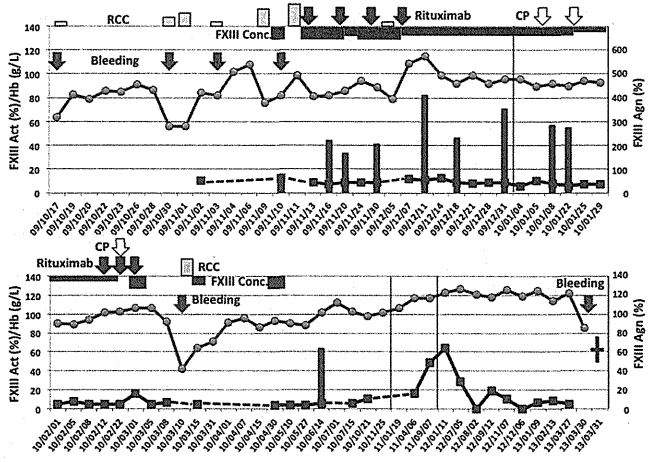

Fig. 1 Clinical course of the present AH13 case. Hemoglobin levels (Hb, *closed circle*) corresponded to bleeding episodes and transfusion of red blood cells (red cell concentrates; RCC) in the opposite directions. Plasma-derived FXIII-concentrates were given at 1,200 U first daily, secondly ever other day, and third every 3 days. The patient underwent immune-suppressive therapies firstly with rituximab and secondly cyclophosphamide (CP) pulse, and thirdly with rituximab. Although FXIII antigen (*filled column*) increased up to 416 % of the normal, FXIII activity (*closed square*) remained low at around 10 % following the replacement therapy with FXIII-concentrates. FXIII activity spontaneously rose and gradually decreased to 5 % 3 days before her hemorrhagic death. *Vertical lines* depict the ends of years

Despite treatment, the patient's FXIII level remained low and her hemoglobin level suddenly dropped to 42 g/L again in Mar 2010. CT imaging revealed an intramuscular hematoma in her left thigh that was successfully controlled by a daily infusion of FXIII-concentrates (Fig. 1, bottom). She underwent no further immunosuppressive treatment, such as steroids, cyclosporine, etc., in order to avoid infection, because of her old age and low capacity for daily living: she was bedridden as well. In addition, there is no gold standard by which AH13 patients are treated. Since her general condition stabilized without further bleeding episodes, she was discharged from the hospital to her home 6 months after admission.

Thereafter, she was regularly followed once a month by her original physician in another hospital. Her FXIII activity level started to spontaneously increase 8 months after the final dose of rituximab, reached 64 % of normal in the following 15 months, and then gradually returned to 10 % of normal after another 10 months (Fig. 1, bottom). Nevertheless, no bleeding episodes were observed for about 3 years.

On the morning of Mar 30, 2013, she was found lying on the floor. She complained of abdominal pain and repeatedly vomited for several hours. When she was transferred to a local hospital by ambulance, there were bruises on her right forehead, left elbow, and left knee. She had tenderness at the left hypochondrium. Laboratory examinations revealed moderate anemia (hemoglobin 85 g/L) and an essentially normal platelet count and PT, and slightly shortened APTT (Table S2). CT scanning revealed a subcortical hemorrhage in the right frontal lobe and a splenic laceration with intraperitoneal hemorrhage (Fig. S1 C, D). She developed shock and died shortly thereafter.

Results and discussion

Laboratory coagulation tests

All coagulation-fibrinolysis factors, except for FXIII were within normal ranges on Nov 2 (Table S1). D-dimer, thrombin–antithrombin complex, and von Willebrand factor antigen levels increased slightly or moderately, according to the patient's severe bleeding condition.

Because the patient's FXIII activity and antigen were 13 and 80 % of the normal values, respectively, on Nov 10, the specific activity was as low as 0.16 (normal value, 1.0). In addition, a 1:1 cross-mixing test between the patient's and a healthy control's plasma clearly demonstrated an "inhibitor" pattern (patient, 19 %; control, 104 %; and mixed, 17 %). All these results suggested the presence of an FXIII inhibitor.

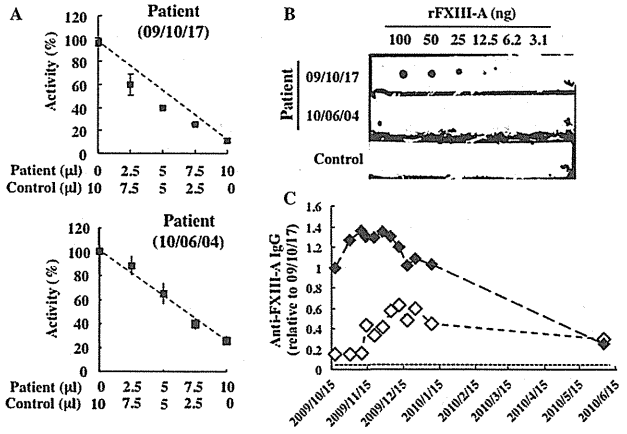

Fig. 2 JCRG analyses of the patient's plasma FXIII and detection of anti-FXIII antibodies. **a** A 5-step dilution cross-mixing test by an amine incorporation assay was performed using the patient's plasma at the ratios of 0:1, 1:3, 1:1, 3:1, and 1:0 with a normal plasma. The mixed samples showed an "inhibitor" pattern, because there was a downward deviation (Oct. 17, 2009 or hospital day 1). A broken line depicts a theoretical 'deficient' pattern. The patient's plasma of June 4, 2010 demonstrated the deficiency pattern. **b** A dot blot assay was performed using recombinant FXIII-A (rFXIII-A) at the indicated amounts shown as antigen (ng). The results showed the presence of anti-FXIII-A antibodies (on Oct. 17, 2009). The negative control stands for healthy individual's plasma. The patient's plasma of June 4, 2010 showed a negative result. **c** After the immunosuppressive therapy, her anti-FXIII-A immunoglobulin G (IgG) had significantly decreased, judging from our ELISA results. Although the dot blot assay did not detect anti-FXIII-A autoantibodies in her sample of June 4, 2010 (b), a small amount of anti-FXIII-A IgG was still clearly detected by the ELISA (*filled diamonds*). A similar amount of anti-FXIII-A IgG bound to own FXIII-A was also detected by the ELISA without adding rFXIII-A (*open diamonds*). A dashed line represents an average level of normal controls

Experimental FXIII tests

The patient's plasma on Oct 17 (hospital day 1) was analyzed by the JCRG in detail; the FXIII-A antigen was 23 % of normal and the FXIII amine incorporation activity was 12 %, thus its specific activity was 0.47 (Table S3). Her FXIII-B antigen was 85 % of normal. Although her FXIII activity was extremely low during this acute stage, both FXIII-A and FXIII-B proteins retained their normal molecular weights as shown by western blotting (Fig. S2 A).

Our 5-step dilution cross-mixing test of the patient's plasma demonstrated a concave "inhibitor" pattern (Fig. 2a, top). An in-house dot blot test using rFXIII-A, rFXIII-B, and rA_2B_2 tetramer detected anti-FXIII-A autoantibodies (Fig. 2b, top; Fig. S2 C). In addition, our ELISA method clearly detected anti-FXIII-A antibodies (Fig. 2c). Accordingly, she was definitely diagnosed with AH13. A fibrin cross-linking test also visualized a drastic retardation in γ-chain dimerization and the absence of α-chain polymerization (Fig. S2 B).

Approximately 3 months after the final administration of rituximab, the patient's plasma showed a straight "deficient" pattern in the 5-step dilution cross-mixing test (Fig. 2a), suggesting that her FXIII inhibitor had virtually disappeared. This finding agreed with the fact that the dot blot analysis did not detect anti-FXIII-A antibodies. This partial recovery may be associated with the slower effect of rituximab [14] or spontaneous antibody regression [15].

Our ELISA, however, detected small amounts of total and FXIII-A-bound anti-FXIII-A antibodies (Fig. 2c). These findings are quite in agreement with the fact that the patient had 64 and 6 % of FXIII antigen and activity, respectively (Fig. 1, bottom), indicating the presence of the FXIII/13 antigen–antibody complex in the patient's sample.

Extended coagulation screening tests (Table S4) were also conducted by the JCRG to take an overall look at the current status of coagulation–fibrinolysis system in this AH13 case. All test results were consistent with those discussed above.

It was interesting to note that the patient's FXIII activity transiently increased in late 2011 and gradually decreased in early 2012: It may be attributable to the rather late effect of rituximab because it may require more time than other regimens [14]. Alternatively, the amount of her

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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Complete remission* achieved by steroid pulse therapy following rituximab treatment in a case with autoimmune haemorrhaphilia due to anti-factor XIII antibodies

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Dear Sirs,

Coagulation factor XIII (FXIII or F13) is a plasma fibrin-stabilising factor which crosslinks fibrin monomers, and fibrin and α_2-plasmin inhibitor, and thus plays an essential role in haemostasis. Therefore, its congenital deficiency results in a life-long haemorrhagic tendency (1, 2). In contrast, acquired coagulation FXIII deficiency is a common abnormal condition secondary to its hyper-consumption or hypo-synthesis (2, 3), while autoimmune haemorrhaphilia due to anti-FXIII antibodies (AHXIII) is a rare life-threatening bleeding disorder mostly in the elderly (3, 4). In the 21st century, the number of AHXIII cases has been on the increase at least in Japan (unpublished data), because Japan has become a super-aging society first in the world. Therefore, it is most important to save patients' lives by its prompt examinations, correct diagnosis, and proper treatment. Here, we report such an excellent example.

A 70-year-old Japanese male had no personal and family history of bleeding tendency. He had general fatigue and arthralgia of the right wrist from the second half of May 2013, and then diffuse swelling developed in his right back in the middle of June. Because the swelling and pain of his right back increased, he visited a local hospital. Since a hematoma was found by CT imaging and severe anaemia (Haemoglobin 4.7g/dl) was detected by haematological analyses, he was referred to our hospital. Hand X-ray showed non-destructive synovitis and laboratory examinations revealed severe anaemia (haemoglobin 6.7 g/dl) and haemolysis (LDH 1,469 IU/l; total bilirubin 1.9 mg/dl; haptoglobin, <6.6 mg/dl) as well as positive Coombs' tests (direct 3+, indirect 1+), antinuclear antibody (ANA, 1:2,560 dilution) and anti-DNA antibody (34 IU/ml). Accordingly, he was diagnosed as having systemic lupus erythematosus and autoimmune haemolytic anaemia.

Furthermore, a physical examination on admission confirmed extensive swelling from the right scapular to lumber portions and CT imaging revealed a huge intramuscular haematoma in his right back (data not shown). Although PT, APTT and platelet count were normal, his FXIII activity and antigen turned out to be as low as 4% and 5% of normal, respectively. The reduced activity was not corrected (6% residual FXIII activity) by a normal plasma (97% FXIII activity) in a 1:1 cross-mixing test, and a five-step dilution cross-mixing test of the patient's plasma showed a concaved "inhibitor" pattern (Suppl. Figure 1A, available online at www.thrombosis-online.com), clearly indicating the presence of anti-FXIII inhibitor. Finally, all three immunological tests, ELISA and dot blot assay (▶Figure 1 and Suppl. Figure 1B, available online at www.thrombosis-online.com), and immuno-chromatography (data not shown), detected anti-FXIII A subunit (FXIII-A) autoantibodies. Thus, he was definitely diagnosed as AHXIII. Detailed experimental studies also demonstrated the absence of both γ-dimerisation and α-polymerisation of fibrin (Suppl. Figure 1C, available online at www.thrombosis-online.com), indicating that fibrin-stabilisation was highly impaired.

Whereas he was initially treated with prednisone (PSL; 0.8 mg/kg/day) from hospital day 2, there was no evidence of its efficacy on either his FXIII activity or anti-FXIII inhibitor potency (residual FXIII activity in the 1:1 cross-mixing test), for 28 days (▶Figure 1). Because he had repeated bleeding episodes, he was infused with plasma-derived FXIII concentrates (27–45 U/kg). Pharmacokinetic studies revealed extremely low recovery rates and shortened half-lives of exogenous FXIII (Suppl. Figure 2, available online at www.thrombosis-online.com), suggesting the presence of a large amount of anti-FXIII inhibitor (i.e. "free" neutralising anti-FXIII-A autoantibodies). Accordingly, he was treated with rituximab (375 mg/m²/week) for four consecutive weeks. Since his plasma FXIII activity and residual FXIII activity in the cross-mixing test only slightly increased after the rituximab therapy, he was given methyl-PSL (1,000 mg/day) for three days (steroid pulse therapy) from day 57. Thereafter, residual FXIII activity in the cross-mixing test started to increase rapidly, indicating a rapid decrease in the neutralising anti-FXIII-A autoantibodies. The patient's FXIII activity also gradually increased and reached to normal levels about 50 days after the steroid pulse therapy (▶Figure 1). All immunological tests confirmed the disappearance of the anti-FXIII-A autoantibodies (▶Figure 1 and Suppl. Figure 1D, available online at www.thrombosis-online.com), and thus he was discharged on day 116 (hospital week 17, ▶Figure 1). Until the time of submission of this revised manuscript, he is still in complete remission (CR) for more than six months.

Haemostatic therapy is essential in the management of AHXIII, because it is a life-threatening disease. In fact, three out of 12 Japanese AHXIII cases had deceased before the arrival of test plasma samples to the last author's laboratory („dead-on-arrival" of test samples) in 2013 (unpublished data). Fortunately, the present case was promptly diagnosed and properly treated under continuing close monitoring of FXIII activity, antigen, cross-mixing tests, anti-FXIII-A antibodies, etc. Especially, pharmacokinetic analyses were carried out as many as four times to estimate recovery rates, peak times, as well as half-lives of administered FXIII. These results clearly indicated the rapid neutralisation and enhanced clearance of the infused exogenous FXIII in the present patient *in vivo* (Suppl. Figure 2, available online at www.thrombosis-online.com). All these detailed examinations made it possible to rapidly achieve CR of AHXIII without any further bleeding episodes or complications after the steroid pulse therapy.

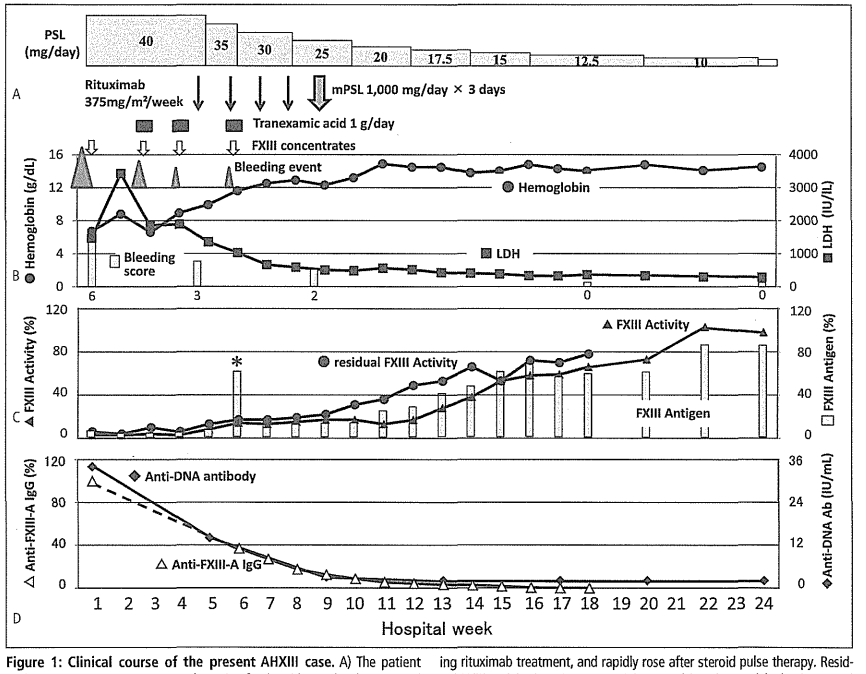

Figure 1: Clinical course of the present AHXIII case. A) The patient underwent immune-suppressive therapies firstly with regular-dose steroid (PSL), secondly rituximab, and thirdly steroid pulse (methyl-PSL; mPSL). Plasma-derived FXIII concentrates were given together with an anti-fibrinolytic agent (tranexamic acid). B) A Japanese version of ISTH/SSC Bleeding Assessment Tool (JBAT; shadowed column) was applied to objectively evaluate the patient's bleeding symptoms. Bleeding score was 6 at admission to our hospital and gradually deceased to 0 following medication, in the opposite direction to haemoglobin levels (closed circle). LDH (closed square) rapidly decreased following the immune-suppressive therapies. C) Both FXIII activity (closed circle) and antigen (shadowed column) only slightly increased following rituximab treatment, and rapidly rose after steroid pulse therapy. Residual FXIII activity in a 1:1 cross-mixing test (closed triangle) also increased after steroid pulse therapy. *; This relatively high FXIII antigen level was obtained 24 hours after the infusion of FXIII concentrates (2,400 U), and the resulting low FXIII specific activity (i.e. FXIII activity/antigen ratio) indicated the formation of FXIII antigen-antibody complexes between "free" anti-FXIII-A autoantibodies and exogenous FXIII concentrates, as shown in Suppl. Figure 2D (available online at www.thrombosis-online.com). D) Anti-FXIII-A IgG (open triangle) measured by ELISA decreased in parallel with anti-DNA antibody (closed diamond). The amount of anti-FXIII-A IgG on June 28 (hospital day 1), 2013 is defined as 100%.

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* Note: In this manuscript complete remission is defined as the disappearance of all anti-FXIII autoantibodies.

† Both YN and AI contributed to this research project as senior authors.

Steroid pulse was given (in combination with calcineurin inhibitors) to acquired haemophilia A (AHA) cases, and sustained response was achieved in 10 of 11 patients in a median time of three weeks (5). Several AHA cases were also successfully treated with steroid pulses alone (6) or followed by regular-dose steroid therapy (7–9). In contrast, two AHXIII cases were treated with steroid pulse, but there was no evidence of response (10, 11). Since FXIII activity had not improved following rituximab treatment in the present case, steroid pulse was added (▶ Figure 1). However, we cannot attribute the patient's CR to steroid pulse alone, because rituximab may require more time than other regimens to achieve remission, as reported in 51 AHA cases (12). Complete (13) and partial responses (14, 15) were obtained by rituximab treatment in one and two AHXIII cases, respectively, while no response was achieved in two cases (11, 16). Accordingly, it would be proper to conclude that a combination of steroid pulse and rituximab treatment achieved CR in this AHXIII case.

It is also important to note that both ANA and anti-DNA antibodies disappeared in concert with anti-FXIII autoantibodies (▶ Figure 1), indicating the efficacy of the combination therapy on all the aforementioned autoimmune comorbidities.

Finally, it is essential to raise the awareness of AHXIII to save its patients' lives by prompt diagnosis and treatment. We would also like to emphasise that detailed characterisation of AHXIII, especially by both the mixing study for the detection of neutralising antibodies against FXIII-A (e.g. functional 1:1 and five-step dilution tests) and the binding assays for the detection of non-neutralising antibodies against FXIII-A and FXIII-B (e.g. immunological tests), "must" be carried out, as per recommendation of the FXIII/Fibrinogen subcommittee of ISTH/SSC (1), in order not to overlook any AHXIII patients.

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Conflicts of interest
None declared.

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症例報告

遺伝子組換えトロンボモジュリン製剤が奏効した維持透析中の解離性大動脈瘤に合併した慢性播種性血管内凝固

早川佳奈[1]，田村志宣[2]，義間大也[1]，早川隆洋[1]，
栗原稔男[3]，大浦真紀[4]，中野好夫[1]，惣宇利正善[5]，
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症例は62歳男性。3年前に多発性嚢胞腎，急性大動脈解離（Stanford A型）の既往あり。急性大動脈解離の手術以降，慢性腎不全が進行し透析導入となった。透析開始から間もなくして，シャント穿刺部位の止血困難・後出血を認めたため，当科受診となった。血液検査上，軽度の血小板減少と線溶系マーカーの上昇を認め，慢性播種性血管内凝固が疑われた。さらに，第XIII因子活性の低下も認めたが，そのインヒビターは検出されなかった。造影CTでは，解離性大動脈瘤の増悪を確認した。以上より，解離性大動脈瘤に合併した慢性播種性血管内凝固ならびに二次性出血性第XIII因子欠乏症と診断した。治療として，維持透析中であったこと，手術までの期間があることより，遺伝子組み換えトロンボモジュリンを選択。その定期投与を繰り返すことで，止血機能は改善に転じ，術前まで良好なコントロールを得ることができた。（臨床血液 55（11）：2300～2305，2014）

Key words : Chronic disseminated intravascular coagulation, Recombinant human soluble thrombomodulin, Dissecting aortic aneurysm, Factor XIII

緒　言

大動脈瘤や大動脈解離に慢性の播種性血管内凝固（Disseminated Intravascular Coagulation, DIC）をきたすことはよく知られているが，著明な出血傾向や血小板減少をきたす症例は約4%程度といわれている[1]。根本的な治療としては原因部位の手術であるが，様々な理由により内科的コントロールを必要とする症例が少なからず存在する。一方で，内科的コントロールを要する解離性大動脈瘤に伴う慢性DICの症例報告はあるものの，定まった治療法は未だ確立していない。今回，我々は，解離性大動脈瘤増悪に伴う慢性DICを合併し，止血困難・後出血をきたした維持透析患者に対し，遺伝子組換えトロンボモジュリン製剤（recombinant human soluble thrombomodulin, rTM）を投与することで，慢性DIC治療を内科的に成功し得た1例を経験したので報告する。

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症　例

患　者：62歳，男性。

主　訴：シャント穿刺部の止血困難・後出血

既往歴：虫垂炎（20歳），急性大動脈解離 Stanford A型・多発性嚢胞腎（59歳）

家族歴：母・妹；多発性嚢胞腎，兄；多発性嚢胞腎・大動脈解離・狭心症

現病歴：3年前より多発性嚢胞腎のため，当院腎臓内科を受診し，以後外来通院となった。しかし，徐々に腎機能の悪化を認め，透析導入，左前腕内シャント設置術を施行された（この時点で止血異常を認めず）。翌月より近医で維持透析を開始。転医以降，シャント穿刺部の止血に1時間程度必要となった。さらに，穿刺部の止血後の後出血も徐々に認めるようになった。症状の改善を認めないため，透析導入7か月後に精査・加療目的で当科紹介となった。

初診時現症：身長160.7 cm，体重70.8 kg，体温36.3度，血圧133/80 mmHg，脈拍60回/分。眼球結膜黄染なく，眼瞼結膜貧血を認めず。前胸部から右下腹部，左前腕に手術痕を認める。左前腕の内シャント吻合部の手術痕直下に多数の皮下出血を認めた。

初診時検査所見（Table 1）：血小板8.8万/μlと減少し，プロトロンビン68%と軽度低下を認めるも，活性化部分トロンボプラスチン31.4秒と正常。フィブリノーゲン128 mg/dlと低下，fibrin and fibrinogen degradation products 78.49 μg/mlとD-ダイマー15.71 μg/mlと共に上昇。旧厚生省DIC診断基準ではDICスコア7点であり，かつthrombin-antithrombin complex（TAT）31.4 ng/mlとplasmin α2-plasmin inhibitor complex（PIC）11.8 mg/mlと共に高値であったことから，線溶亢進型DICと診断した[2]。

その他の凝固因子に関しては，合成基質法で，第XIII因子活性33%，第XIII因子抗原30%と低下を認めた。さらに，本例は，軽症のDICであったことに加え，後出血という特徴的な出血症状をきたしていたことから，第XIII因子の詳細な解析を続けた。ELISA法で測定した第XIII因子-Aサブユニット抗原，Aサブユニットと結合したBサブユニット抗原量は共に軽度低下するも，第XIII因子-Bサブユニット抗原量は正常であった（Fig. 1a）。なお，健常者と患者の第XIII因子の比活性は正常であった。さらに，アミン取り込み試験で測定した第XIII因子の活性は低下（Fig. 1a）。5段階希釈混合試験は，インヒビター陰性パターンを示した（Fig. 1b）。さらに，SDS-ポリアクリルアミドゲル電気泳動によるフィブリン架橋試験では健常者と比較しても有意差なく，ドットブロット法でも第XIII因子の抗体は陰性であった（Fig. 2）。以上より，自己免疫性血友病XIIIは否定された。

大血管造影CT（Fig. 3）：大動脈弓部から下行大動脈にかけて偽腔開存型の大動脈解離を認め，弓部の瘤に関しては透析導入前と比較し拡大傾向を認めた。

臨床経過（Fig. 4）：心臓血管外科との協議の結果，内科的にDICコントロールを優先する方針となった。維持透析中であり，手術までの準備期間を要するため，rTMを選択した。rTM 130 U/kgを6日間連日投与（透析日は透析後に点滴）。当科加療中に新鮮凍結血漿は一度も使用しなかった。また，透析中の抗凝固薬とrTMの作用が重なることがないように，透析時，半減期が非常に短いメシル酸ナファモスタットを使用した。本例では，抗凝固薬併用による出血傾向の副作用などは特に認めなかった。投与開始1週間で内シャント吻合部の手術痕直下の皮下出血は認めなくなり，シャント穿刺部の止血は15分程度まで短縮することができた。後出血も認めなくなった。さらに，血液検査では，TATとPICは共に低下し，第XIII因子の活性の改善を認めた。以上の結果から，二次性出血性後天性第XIII因子欠乏症と診断した。退院後，非透析日に週1回のrTM 130 U/kg投与の維持療法に切り替えるも，徐々に慢性DICの増悪を認めたため，治療開始3週間目から非透析日に週2回のrTM 130 U/kg投与に変更。rTMの週2回投与に変更以降，慢性DICの再増悪は認めていない。術前までのDICコントロールは良好であり，治療開始4ヶ月後に解離性大動脈瘤の手術を無事行うことができた。

考　察

DICを診断・治療するうえで，病型分類が重要とされており，基本的には「線溶亢進型」「線溶均衡型」「線溶抑制型」の3つに分類される[2]。その中で線溶亢進型

Table 1　Laboratory data in our patient's peripheral blood.

White Blood Cells	5,600/ml	AST	23 IU/l	ANA	<40
Red Blood Cells	346×10⁴/ml	ALT	17 IU/l	MPO-ANCA	<1.3 U/ml
Hemoglobin	11.2 g/dl	Total protein	7.1 g/dl	PR3-ANCA	<1.3 U/ml
Hematocritt	31.0%	Albumin	4.4 g/dl	HIT antibody	negative
Platelet count	8.1×10⁴/μl	ALP	234 IU/l	Factor VIII activity	52%
		LDH	229 IU/l	Factor IX activity	100%
Prtothrombin	68%	Creatine kinase	88 IU/l	Factor XIII activity	33%
APTT	31.4 sec	Creatinine	7.02 mg/dl	Factor XIII antigen	30%
Fibrinogen	128 μg/dl	BUN	35.3 mg/dl	Factor VW activity	82%
FDP	78.49 μg/ml	CRP	0.37 mg/dl	ADAMTS-13	103%
D-dimer	15.71 μg/ml			α2 plasmin inhibitor	57%
Antithrombin III	87%				

APTT, activated partial thromboplastin time; FDP, fibrin and fibrinogen degradation products;
BUN, blood urea nitrogen; ANA, antinuclear antibodies; ANCA, antineutrophil cytoplasmic antibody; MPO, myeloperoxidase; PR3, proteinase 3; HIT, heparin-induced thrombocytopenia; ADAMTS-13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13.

a

	FXIII-A		FXIII-B		A-bound FXIII-B		Amine incorporation	
	U/ml	(%)	U/ml	(%)	U/ml	(%)	(pmol/min/ml)	(%)
Control	1.03 ± 0.07	(100)	0.85 ± 0.08	(100)	1.32 ± 0.15	(100)	4.95 ± 0.10	(100)
Patient	0.43 ± 0.01	(42)	0.87 ± 0.01	(102)	0.51 ± 0.03	(39)	1.96 ± 0.05	(40)

b

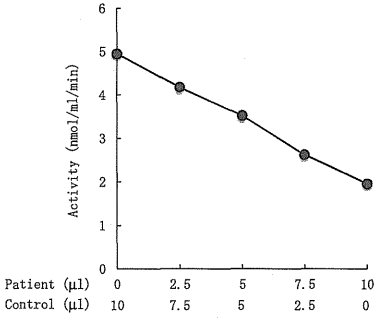

Fig. 1 a. The antigen levels of FXIII-A, FXIII-B, A-bound FXIII-B were analysed by enzyme-linked immunosorbent assay. The enzymatic activity of FXIII was measured by amine incorporation test. b. 5-Step dilution cross-mixing test of amine-incorporation activity was performed using our patient's plasma at the ratios of 0:1, 1:3, 1:1, 3:1, and 1:0 with normal plasma.

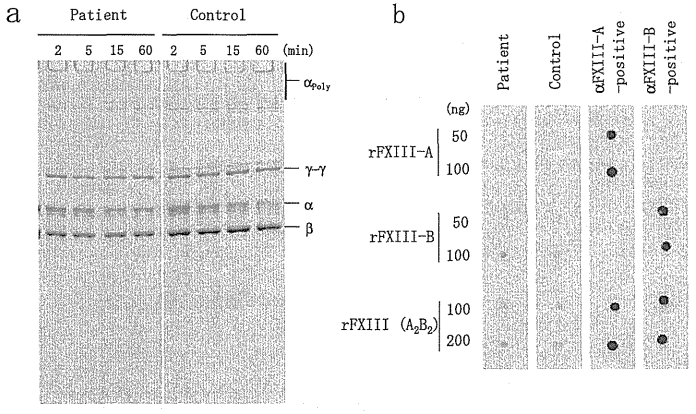

Fig. 2 a. Fibrin cross-linking test was performed using the patient's and normal plasma. The clots were recovered at the indicated time intervals and subjected to SDS-PAGE. b. Dot blot analysis of FXIII-reactive immunoglobulins was performed using recombinant FXIII-A, recombinant FXIII-B, and their complexes at the indicated amounts shown as antigen (ng).

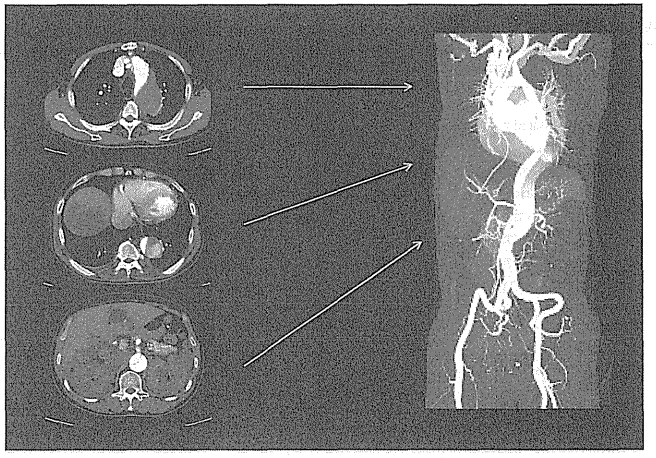

Fig. 3 Contrast-enhanced cardiac vascular computed tomography showed dissecting aortic aneurysm of the aortic arch and the descending aorta.

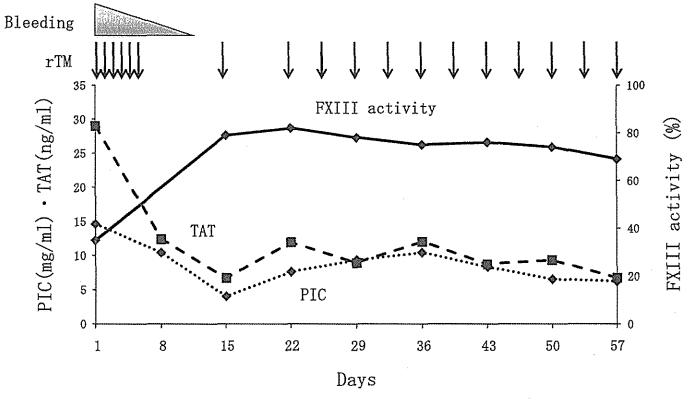

Fig. 4 Clinical outcome of recombinant human soluble thrombomodulin (rTM) for the patient with chronic DIC complicating dissecting aortic aneurysm after referring to our hospital.

は，二次線溶から予測される以上の線溶活性が存在するDICである。臨床的に出血症状は重篤だが，線溶抑制型と比較して臓器障害は目立たない。急性前骨髄球性白血病や大動脈瘤などを基礎疾患とすることが多い。本例は，初診時のTAT・PICが共に高値であり，大動脈瘤に伴う線溶亢進型DICに分類される。さらに，分類に明確な定義はなく基礎疾患は大きく異なるものの，臨床経過より「急性」と「慢性」に分類されることもある[2]。「慢性」は，固形癌や大動脈瘤などが代表疾患として挙げられ，本例は，血液検査・画像検査の結果より，解離

性大動脈瘤の増悪に伴う慢性 DIC と診断した。

大動脈瘤に合併した慢性 DIC は，1967 年に Fine らが 1 例目を報告して以後，数々報告されているが，著明な出血傾向や血小板減少をきたす症例は極僅かである[1,3]。その慢性 DIC を合併した大動脈瘤の外科的な治療方針については，早期の手術を推奨する意見と DIC のコントロールを的確に行ったうえでの手術を推奨する意見と二つに分かれる[4,5]。さらに，内科的治療に関しても，古くからヘパリン類・蛋白合成酵素阻害剤・トラネキサム酸を用いた報告例が幾つかあるが，定まった治療方針は確立されていない[6~9]。本例は，透析患者であり，かつ手術までの準備期間を要するため，瘙爬の副作用報告のあるトラネキサム酸や持続点滴の必要となるヘパリン類・蛋白合成酵素阻害剤は，投与困難と考えた。そこで，井山らの報告に基づき，持続点滴が必要でなく維持透析例でも投与可能な rTM を選択した[10]。

DIC は，基礎疾患によって発生機序は異なるが，トロンビンの過剰産生という点においては共通しており，トロンビン生成を阻害する rTM は，DIC 全般で保険適応を得ている[10]。本例は，rTM の 6 日間連日投与により，DIC の著明な改善を認めた。一方で，維持療法としての rTM の投与法については，症例数が極めて少なく詳細不明である。井山らの報告より，1 週間に 1 回の間隔で投与をしてみたところ，慢性 DIC の再増悪を認めた[10]。そのため，1 週間 2 回の間隔で投与回数を増やしたところ，慢性 DIC の改善を認めた。本例は，1 週間に 1 回の投薬では，rTM の効果が不足していたと考えられる。慢性 DIC を合併した透析例での rTM の投与法について，今後の検討課題と考える。

第 XIII 因子は，A サブユニットと B サブユニットの 4 量体から成り，血液凝固過程の最終段階におけるフィブリンの安定化，止血後の創傷治癒，後出血に大きく関係している[11,12]。本例では，初診時に第 XIII 因子活性 33％と低下（正常 70～130％）を認めていたことから，第 XIII 因子欠乏症と診断した。第 XIII 因子欠乏症は，先天性と後天性に大きく分類される。さらに，後天性は，抗第 XIII 因子抗体による自己免疫性血友病 XIII，または基礎疾患により第 XIII 因子が低下する二次性出血性後天性第 XIII 因子欠乏症に分類される[11,12]。Song らの解析では，DIC 全般で第 XIII 因子活性の低下を認め，DIC スコアと第 XIII 因子活性の逆相関が示されている[13]。さらに，第 XIII 因子活性が 30％以下に低下した上行弓部大動脈瘤に伴う DIC の症例報告もある[14]。以上から，DIC（基礎疾患に大動脈瘤を含む）は，二次性出血性後天性第 XIII 因子欠乏症の代表疾患の一つであると考えられる。抗第 XIII 因子抗体陰性であった本例は，二次性出血性後天性第 XIII 因子欠乏症と診断した。そして，rTM の定期投与を行った本例は，第 XIII 因子活性を 70％以上に維持することができ，加療開始前の出血スコア 4 点から維持療法中は 1 点まで改善できた[15]。

今回，解離性大動脈瘤増悪に伴う慢性 DIC ならびに二次性出血性後天性第 XIII 因子欠乏症の透析患者の一例を経験した。持続投与が必要なく維持透析中であっても投与可能である rTM を選択し，術前までに良好な DIC のコントロールを得ることができた。

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Successful treatment of chronic disseminated intravascular coagulation using recombinant human soluble thrombomodulin in a dialysis patient with dissecting aortic aneurysm

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Key words : Chronic disseminated intravascular coagulation, Recombinant human soluble thrombomodulin, Dissecting aortic aneurysm, Factor XIII

A 62-year-old man had a history of acute aortic dissection (Stanford type A) and had been diagnosed with polycystic kidney disease three years earlier, and then developed end-stage renal failure. He was referred with chief complaints of difficult hemostasis and consecutive hemorrhagic episodes at the puncture site of the shunt soon after dialysis introduction. We suspected chronic disseminated intravascular coagulation (DIC) due to mild thrombocytopenia and a fibrinolytic system abnormality. Plasma factor XIII activity was decreased, but no inhibitor was detected. In addition, contrast-enhanced computed tomography showed exacerbation of a dissecting aortic aneurysm. We finally diagnosed chronic DIC and secondary factor XIII deficiency associated with the aortic aneurysm. We selected treatment involving recombinant human soluble thrombomodulin (rTM) because he was on maintenance dialysis and required long-term follow-up bofore the operation. Hemostatic function improved with regular administration of rTM, and was well-controlled preoperatively.

Inhibitors of Factor XIII/13 in Older Patients

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Abstract

Factor XIII/13 (FXIII or F13) is a plasma protransglutaminase, which stabilizes fibrin clots, and thus plays an important role in hemostasis. Autoimmune hemo(rrha)philia due to anti-F13 autoantibodies (AH13) has been on the rise in Japan, which has become the leading superaging society in the 21st century. The mean age of Japanese AH13 cases has risen to 70.4 years. A total of 83 AH13 cases have been diagnosed in the world as of July 2014. To raise the awareness of AH13, the author and members of the Japanese Collaborative Research Group first proposed "Criterion and Algorithm of Laboratory Tests for anti F13" in February 2012. AH13 is not just an acquired isolated defect of F13 molecule itself but a disturbance caused by autoantibodies. Accordingly, AH13 cases are diagnosed in patients with otherwise unexplained hemorrhages by a combination of a severe deficiency of F13 activity and the presence of anti-F13 autoantibodies. As patients with this disease manifest life-threatening bleeding symptoms, prompt diagnosis and proper treatment are essential. Because AH13 tends to become chronic and intractable, affected patients must be closely followed for a prolonged period.

Keywords

- anti-factor XIII/13 autoantibody
- autoimmune hemorrhagic disease
- chronic intractable disease
- hemostatic therapy
- immunosuppressive treatment

Factor XIII/13 (the terms FXIII and F13 are used to avoid confusion with FVIII and FXII) is a fibrin-stabilizing factor in the plasma consisting of A and B subunits (F13-A and F13-B, respectively). F13-A has a catalytic active site, the Cys-314 residue, of a protransglutaminase, whereas F13-B is a carrier of F13-A, and protects it. At the end of the coagulation cascade, thrombin-activated F13 (F13a) cross-links the fibrin monomers, as well as fibrin and α_2-plasmin inhibitor (α_2-PI), the latter being the primary inhibitor of plasmin in plasma, fibronectin, etc.[1–3]

It was reported that both F13-A and F13-B levels were correlated significantly with age in both men and women, and that the F13-A subunit was observed to be significantly increased with increasing age in a multiple regression model.[4] Accordingly, elevated F13-A levels may play an important role(s) in the pathogenesis of thrombotic vascular diseases, such as cerebral infarction, acute myocardial infarction, in the older adult. It is also interesting to note that a common polymorphism in the *F13-A* gene (F13-A Val34Leu) has long been considered as a protective genetic factor against arterial and venous thrombosis, and that there is a trend toward a higher mean age of the clinical onset in homozygotes for the Leu allele than in the Val wild types among patients with ischemic diseases.[5]

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Autoimmune Hemo(rrha)philia due to Anti-F13 Antibodies among Hemorrhagic Acquired F13 Deficiency

Because F13 cross-links fibrin monomers, as well as fibrin and α_2-PI, congenital F13 deficiency results in a lifelong tendency for severe bleeding.[1–3] Although congenital F13 deficiency is rare, with a prevalence of about one case per one million population, acquired F13 deficiency is fairly common,[6] and it is secondary to underlying diseases/disorders resulting in hyposynthesis and/or hyperclearance of F13-A and/or F13-B. However, patients with acquired F13 deficiency rarely bleed because their F13 levels remain only moderately reduced.[7,8] In contrast, patients with autoimmune hemophilia-like disease (i.e., hemorrhophilia) due to anti-F13 antibodies (AH13) experience severe bleeding due to drastically decreased F13 levels.

There are two types of anti-F13 autoantibodies; neutralizing/inhibitory and nonneutralizing/noninhibitory types, respectively. The former type of antibodies inhibits either the activation of F13 to F13a or the catalytic activity of F13a, while the latter enhances the clearance of F13, F13-A, and/or F13-B from the circulation.

Increasing AH13 Cases in Japan

Although AH13 used to be rare in Japan, its incidence has been on the rise in the 21st century.[3] In view of that, a nationwide survey supported by the Japanese Ministry of Health, Labor and Welfare was conducted and identified 12 cases with

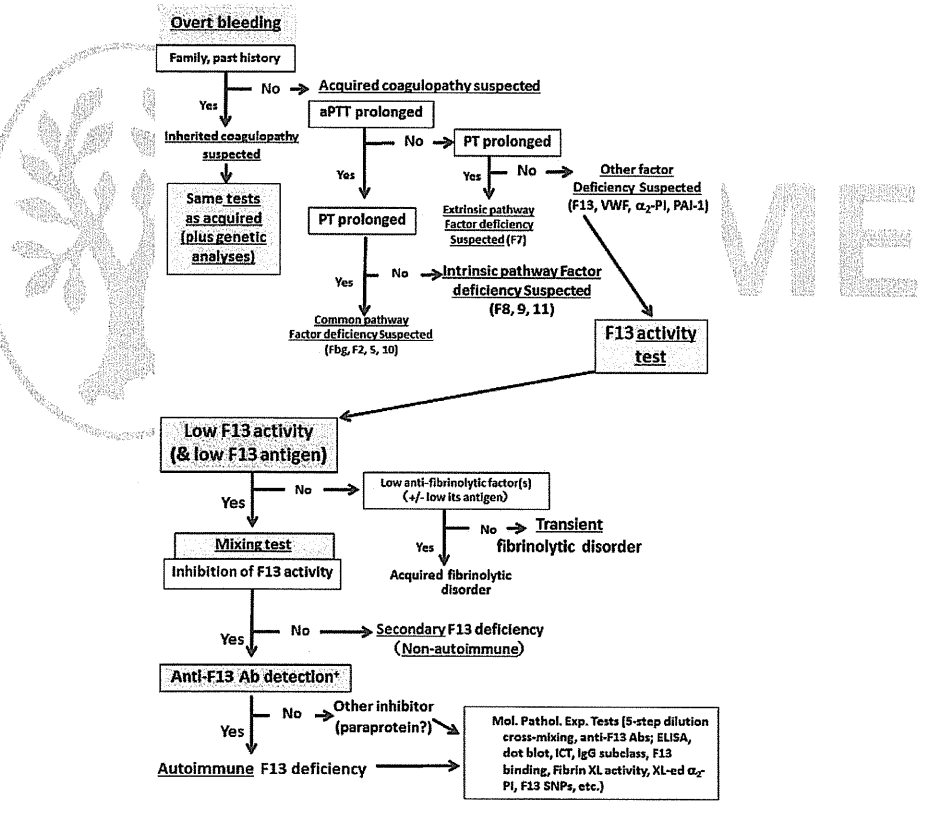

Fig. 1 Algorithm for laboratory tests to diagnose AH13. (Top) Physicians in charge of AH13 suspected cases perform screening tests before consultation. (Bottom) Coagulation-specialists carry out detailed experimental examinations. aPTT, activated partial thromboplastin time; ICT, immunochromatography; PI, plasmin inhibitor; PT, prothrombin time; PAI-1, plasminogen activator inhibitor-1; SNP, single nucleotide polymorphism; VWF, von Willebrand factor; XL, cross-linked.