Ⅴ．研究成果の刊行に関する一覧表

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Regular Article

PLATELETS AND THROMBOPOIESIS

Clot retraction is mediated by factor XIII-dependent fibrin-αIIbβ3-myosin axis in platelet sphingomyelin-rich membrane rafts

Kohji Kasahara,[1] Mizuho Kaneda,[1] Toshiaki Miki,[1] Kazuko Iida,[1] Naoko Sekino-Suzuki,[1] Ikuo Kawashima,[2] Hidenori Suzuki,[1,3] Motoyuki Shimonaka,[4] Morio Arai,[5] Yoshiko Ohno-Iwashita,[6] Soichi Kojima,[7] Mitsuhiro Abe,[8] Toshihide Kobayashi,[8] Toshiro Okazaki,[9] Masayoshi Souri,[10] Akitada Ichinose,[10] and Naomasa Yamamoto[11]

[1]Laboratory of Biomembrane and [2]Molecular Medicine Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; [3]Division of Morphological and Biomolecular Research, Nippon Medical School, Tokyo, Japan; [4]Department of Chemistry, Tokyo University of Science, Tokyo, Japan; [5]CMR Development Division, Novo Nordisk Pharma Ltd., Tokyo, Japan; [6]Faculty of Pharmacy, Iwaki Meisei University, Fukushima, Japan; [7]Molecular Ligand Biology Research Team and [8]Lipid Biology Laboratory, RIKEN Advanced Science Institute, Saitama, Japan; [9]Department of Hematology and Immunology, Kanazawa Medical University, Ishikawa, Japan; [10]Department of Molecular Patho-Biochemistry and Patho-Biology, Yamagata University School of Medicine, Yamagata, Japan; and [11]Department of Biochemistry, Faculty of Pharmaceutical Sciences, Ohu University, Fukushima, Japan

Key Points

- Clot retraction of sphingomyelin-rich raft-depleted platelets from sphingomyelin synthase knockout mouse is delayed.
- Translocation of fibrin to sphingomyelin-rich rafts in platelet membrane is induced by thrombin in the presence of FXIII crosslinking activity.

Membrane rafts are spatially and functionally heterogenous in the cell membrane. We observed that lysenin-positive sphingomyelin (SM)–rich rafts are identified histochemically in the central region of adhered platelets where fibrin and myosin are colocalized on activation by thrombin. The clot retraction of SM-depleted platelets from SM synthase knockout mouse was delayed significantly, suggesting that platelet SM-rich rafts are involved in clot retraction. We found that fibrin converted by thrombin translocated immediately in platelet detergent-resistant membrane (DRM) rafts but that from Glanzmann's thrombasthenic platelets failed. The fibrinogen γ-chain C-terminal (residues 144-411) fusion protein translocated to platelet DRM rafts on thrombin activation, but its mutant that was replaced by A398A399 at factor XIII crosslinking sites (Q398Q399) was inhibited. Furthermore, fibrin translocation to DRM rafts was impaired in factor XIII A subunit-deficient mouse platelets, which show impaired clot retraction. In the cytoplasm, myosin translocated concomitantly with fibrin translocation into the DRM raft of thrombin-stimulated platelets. Furthermore, the disruption of SM-rich rafts by methyl-β-cyclodextrin impaired myosin activation and clot retraction. Thus, we propose that clot retraction takes place in SM-rich rafts where a fibrin-αIIbβ3-myosin complex is formed as a primary axis to promote platelet contraction. (*Blood.* 2013;122(19):3340-3348)

Introduction

Membrane rafts are dynamic assemblies of sphingolipids, cholesterol, and proteins that can be stabilized into platforms involved in the regulation of a number of vital cellular processes.[1] The important role of rafts at the cell surface may be their function in signal transduction. A number of studies provide considerable evidence that rafts are integral to the regulation of immune and neuronal signaling. Membrane rafts are also involved in hemostasis and thrombosis. Among blood cells, platelets are critical for maintaining the integrity of the blood coagulation system. Platelet rafts are critical membrane domains in physiological responses such as adhesion and aggregation.[2] The localization of the adhesion receptor glycoprotein (GP)Ib-IX-V complex to membrane rafts is required for platelet adhesion to the vessel wall by binding the von Willebrand factor.[3] Membrane rafts are also required for platelet aggregation via the collagen receptor GPVI,[4] the adenosine 5′-diphosphate (ADP) receptor P2Y12,[5] the Fcγ receptor FcγRIIa,[6] and the C-type lectin-like receptor CLEC-2.[7] Detergent-resistant membrane (DRM) rafts of platelets show round vesicles of heterogeneous sizes ranging from 20 to 500 nm, which are enriched in CD36 (GPIV).[8,9] Recent reports have demonstrated that membrane rafts are spatially and compositionally heterogeneous in the cell membrane.[10,11] However, little is known about raft heterogeneity in platelet membranes.

We have identified glycosphingolipid-binding proteins and investigated the signaling in membrane rafts.[12-16] Previously, we reported on translocation of the heterotrimeric G protein Gαo to the DRM raft in the developing cerebellum.[17] In this study, we demonstrated that sphingomyelin (SM)-rich rafts are localized in the central region of adhered platelet membranes where fibrin translocates on thrombin stimulation in combination with a coagulation factor XIII (FXIII), and this FXIII crosslinking occurs on SM-rich rafts of platelets and is involved in clot retraction.

Methods

The study was approved by the institutional ethics committee. The patient gave informed consent in accordance with the Declaration of Helsinki.

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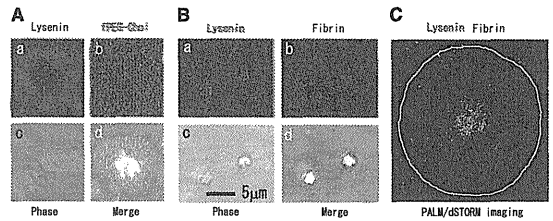

Figure 1. Immunocytochemical colocalization of fibrin(ogen) with sphingomyelin-rich rafts in central region of adhered platelets in the presence of thrombin. (A) (a) mCherry-lysenin-positive sphingomyelin (SM)-rich rafts (red), (b) fPEG-Chol-positive cholesterol-rich rafts (green), (c) phase-contrast cell morphology, (d) merged images of (a-c). (B) colocalization of fibrin (red) with green fluorescent protein–lysenin-positive SM-rich rafts (green). Scale bar, 5 μm. (C) Photoactivated localization microscopy and direct stochastic optical reconstruction microscopy (PALM/dSTORM) imaging of lysenin-positive SM-rich rafts (green) and fibrin(ogen) (red). The white line represents the shape of an adhered human platelet.

The experimental protocols were approved by the Animal Use and Care Committee.

Platelet preparation

Blood was collected into 3.8% sodium citrate at a ratio of 9:1. The blood was centrifuged (140× *g*) to prepare platelet-rich plasma (PRP). To prepare washed platelets, we incubated PRP with 4 mM citric acid and washed with Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 3.75 mM NaH_2PO_4, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 0.35% bovine serum albumin, and 5 mM glucose; pH 6.8) containing 1 mM PGE1 and 1 U/mL heparin. Finally, the platelets were resuspended in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 3.75 mM NaH_2PO_4, 5 mM HEPES, 0.35% bovine serum albumin, and 5 mM glucose; pH 7.4) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

Cell staining

To stain SM-rich rafts, we incubated platelets with 10 μg/mL nontoxic mCherry-lysenin or nontoxic enhanced green fluorescent protein (EGFP)–lysenin in Tyrode's buffer (pH 7.4) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, and platelets were adhered onto a glass-bottomed culture dish coated with 100 μg/mL human fibronectin by treatment with 1 U/mL thrombin for 15 minutes and were fixed in 4% paraformaldehyde. To stain cholesterol-rich rafts, we incubated washed platelets with 2 μM fluorescent esters of (polyethylene glycol) cholesteryl ether (fPEG-Chol). To stain fibrin(ogen) or integrin β3 on the platelet surface, we adhered platelets onto a glass-bottomed culture dish coated with 100 μg/mL fibronectin by 1 U/mL thrombin, fixed in 4% paraformaldehyde, and incubated with the antifibrinogen rabbit polyclonal antibody or antiintegrin β3 monoclonal antibody TM83[18] for 1 hour, and then with the Alexa Fluor 594 or 488-labeled secondary antibody. To stain myosin or activated myosin, we fixed adhered platelets in 4% paraformaldehyde with 0.05% Triton X-100, 1 mM Na_3VO_4, and 50 mM NaF, and incubated with the antimyosin rabbit polyclonal antibody or antiphosphomyosin light chain 2 (Ser19) monoclonal antibody for 1 hour, and then with the Alexa Fluor 488–labeled secondary antibody. The images were captured using a Carl Zeiss (Oberkochen, Germany) confocal imaging system (LSM510 META).

Sucrose density gradient analysis

We homogenized 600 000 000 platelets using a Teflon glass homogenizer in 2 mL of Tris/Triton buffer (0.05% Triton X-100, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM EGTA). The sucrose content was then adjusted to 40% by adding 80% sucrose. A sucrose gradient (5% to 30%) in 6 mL of Tris buffer without Triton X-100 was layered over the lysate and was centrifuged for 17 hours at 39 000 × *g* at 4°C in a Hitachi (Tokyo, Japan) RPS40T rotor. The 10 fractions were collected from the top of the gradient. DRM raft fraction (fraction no. 5) was analyzed by 2-dimentional (2-D) polyacrylamide gel electrophoresis (PAGE) using a Multiphor II Electrophoresis Unit with Immobiline DryStrip pH 3-10 (GE Healthcare, Fairfield, CT).

Fibrin clot retraction

PRP was activated with 1 U/mL thrombin and 5 mM $CaCl_2$, and the reaction mixtures were left unstirred at 37°C in siliconized tubes. The extent of clot retraction was monitored by taking photographic images, and measurement of clot areas was performed using ImageJ (National Institutes of Health, Washington, DC). Clot retraction was expressed as a percentage by ratio between a clot area and that of the entire reaction mixture (area ratio). Results are presented as mean ± standard deviation (SD) of 3 independent experiments, and analyzed using an unpaired Student *t* test. The area ratio method was comparable to the weight ratio method.[19] Plasma-free clot retraction assay was performed with 300 000/μL washed platelets, 2 mg/mL fibrinogen, 0.5 U/mL thrombin, 1 mM $CaCl_2$, and 5 mM glucose in HEPES-Tyrode's buffer (pH 7.4). They were solubilized by adding an equal volume of a 2× solubilization buffer (252 mM Tris pH 6.8, 40% glycerol, 4% sodium dodecyl sulfate (SDS), 8 M urea, 32.5 mM dithiothreitol, 2 mM Na_3VO_4, and 0.25% Bromophenol Blue) at 95°C for 60 minutes. Protein phosphorylation was analyzed by immunoblotting with a phosphospecific antibody to myosin light chain (Ser19), phosphotyrosine (PY20).

Results

SM-rich rafts colocalize with fibrin in the central region of adhered platelets induced by thrombin

The perfringolysin O derivative BCθ binds selectively to a subpopulation of platelet DRM rafts (cholesterol-rich rafts), suggesting that a heterogeneous population of membrane rafts exists in platelets.[20] SM is a major component of raft lipids in platelets.[2,20] Lysenin is a specific probe of SM-rich rafts.[21] A previous study showed that BCθ-positive cholesterol-rich rafts are uniformly distributed on the cell surface or at the leading edge of spreading platelets.[22] Therefore, we investigated a subcellular distribution of SM-rich rafts in spreading platelets. Lysenin-positive SM-rich rafts were mainly localized in the central region of adhered platelets by thrombin (Figure 1Aa, red). In contrast, polyethylene glycol-derivatized cholesterol (PEG-Chol), a probe for cholesterol-rich rafts,[21] was localized evenly on the membrane (Figure 1Ab, green). These observations suggest that SM-rich rafts are a subset of cholesterol-rich rafts at the plasma membrane of adhered platelets by thrombin (Figure 1Ad). To clarify the specific function of SM-rich rafts, we first focused on the distribution of fibrinogen and SM clusters. Previous immunocytochemical studies showed that fibrin (ogen) is present at the center of spreading platelets treated by thrombin.[23] Lysenin-positive SM-rich rafts (Figure 1Ba, green) and fibrin(ogen) (Figure 1Bb, red) were mostly colocalized as a patch in double-staining the central region of adhered platelets by thrombin (Figure 1Bd). Superresolution photoactivated localization microscopy and direct stochastic optical reconstruction microscopy (PALM/dSTORM) also indicated the colocalization between fibrin(ogen) and SM-rich rafts (Figure 1C), suggesting that SM-rich rafts act as platforms of fibrin(ogen)-mediated outside-in signal, leading to clot retraction.

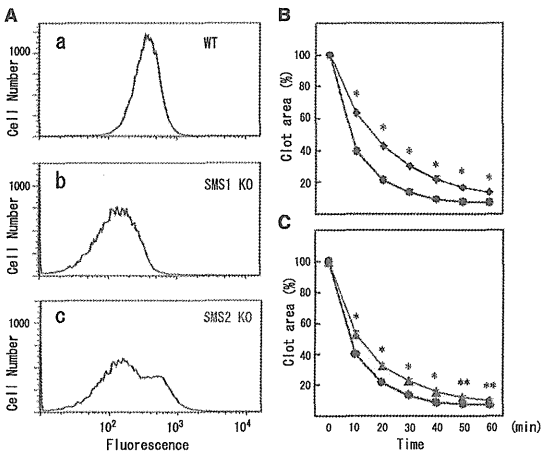

Figure 2. Reduced SM-rich rafts and delayed clot retraction in SM synthase-deficient platelets. (A) Flow cytometry of SM-rich rafts of (a) wild-type, (b) SMS1-deficient, and (c) SMS2-deficient mouse platelets. (B) Time-dependent clot retraction of SMS1-deficient PRP (red diamonds) and wild-type PRP (black circles). The extent of clot retraction was assessed at the indicated times by measuring clot area. (C) Time-dependent clot retraction of SMS2-deficient PRP (blue triangles) and wild-type PRP (black circles). Data are presented as the means plus or minus SD of quadruplicates. *Statistically significant difference ($P < .001$). **Statistically significant difference ($P < .005$).

SM-depleted platelets exhibit delayed fibrin clot retraction

We investigated the possible involvement of SM-rich rafts in clot retraction. In mammals, 2 sphingomyelin synthase (SMS) isoforms (SMS1 and SMS2) have been shown to account for SM synthesis. SMS1 is responsible for the bulk of SM production. SMS1 or SMS2 deficiency exhibits a dysfunction of membrane rafts.[24-26] Flow cytometry demonstrated that the remaining quantity of lysenin-positive SM-rich rafts in SMS1- and SMS2-deficient platelets was 43.5% (Figure 2Ab) and 73.8% (Figure 2Ac) of normal platelets, respectively. SMS1- or SMS2-deficient platelets aggregated in response to 1 U/mL thrombin (data not shown). However, clot retraction was significantly delayed in SMS1-deficient PRP (Figure 2B, red) and SMS2-deficient PRP (Figure 2C, blue). In proportion to quantity of SM-rich rafts, the average time of 50% retraction was prolonged by 2- and 1.3-fold in SMS1-deficient and SMS2-deficient PRPs, respectively. These results suggest that platelet SM-rich rafts are required for normal clot retraction.

Fibrin translocation to platelet DRM raft on thrombin-stimulation

To confirm the association of fibrin(ogen) with platelet membrane rafts, we attempted to identify DRM-raft-specific proteins from activated platelets. We isolated the DRM raft fraction of thrombin-stimulated platelets by sucrose density gradient centrifugation. Several specific proteins (Figure 3Aa, right panel) were detected in the DRM fraction (lane 5) of thrombin-stimulated platelets. Three specific proteins of 65, 50, and 47 kDa were isolated by 2D-PAGE from the DRM fraction (Figure 3Bb, arrows). By mass spectrometry, they were identified as fibrins α, β, and γ, respectively, because no sequences of fibrinopeptide A (Ala1-Arg16) or fibrinopeptide B (PyroGlu1-Arg14) were detected (supplemental Figure 1, found on the *Blood* Web site). These results were supported by immunoblotting with an antifibrinogen antibody (Figure 3C-D) and an antifibrin antibody (Figure 3E). In resting platelets, fibrinogens Aα (67 kDa), Bβ (52 kDa), and γ (47 kDa) were detected in the nonraft fraction (Figure 3C, lanes 7-10). In contrast, fibrins α(65 kDa), β(50 kDa), and γ(47 kDa) were detected in the DRM fraction of thrombin-stimulated platelets (Figure 3C). The fibrinogen translocation to the DRM fraction was not detected in collagen- or ionophore-stimulated platelets (data not shown). Thrombin caused the dose-dependent translocation of not only 340 kDa fibrin, [αβγ]2 (nonreduced condition), but also the fibrin polymer (Figure 3Dc, asterisk) to the DRM fraction (supplemental Figure 2). Thrombin caused the rapid translocation of fibrin to the DRM fraction within 30 seconds (Figure 3D), and the time-dependent conversion of the fibrin monomer to the fibrin polymer (Figure 3Dc, asterisk). Fibrin was detected in only the DRM fraction of thrombin-stimulated platelets (Figure 3E, lane 3). Moreover, immunoelectron microscopy revealed that the fibrin fiber associated with the DRM (~300 nm) of thrombin-stimulated platelets (Figure 3F). The immunogold-positive fibrin fiber was directly associated with the surface of DRM (Figure 3G). These data suggest that fibrinogen [AαBβγ]2 is released from α-granules of platelets, converted to fibrin [αβγ]2 by the cleavage of fibrinopeptides A and B with thrombin, and translocated to membrane rafts of thrombin-stimulated platelets.

Integrin αIIbβ3 is required for fibrin translocation to DRM rafts of thrombin-stimulated platelets

To investigate the role of integrin αIIbβ3 in fibrin translocation to the DRM fraction, we used platelets from a type I Glanzmann's thrombasthenia patient, a disease characterized by the absence of αIIbβ3, a fibrin receptor.[27] In αIIbβ3-deficient platelets, a small amount of fibrinogen is detectable by western blotting, and almost no fibrin is translocated to the DRM fraction even with thrombin stimulation (supplemental Figure 3Aa, upper panel). This result suggests that αIIbβ3 is required for the fibrin translocation to the DRM fraction. The HHLGGAKQAGDV sequence at the carboxyl

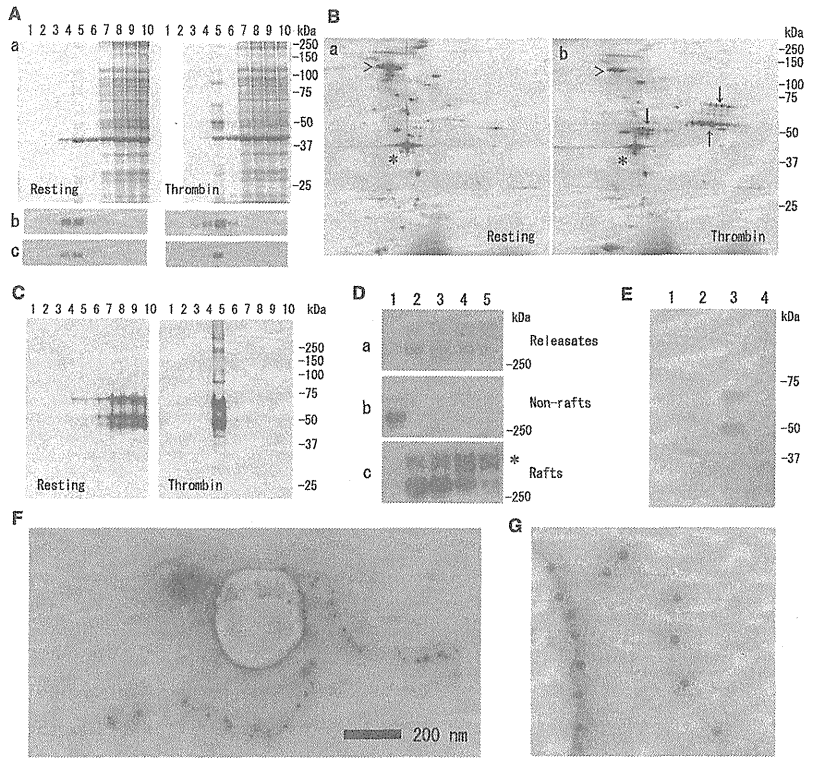

Figure 3. Fibrin translocation to DRM raft fraction of human platelets by thrombin stimulation. (A) Sucrose density gradient analysis of proteins in washed human platelets. Resting platelets (left panel) and platelets stimulated for 3 minutes with 1 U/mL thrombin (right panel) were lysed in Triton X-100, and sucrose gradients (5% to 30%) were formed over them. Ten fractions were collected from top to bottom after centrifugation. The proteins were subjected to SDS-PAGE and stained with Coomassie brilliant blue (a). Immunoblotting of raft marker proteins with anti-CD36 antibody (b) and anti-Lyn antibody (c). A 40-kDa major protein was identified as actin by immunoblotting. (B) Two-dimensional PAGE analysis of DRM raft fraction (fraction 5) in resting platelets (a) and thrombin-stimulated platelets (b). The asterisk indicates actin. The arrowhead indicates integrin αIIb. (C) Immunoblotting with antifibrinogen antibody of panel A. (D) Time-dependent translocation of fibrin(ogen) by thrombin for 0 seconds (lane 1), 30 seconds (lane 2), 1 minute (lane 3), 5 minutes (lane 4), and 15 minutes (lane 5). Immunoblotting under nonreduced condition by antifibrinogen antibody of releasates (a), nonraft fraction (b), and DRM raft fraction (c). The asterisk indicates the fibrin polymer. (E) Immunoblotting with antifibrin specific antibody of DRM raft fraction (lanes 1 and 3), nonraft fraction (lanes 2 and 4) of resting platelets (lanes 1 and 2), and thrombin-stimulated platelets at 3 minutes (lanes 3 and 4). (F) Association of fibrin fiber with DRM of thrombin-stimulated platelets in immunoelectron microscopy. The DRM of thrombin-stimulated human platelets was incubated with an antifibrinogen antibody and then anti-IgG-labeled with colloidal gold. Scale bar, 200 nm. (G) Magnification of gold-attached area on DRM in panel F. The gold-positive fibrin fiber directly associated with the surface of DRM.

termini of the γ-chains of human fibrin provides recognition sites for the binding of fibrin protofibril to αIIbβ3 on activated human platelets. The dodecapeptide, γ 400-411, inhibited the fibrin translocation to the DRM fraction (supplemental Figure 3B). To confirm the possible involvement of γ-chains in fibrin translocation to the DRM fraction, we used the fibrinogen γ-chain C-terminal (residues 144-411) fusion protein with a human growth hormone (supplemental Figure 3C). The fibrinogen γ-chain C-terminal (144-411) fusion protein, but not the C-terminal deletion mutant (144-399) in the fibrinogen γ-chain, bound to the DRM of thrombin-stimulated platelets (supplemental Figure 3Cd, lane R). These results suggest that the carboxyl termini of human fibrin γ-chains are involved in fibrin translocation to the DRM fraction. We observed that a small amount of αIIbβ3 is present in DRM rafts, and a large amount of αIIbβ3 is present in the nonraft fraction (supplemental Figure 4). These data suggest that αIIbβ3 is necessary for the initial binding to fibrinogen on a thrombin-activated platelet surface, but not sufficient for the fibrin translocation to DRM rafts.

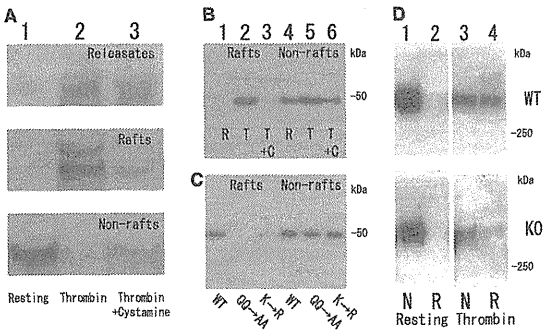

Figure 4. Requirement of FXIII transglutaminase for fibrin translocation to DRM raft fraction of thrombin-stimulated platelets. (A) Inhibition of fibrin translocation to DRM raft fraction by transglutaminase inhibitor cystamine. Immunoblotting under nonreduced condition by antifibrinogen antibody of releasates (upper panel), DRM raft fraction (middle panel), and nonraft fraction (lower panel) in resting (lane 1) and thrombin-stimulated human platelets in the absence (lane 2) or presence (lane 3) of cystamine. Total amount of fibrin in releasate, raft, and nonraft fraction of thrombin-stimulated human platelets in the presence of cystamine (lane 3) is comparable to that of fibrinogen in nonraft fraction of resting platelets (lane 1). (B) Transglutaminase-dependent association of fibrinogen γ-chain fusion protein with DRM raft fraction in thrombin-stimulated platelets. Immunoblotting of DRM raft fraction (lanes 1, 2, and 3) and nonraft fraction (lanes 4, 5, and 6) in resting (lanes 1 and 4) and thrombin-stimulated platelets in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of cystamine. (C) FXIII-dependent association of fibrinogen γ-chain fusion protein with DRM raft fraction in thrombin-stimulated platelets. Immunoblotting by anti-His tag antibody of DRM raft fraction (lanes 1, 2, and 3) and nonraft fraction (lanes 4, 5, and 6) using fibrinogen γ-chain fusion protein (lanes 1 and 4), fibrinogen γ-chain fusion protein mutants of FXIII-crosslinking site Q398Q399 (lanes 2 and 5), or K406 (lanes 3 and 6). (D) Impaired fibrin translocation to DRM raft fraction of thrombin-stimulated platelets in FXIIIA subunit-deficient mice. Immunoblotting under nonreduced condition by antifibrinogen antibody of DRM raft fraction (lanes 2 and 4) and nonraft fraction (lanes 1 and 3) in resting platelets (lanes 1 and 2) and thrombin-stimulated platelets (lanes 3 and 4) of wild-type mice (upper panel) and FXIIIA-deficient mice (lower panel).

Requirement of FXIII crosslinking activity in fibrin translocation to DRM raft of thrombin-stimulated platelets

We found that the thrombin receptor-activating peptide (TRAP) did not cause fibrinogen translocation to the DRM fraction (supplemental Figure 5), suggesting that the protease activity of thrombin is necessary for fibrin translocation to the DRM fraction. To investigate the possible involvement of FXIII in that fibrin translocation to DRM fraction, we investigated whether the fibrin translocated to the DRM fraction is a substrate for FXIII. 5-(Biotinamido) pentylamine (5BAPA)–incorporated proteins induced by transglutaminase with molecular weights of 65 and 47 kDa are predominantly present in the DRM of platelets stimulated with thrombin for 15 minutes (supplemental Figure 6A, lane 4), but not TRAP or collagen. The 5BAPA-incorporated 65-kDa and 47-kDa proteins (supplemental Figure 6B, lane 3) comigrated with fibrin in the DRM fraction (supplemental Figure 6B, lane 7), suggesting that they are fibrin α- and γ-chains, respectively. Another major 5BAPA-incorporated 95-kDa protein was identified as a cytosolic protein (data not shown). Cell-surface 5BAPA incorporation was analyzed by flow cytometry (supplemental Figure 6C). 5BAPA was incorporated to the surface of thrombin-stimulated human platelets (supplemental Figure 6Cb). Pretreatment with cystamine, a transglutaminase inhibitor, just before thrombin stimulation completely inhibited the 5BAPA incorporation (supplemental Figure 6Cc). 5BAPA was not incorporated to the surface of TRAP-stimulated human platelets (supplemental Figure 6Cd). Fibrin γ 400-411, the HHLGGAKQAGDV dodecapeptide, inhibited the 5BAPA incorporation to fibrin α- and γ-chains in thrombin-activated platelets (supplemental Figure 6D, lane 3). Confocal imaging showed that cell-surface 5BAPA incorporation to fibrin was colocalized with lysenin-positive SM-rich rafts (supplemental Figure 6E). Furthermore, 5BAPA incorporation was impaired in FXIII A subunit-deficient mouse platelets (supplemental Figure 6F, lane 2) but not in tissue transglutaminase-deficient mouse platelets (supplemental Figure 6F, lane 3). A substantial amount of FXIII was identified in the raft fraction of thrombin-stimulated platelets (supplemental Figure 7) but not in resting platelets, suggesting that platelet-derived FXIII was expressed on the surface. These results suggest that fibrin is a specific substrate for FXIII in SM-rich rafts on thrombin-stimulated platelet surface. However, it is a question whether FXIII is involved in fibrin translocation to rafts.

Next we examined the effect of cystamine on fibrin translocation to DRM fraction of thrombin-stimulated platelets (Figure 4A). Pretreatment with cystamine just before thrombin stimulation inhibited fibrin translocation to DRM fraction and polymer formation (Figure 4A, lane 3), binding the fibrinogen γ-chain C-terminal fragment (144-411) fusion protein to DRM fraction (Figure 4B, lane 3). Furthermore, the fibrinogen γ-chain C-terminal fragment (144-411) fusion protein mutants of FXIII-crosslinking site Q398Q399 or K406 hardly bound to the DRM (Figure 4C, lanes 2 and 3) but still bound to the nonraft fraction of thrombin-stimulated platelets (Figure 4C, lanes 5 and 6). Finally, the thrombin-induced fibrin translocation to DRM fraction was impaired in FXIII A subunit-deficient platelets (Figure 4D, lane 4). These results suggest that transglutaminase activity of FXIII is required for the fibrin translocation to DRM fraction of thrombin-stimulated platelets. A precise role of FXIII in fibrin translocation to rafts remains to be explored.

Involvement of fibrin and myosin translocation to SM-rich rafts in clot retraction

Clot retraction is mediated by the interaction of the fibrin fiber and actomyosin via the integrin αIIbβ3, together with the activation of the platelet contractile apparatus. Consistent with this idea, clot retraction was impaired in type I Glanzmann's thrombasthenia (Figure 5A). Therefore, we investigated the distribution of myosin in thrombin-stimulated platelets on sucrose density gradient. Thrombin caused a transient (within 5 minutes) increase in myosin level in the

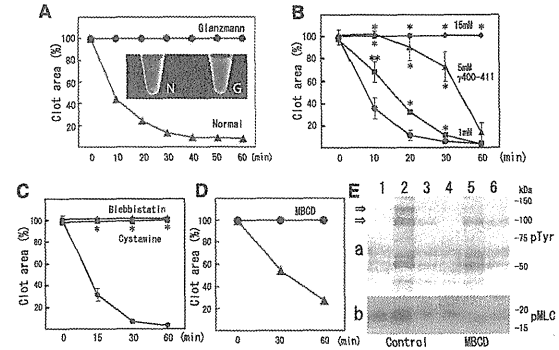

Figure 5. Implication of fibrin and myosin translocation to DRM raft fraction in clot retraction. (A) Impairment of clot retraction in type I Glanzmann's thrombasthenia. Time-dependent clot retraction of Glanzmann's thrombasthenia PRP (circles) and normal PRP (triangles). PRP was incubated with 1 U/mL thrombin and 2 mM $CaCl_2$. The extent of clot retraction was assessed at the indicated times by measuring clot area. Photo image of clot retraction assay after 120 minutes: N, normal PRP; G, Glanzmann's thrombasthenia PRP (inset). (B) Inhibition of clot retraction by fibrinogen γ-chain 400-411 dodecapeptide. PRP was incubated with 1 U/mL thrombin, 2 mM $CaCl_2$ with no peptide (circles), and 1 mM (squares), 5 mM (triangles), and 15 mM (diamonds) fibrinogen γ-chain 400-411 dodecapeptide. Data are presented as means plus or minus SD of quadruplicates. *Statistically significant difference ($P < .01$). **Statistically significant difference ($P < .05$). (C) Inhibition of clot retraction by cystamine or blebbistatin. PRP was incubated with 1 U/mL thrombin, 2 mM $CaCl_2$ with buffer (circles), 10 mM cystamine (squares), or 100 μM blebbistatin (triangles). Data are presented as means plus or minus SD of triplicates. *Statistically significant difference ($P < .001$). (D) Inhibition of clot retraction by raft disruption by MBCD. Mixture of washed platelets and purified fibrinogen was incubated with 1U/ml thrombin in the presence (circle) and absence (triangle) of 2% MBCD. The photograph was taken after 30 and 60 minutes. (E) Raft disruption by MBCD inhibits transient increase in degree of tyrosine phosphorylation (a) and phosphorylation of myosin light chain at serine residue 19 (b). A mixture of washed platelets and purified fibrinogen was incubated with 1 U/mL thrombin for 0 minutes (lanes 1 and 4), 5 minutes (lanes 2 and 5), and 60 minutes (lanes 3 and 6) in the presence (lanes 4-6) and absence (lanes 1-3) of 2% MBCD. Arrows indicate the tyrosine phosphorylation of 125-kDa and 100-kDa proteins.

DRM fraction (from 3.4% to 26.5%) and a transient decrease in the nonraft fraction (from 51.0% to 26.8%) (Figure 6A). The increase in myosin level in the DRM fraction was inhibited by blebbistatin, a myosin adenosine triphosphatase (ATPase) inhibitor (Figure 6B). Furthermore, TRAP also caused an increase in myosin in the DRM fraction (Figure 6C, lane 3). These results suggest that the activation of thrombin receptors induced the ATPase activity-dependent translocation of a significant amount of myosin to the DRM raft fraction.

Next, we confirmed the involvement of fibrin and myosin translocation to the DRM fraction in clot retraction. The γ 400-411 dodecapeptide inhibited not only fibrin translocation to the DRM fraction (supplemental Figure 3B) but also clot retraction in a dose-dependent manner (Figure 5B). Furthermore, cytamine or blebbistatin inhibited not only fibrin (Figure 4A) or myosin (Figure 6B) translocation to the DRM fraction but also clot retraction (Figure 5C). These results suggest that the translocation of fibrin and myosin to SM-rich rafts is involved in clot retraction. We investigated the effect of cholesterol depletion with methyl-β-cyclodextrin (MBCD) on the disruption of SM-rich rafts, the tyrosine phosphorylation of cellular proteins, and the phosphorylation at serine 19 of the myosin light chain (which increases actin-activated myosin ATPase activity) using a plasma-free clot retraction system with washed platelets in purified fibrinogen. Flow cytometry demonstrated that both lysenin-positive SM-rich and BC8-positive cholesterol-rich rafts were prerequisitely present on resting platelets, that the amounts of both rafts were

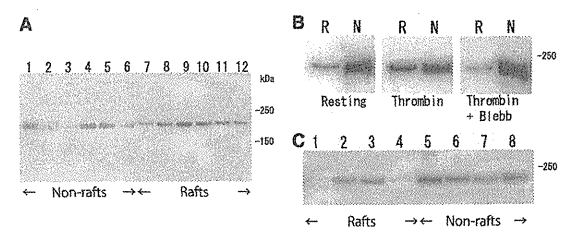

Figure 6. Transient translocation of myosin to DRM raft fraction. (A) Time-dependent translocation of myosin from nonraft fraction (lanes 1-6) to DRM raft fraction (lanes 7-12) in platelets by thrombin stimulation for 0 seconds (lanes 1 and 7), 30 seconds (lanes 2 and 8), 1 minute (lanes 3 and 9), 5 minutes (lanes 4 and 10), 15 minutes (lanes 5 and 11), and 60 minutes (lanes 6 and 12). Immunoblotting was performed with a myosin heavy-chain antibody. (B) ATPase-activity-dependent translocation of myosin to DRM raft fraction. Immunoblotting with antimyosin antibody of DRM raft fraction (lane R) and nonraft fraction (lane N) in resting platelets (left panel) and thrombin-stimulated platelets in the absence (middle panel) or presence (right panel) of 100 μM myosin ATPase inhibitor blebbistatin. (C) PAR-dependent translocation of myosin to DRM raft fraction. Immunoblotting with antimyosin antibody of DRM raft fraction (lanes 1-4) and nonraft fraction (lanes 5-8) in resting platelets (lanes 1 and 5) and platelets stimulated with 0.2 U/mL thrombin (lanes 2 and 6), 25 μM TRAP (lanes 3 and 7), and 20 μM adenosine 5′-diphosphate (lanes 4 and 8) for 3 minutes.

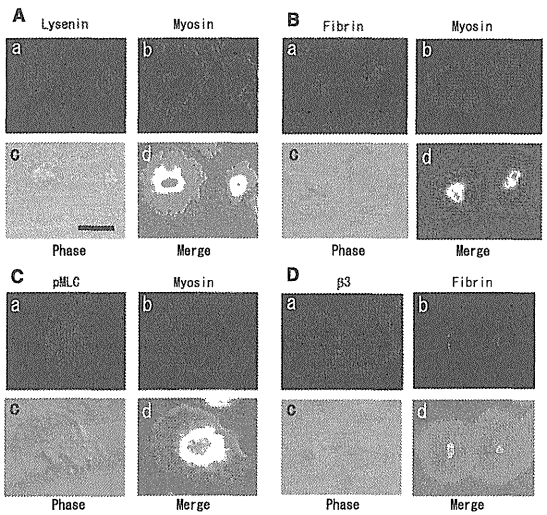

Figure 7. Immunocytochemical colocalization of myosin and Ser19-phosphorylated myosin light chain with fibrin on SM-rich rafts. (A) Localization of SM-rich rafts (green) and myosin (red). Scale bar, 5 μm. (B) Localization of fibrin (green) and myosin (red). (C) Localization of Ser19-phosphorylated MLC (green) and myosin (red). (D) Integrin β3 (green) and fibrin (red).

unchanged with thrombin stimulation (supplemental Figure 9), and that treatment with MBCD reduced the amount of SM-rich rafts to 28.6%, suggesting that SM-rich rafts are cholesterol-dependent (supplemental Figure 9A). Pretreatment with MBCD inhibited clot retraction (Figure 5D). Thrombin caused a rapid (within 5 minutes) tyrosine phosphorylation of the 125-kDa and 100-kDa proteins, and such phosphorylation returned to the control level at 60 minutes (Figure 5Ea). A previous study demonstrated a close relationship between clot retraction and a transient tyrosine phosphorylation.[28] The level of phosphorylation of the myosin light chain (MLC) at Ser19 was increased by thrombin treatment of 5 minutes, which returned to control level at 60 minutes (Figure 5Eb). MBCD inhibited the transient phosphorylation of the 125-kDa and 100-kDa proteins and MLC. These results suggested that SM-rich rafts act as platforms of fibrin-mediated outside-in signal, leading to clot retraction.

Colocalization of activated myosin with fibrin in SM-rich rafts of adhered platelets by thrombin

We investigated the distributions of fibrin, myosin, αIIbβ3, and SM-rich rafts by immunofluorescence analysis. Myosin was localized around a central region of spreading platelets (Figure 7A, red). This is consistent with previous findings of a myosin-containing spherical structure surrounding a central zone in spreading platelets.[29] A subpopulation of myosin was shown to colocalize in part with SM-rich rafts (Figure 7Ad) and fibrin (Figure 7Bd) inside the myosin-containing spherical structure. Interestingly, Ser19-phosphorylated MLC (activated myosin) was mostly localized in the central region (Figure 7Ca) and colocalized in part with myosin (Figure 7Cd). These data suggest that myosin is activated beneath SM-rich rafts and that activated myosin is colocalized with fibrin. In contrast to fibrin, integrin β3 was localized evenly on the membrane (Figure 7Da), suggesting the selective association of fibrin with a subpopulation of αIIbβ3 in SM-rich rafts. In conclusion, we propose that fibrin is translocated to SM-rich rafts in combination with FXIII and that platelet SM-rich rafts act as platforms where extracellular fibrin and the intracellular actomyosin efficiently join via integrin αIIbβ3 to promote clot retraction.

Discussion

In this study, we demonstrated that fibrin translocation in platelet DRM rafts is a specific event in platelet activation by thrombin. This is because (1) fibrin translocation did not occur in type I Glanzmann's thrombasthenic platelets, (2) the fibrinogen γ-chain dodecapeptide (residues 400-411) or cystamine significantly inhibited fibrin translocation, (3) the fibrinogen γ-chain C-terminal fusion protein, which has no ability to form fibers, also bound to the DRM of thrombin-stimulated platelets, and (4) immunocytochemical study showed the colocalization of fibrin with raft markers. In PALM image, fibrin and SM-rich rafts were not exactly colocalized (Figure 1C). When we stained SM with Dronpa-lysenin and Alexa 647-lysenin simultaneously, SM-rich domains stained with Dronpa-lysenin and Alexa 647-labeled lysenin were adjacent to one another, as described previously.[30] Therefore, this result implies that the problem is caused by the chromatic aberration and suggests that fibrin and SM-rich rafts are indeed in very close proximity.

Membrane rafts are spatially and functionally heterogenous in the cell membrane.[10,11] In a migrating T cell, a ganglioside GM3-rich raft containing a chemokine receptor is present at the leading edge, whereas a GM1-rich raft containing integrin β1 is present at the uropod.[31] In Jurkat T cells, SM-rich rafts are spatially distinct from GM1-rich rafts.[32] SMS1 deficiency impairs T-cell receptor signaling through the dysfunction of membrane rafts[24] and the raft-dependent apoptosis of lymphoma.[25] SMS2 deficiency impairs receptor clustering to membrane rafts.[26] SM-rich rafts are required for cytokinesis.[30] In this study, we demonstrated that SM-rich rafts were colocalized with fibrin in the central region of adhered platelets by thrombin. SMS1- and SMS2-deficient platelets exhibit a reduced fraction of SM-rich rafts and delayed fibrin clot retraction, suggesting that SM-rich rafts are involved in clot retraction.

What is the mechanism of SM-rich raft-mediated clot retraction? The retraction results from the actomyosin contraction of a platelet pseudopod attached to a fibrin strand.[33] The thrombin-induced phosphorylation of tyrosines 747 and 759 in an integrin β3 results in the linkage of integrin αIIbβ3 to myosin to mediate the transmission of force to the fibrin clot during clot retraction.[34] Activated αIIbβ3 connects with actin through talin and vinculin. In this study, we demonstrated the translocation of fibrin and myosin to the platelet DRM raft fraction and the microscopic colocalization of fibrin and myosin with SM-rich rafts in thrombin-stimulated platelets. We observed that the fibrinogen γ-chain 400-411 dodecapeptide and blebbistatin inhibited not only the fibrin and myosin translocation to the DRM fraction, respectively, but also clot retraction. Furthermore, we also demonstrated that αIIbβ3 is required for fibrin translocation to the DRM raft fraction and that αIIbβ3 (Figure 3B, arrowhead, and supplemental Figure 4) and actin (Figure 3A, 40 kDa protein, and Figure 3B, asterisk) are partially present in the platelet DRM raft fraction. These observations suggest that platelet SM-rich rafts, restricted areas on the platelet membrane, act as platforms where extracellular fibrin and the intracellular actomyosin system efficiently join via αIIbβ3 for clot retraction. To support this idea, a previous study demonstrated that DRM rafts specifically associate with the actin cytoskeleton upon platelet activation in an αIIbβ3-dependent manner.[35]

Clot retraction is regulated through multiple signaling pathways. Thrombin receptors, coupled to Gq and G13 heterotrimeric G proteins, can regulate clot retraction through the activation of phospholipase C (PLC)β and Rho kinase, which activates MLC kinase and inhibits MLC phosphatase, respectively.[36] Integrin αIIbβ3 outside-in signal is also required for optimal clot retraction. The engagement of αIIbβ3 is known to activate the c-src and tyrosine phosphorylation of PLCγ2.[37] The src-dependent activation of PLCγ2 induces calcium mobilization, MLC kinase activation, MLC phosphorylation, and actomyosin contraction. The src-dependent actomyosin contraction mediates clot retraction.[36] During clot retraction, the level of tyrosine phosphorylation of the 125-kDa and 100-kDa proteins increased to its peak after 5 minutes and returned to its control level after 60 minutes, in parallel to retraction.[28] Thus, clot retraction is associated with a transient tyrosine phosphorylation. In this study, MBCD treatment reduced the fraction of SM-rich rafts and inhibited the transient increase in the tyrosine phosphorylation of the 125-kDa and 100-kDa proteins and the transient phosphorylation of MLC at Ser 19. Immunofluorescence study showed the colocalization of Ser19-phosphorylated MLC with fibrin in SM-rich rafts of thrombin-stimulated platelets. Therefore, fibrin translocation to SM-rich rafts by thrombin may help to cluster αIIbβ3 and enhance outside-in signaling pathway, contributing to efficient clot retraction.

We have considerable evidence to support that FXIII is required for the fibrin translocation to SM-rich rafts, leading to clot retraction: (i) cystamine inhibited the 5BAPA incorporation to fibrin, translocation of fibrin, and the fibrinogen γ-chain C-terminal (144-411) fusion protein to membrane rafts and clot retraction; (ii) the fibrinogen γ-chain C-terminal (144-411) fusion protein translocated to platelet rafts on thrombin activation, but its mutant that was replaced by A398A399 at FXIII-crosslinking sites (Q398Q399) was inhibited; and finally (iii) fibrin translocation to membrane rafts and clot retraction were impaired in FXIII-A-subunit-deficient mouse platelets.[19] Furthermore, we found that batroxobin, a thrombin-like enzyme (0.25 μg/mL), caused fibrin clot formation but not clot retraction (data not shown). FXIII is activated by the cleavage of FXIIIA with thrombin but not batroxobin. Therefore, this result supports that transglutaminase activity of FXIII is required for clot retraction.

In summary, our results suggest that translocation of fibrin and myosin into platelet SM-rich rafts via integrin αIIbβ3 is an important process for clot retraction signaling. We propose a working hypothesis that the FXIII-dependent fibrin-αIIbβ3-myosin axis in platelet SM-rich rafts is required for efficient clot retraction.

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Authorship

Contribution: K.K. designed and performed research, analyzed data, performed statistical analysis, and wrote the paper; M.K., T.M., K.I., N.S.-S., H.S., I.K., M. Shimonaka, and M. Abe performed research; M. Arai, Y.O.-I., S.K., T.K., T.O., and M. Souri contributed analytical tools; and A.I. and N.Y. designed research, contributed analytical tools, analyzed data, wrote the paper, and worked as senior authors.

Conflict-of-interest disclosure: M. Arai is an employee of Novo Nordisk Pharma, Ltd. The remaining authors declare no competing financial interest.

Correspondence: Kohji Kasahara, Laboratory of Biomembrane, Tokyo Metropolitan Institute of Medical Science, Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan; e-mail: kasahara-kj@igakuken.or.jp.

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Severe inhibitor-negative acquired factor XIII/13 deficiency with aggressive subdural haemorrhage

Hiroki Kawano[a], Daisuke Yamamoto[b], Yoshito Uchihashi[b], Kanako Wakahashi[a], Yuko Kawano[a], Akiko Sada[a], Kentaro Minagawa[a], Yoshio Katayama[a], Eiji Kohmura[b], Masayoshi Souri[c] and Akitada Ichinose[c]

Acquired factor XIII (FXIII) deficiency is a common disease and seldom causes bleeding. However, severe FXIII deficiency may result in life-threatening bleeding. Although the inhibitor against FXIII has recently been focused as the cause of haemorrhagic acquired FXIII deficiency, the pathophysiology of inhibitor-negative cases could also be involved. We report a case of an 85-year-old Japanese man with serious subdural haemorrhage showing a remarkable decreased level of FXIII activity. He also manifested complications of compensated disseminated intravascular coagulation (DIC) with chronic renal failure, abdominal aortic aneurysm (AAA) and right renal carcinoma. Despite the successful evacuation of the haemorrhage, acute subdural haemorrhage subsequently developed that necessitated further craniotomies. Plasma cross-mixing studies and dot blot assay revealed no inhibitors against FXIII. We speculated that the decreased FXIII activity could be mainly due to hyperconsumption by DIC and surgery. Because plasma-derived FXIII concentrates are available to stop bleeding, clinicians should be aware of severe acquired inhibitor-negative FXIII deficiency in cases of unexplained excessive bleeding. ***Blood Coagul Fibrinolysis*** **24:638–641 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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[a]Department of Medicine (Hematology), [b]Department of Neurosurgery, Kobe University Graduate School of Medicine, Kobe and [c]Department of Molecular Patho-Biochemistry and Patho-Biology, Yamagata University School of Medicine, Yamagata, Japan

Correspondence to Hiroki Kawano, MD, PhD Hematology, Department of Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
Tel: +81 78 382 6912; fax: +81 78 382 6910;
e-mail: hkawano@med.kobe-u.ac.jp

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Introduction

Factor XIII (FXIII) is a tetrameric zymogen (FXIII-A2B2), which is converted into an active transglutaminase, FXIIIa, by thrombin and Ca^{2+} in the terminal coagulation cascade. FXIIIa is responsible for fibrin stabilization by forming fibrin γ-chain dimers and cross-linking its α-chains into high-molecular weight polymers [1]. The significant decrease in FXIII levels could result in severe bleeding caused by defective fibrin stabilization. The normal range of plasma FXIII levels is generally 70–130%. Thus, FXIII deficiency (<70%) is classified into congenital and acquired types. The acquired FXIII deficiency is usually caused by hyposynthesis and/or hyperconsumption from underlying diseases, such as leukemia, myelodysplastic syndrome, liver dysfunction, disseminated intravascular coagulation (DIC), major surgery and chronic inflammatory bowel diseases [2]. However, a remarkable decrease in the level of FXIII activity that leads to severe lethal haemorrhage has rarely been reported in this acquired type of deficiency except for patients with autoantibodies against FXIII [2].

We experienced a case of a patient with severe subdural haemorrhage and remarkably decreased FXIII activity without inhibitors. Our case has raised the possibility that acquired inhibitor-negative FXIII deficiency could result in life-threatening haemorrhage when several complications, such as DIC and uremia, were concurrent.

Case report

An 85-year-old Japanese man with chronic subdural haematoma was referred to our hospital. His medical history was pertinent for chronic renal failure, abdominal aortic aneurysm and right renal carcinoma but with no prior history of bleeding or familial bleeding. Despite a successful evacuation of the subdural haematoma, he subsequently experienced an acute subdural haemorrhage with midline shift on cranial computed tomography (CT) images that necessitated further surgical drainage (Fig. 1). Although several blood transfusions (16 units of fresh frozen plasma and 20 units of platelet concentrate) were given during the perioperative period, the subdural haemorrhage was uncontrolled, and oral haematoma and generalized purpura were observed. Blood tests showed moderate anaemia (Hb 8.4 g/dl), mild thrombocytopenia (Plt $9.6 \times 10^4/\mu l$), and normal prothrombin time (PT) and activated partial thromboplastin time (APTT). Furthermore, normal fibrinogen (247 mg/dl) and high levels of fibrin/fibrinogen degradation product (FDP) and D-dimer (33.8 and

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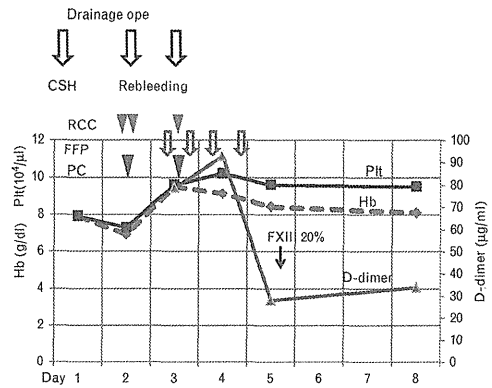
Fig. 1



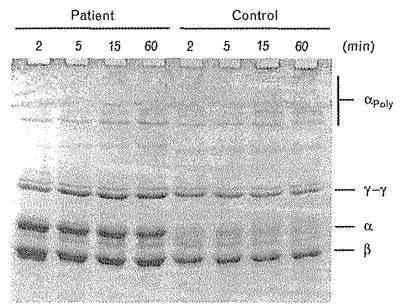
Clinical course of the patient during three craniotomies for the evacuation of subdural haemorrhage. The triangle indicates red cell concentrate (RCC; 4 units) or platelet concentrate (PC; 10 units). Small arrow indicates fresh frozen plasma transfusion (FFP; 4 units). Hb, haemoglobin; Plt, platelet.

28.0 μg/ml, respectively) implied compensated DIC. Other coagulation studies were as follows: AT activity 101% (80–120), thrombin–antithrombin complex (TAT) 69.9 ng/ml (<3), plasmin–plasmin inhibitor complex (PIC) 3.7 μg/ml (<0.8), total plasminogen activator inhibitor-1 (PAI-1) 63 ng/ml (<50), BUN 84 mg/dl, serum creatinine 8.24 mg/dl and triglyceride 113 mg/dl. Platelet function test using traditional turbidimetric platelet aggregometry revealed decreased collagen-induced (1.0 μg/ml) maximal aggregation (51%), whereas ristocetin-induced (1.2 mg/ml) and ADP-induced (2.0 μmol/l) maximal aggregations were within normal limits at 89 and 65%, respectively. Moreover, on the 14th hospital day, we found drastically reduced levels of FXIII activity (measured by ammonia release assay) and antigen (10 and 20%, respectively) in his plasma, and the levels of plasma and serum α2-plasmin inhibitor (α2-PI) were 106% (85–115%) and 123%, respectively. A fibrin cross-linking test by SDS-PAGE showed impaired formation of γ-dimer and α-polymer (Fig. 2). Plasma cross-mixing studies through incubation of patient plasma and normal control revealed that control plasma corrected the patient's decreased FXIII activity (Fig. 3a) [2,3]. A dot blot assay did not detect any immunoglobulins that bind to recombinant FXIII (Fig. 3b). Although the coagulation tests, such as PT and APTT, were within normal limits, and the high levels of D-dimer and FDP improved after the surgeries without anticoagulant therapy, the activity of FXIII remained decreased (day 5 20% and day 14 11%) (Fig. 1). Unfortunately, the patient succumbed to aspiration pneumonia before FXIII replacement therapy.

Discussion

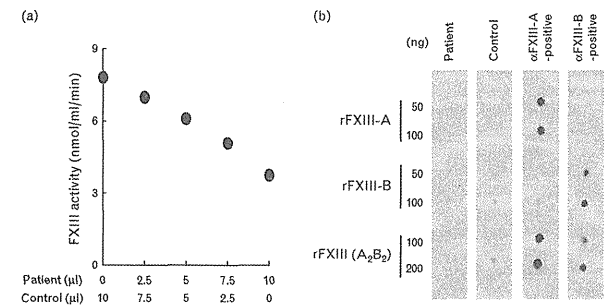
This report presents a case of severe subdural haemorrhage with a significant decrease in FXIII activity, although inhibitors against FXIII were not detected. FXIII deficiency has been known to develop into critical haemorrhages, especially spontaneous intracranial bleeds

Fig. 2



Fibrin cross-linking test. A fibrin cross-linking study was performed by the addition of 1 unit/ml thrombin and 5 mmol/l $CaCl_2$ into the patient's plasma and normal control plasma. The clots were recovered at the indicated time intervals and subjected to SDS-PAGE. The results of the fibrin cross-linking study showed only slight retardation of both γ-dimerization (γ-γ) and α-polymerization (α_{poly}) reactions in the patient's plasma.

Fig. 3



Tests for anti-FXIII antibodies. A 5-step dilution cross-mixing test by 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 normal plasma. The mixed samples containing the patient's plasma and normal plasma clearly showed a 'deficient' pattern, because there was no downward deviation in all of the diluted samples (a). A dot blot assay was performed using rFXIII-A, rFXIII-B and their complexes at the indicated amounts shown as antigen (ng). The results showed the absence of anti-FXIII antibodies in the patient's plasma (b). The positive controls stand for AH13 patients' plasmas identified previously [2,3].

[4]. Moreover, a previous report has implied the association of FXIII deficiency and spontaneous chronic subdural haematoma [5]. In our case, no prior episode of trauma was noted; therefore, we speculated that an asymptomatic acquired FXIII deficiency could develop chronic subdural haematomas.

The α2-PI is cross-linked to fibrin by activated FXIII and has been known to play a significant role in the inhibition of spontaneous fibrinolysis [6]. The difference in α2-PI levels between plasma and serum (plasma-serum α2-PI) *ex vivo* has been proposed as the indicator of reduced FXIII activity *in vivo* [3]. In our case, the value of plasma serum α2-PI was significantly reduced (4.2%), which was consistent with the degree of FXIII activity. A fibrin cross-linking test also showed the decreased pattern of the FXIII-A function in the formation of a stable clot. Together with the result of the dot blot assay that detected no inhibitors against FXIII, we confirmed that our case showed an acquired inhibitor-negative FXIII deficiency. Typically, in a FXIII-deficient patient, the results of standard laboratory clotting tests, including the values of PT, APTT, fibrinogen, platelet count and bleeding time, are all normal. However, in our case, the diagnosis was challenging because some abnormalities were observed in the laboratory examinations, including mild thrombocytopenia and elevated fibrinolytic markers, such as D-dimer and FDP. We have evaluated that our patient had complications of nonovert DIC due to the comorbidities of AAA and renal tumour according to the DIC scoring criteria by the International Society on Thrombosis and Haemostasis (ISTH) [7].

The transient elevation of the levels of fibrinolytic markers, such as FDP and D-dimer, was observed after craniotomy, which indicated a possible exacerbation of DIC as a result of the entry of thromboplastin into the systemic circulation by operative manipulation [8]. DIC has been known to cause mild to moderate reduction of the level of FXIII from overconsumption and was proposed as the useful marker in the diagnosis of DIC [9]. However, the importance of its clinical involvement in haemorrhage has not been fully evaluated. A previous report has shown that FXIII level was also decreased in patients with renal failure from glomerular damage with unknown mechanisms [10]. Thus, acquired inhibitor-negative FXIII deficiency can lead to severe bleeding if several disorders that reduce the levels of FXIII occur as comorbidities. We speculate three reasons for uncontrolled subdural haemorrhage in our case despite several transfusions of FFP and platelet concentrate. Firstly, the overconsumption of FXIII was attributed to exacerbation of DIC during the perioperative period. Secondly, FXIII was lost by perioperative bleeding (total of 1345 ml). Thirdly, the amount of FXIII in FFP might not be enough to replenish the deficiency [11].

Because acquired secondary FXIII deficiency often occurs concomitantly with several diseases that seldom manifest with severe bleeding, it is often overlooked. It is imperative to assess the FXIII level in a case of spontaneous intracranial haemorrhage with several complications, especially in the preoperative period in order to prevent an uncontrolled critical haemorrhage by giving FXIII concentrates.

Acknowledgements

Conflicts of interest

The authors state that they have no conflict of interest.

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Alloantibodies against the B subunit of plasma factor XIII developed in its congenital deficiency

Hideho Wada[1]*; Masayoshi Souri[2]*; Rui Matsumoto[1]; Takashi Sugihara[1]; Akitada Ichinose[2]

[1]Department of Hematology, Kawasaki Medical School, Kurashiki, Japan; [2]Department of Molecular Patho-Biochemistry & -Biology, Yamagata University School of Medicine, Yamagata, Japan

Summary

Factor XIII (FXIII) is a fibrin-stabilising factor consisting of catalytic A subunits (FXIII-A) and carrier B subunits (FXIII-B). FXIII-B prevents the fast clearance of FXIII-A from the circulation. Congenital FXIII-A deficiency is a rare bleeding disorder, and congenital FXIII-B deficiency is even rarer. Through our recent nationwide survey on "acquired haemophilia-like disease due to anti-FXIII autoantibodies," we newly diagnosed severe congenital FXIII-B deficiency in a Japanese man. He developed thrombocytopenia and gingival bleedings at the age of 73, and his FXIII activity was as low as 10% of the normal. When he suddenly developed spontaneous intramuscular haematoma, the bleeding was arrested by infusing FXIII concentrates. However, his FXIII activity remained around 10% of the normal. At the age of 74, ELISA and western blotting assay unexpectedly revealed complete absence of FXIII-B in the patient's plasma. A dot blot assay detected anti-FXIII-B alloantibodies for the first time in this disease, which could be attributed to the infusion of exogenous FXIII. He was found to be homozygous for a Japanese founder-effect mutation of *F13B*. Repeated infusions of exogenous FXIII for hemostasis increased anti-FXIII-B alloantibodies that resisted FXIII substitution. To the best knowledge of the authors, none of the remaining 10 reported cases of congenital FXIII-B deficiency developed alloantibodies to exogenous FXIII-B of plasma FXIII. An originally mild bleeding phenotype of severe congenital FXIII-B deficiency can be exaggerated by additional acquired conditions. Physicians should consider congenital FXIII-B deficiency when they encounter cases of unexplained bleeding disorders.

Keywords

Factor concentrates, factor XIII/transglutaminases, inherited coagulation disorders, alloantibody, factor XIII-resistant state

Correspondence to:
Akitada Ichinose, MD, PhD
Department of Molecular Patho-Biochemistry & -Biology
Yamagata University School of Medicine
2–2–2 Iida-Nishi, Yamagata, 990–9585 Japan
Tel.: +81 23 628 5275, Fax: +81 23 628 5280
E-mail: aichinos@med.id.yamagata-u.ac.jp

* These authors contributed equally to this study.

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Introduction

Coagulation factor XIII (FXIII) is a fibrin-stabilising factor, and is a heterotetramer consisting of two catalytic A subunits (FXIII-A) and two non-catalytic carrier B subunits (FXIII-B) (1-3).

Congenital FXIII deficiency causes a life-long severe bleeding tendency, abnormal wound healing, and recurrent miscarriages. It is a rare haemorrhagic disorder, and more than 500 cases of congenital FXIII deficiency have been identified throughout the world. In most of the reported cases, the congenital FXIII deficiency was caused by defects in the *F13A* gene (1, 4) (http: //www.f13-database.de).

Only a few cases of congenital FXIII-B deficiency, caused by founder-effect mutations, have previously been identified in Japan and Italy (5-8). FXIII-B deficiency may be overlooked by physicians because of the mild bleeding symptoms of this condition (9). A homozygote with congenital FXIII-B deficiency has recently been reported from Germany (10). In an extensive literature survey, we found another reported case of severe FXIII-B deficiency in the USA (11).

During a recent Japanese nationwide survey on "acquired haemophilia-like disease (or haemorrhaphilia) due to anti-FXIII autoantibodies (AH13) (12, 13)," we newly diagnosed severe congenital FXIII-B deficiency (designated as FXIII-B Kurashiki) in a Japanese man, who developed alloantibodies, for the first time, against FXIII-B likely resulting from therapeutic FXIII infusion. Moreover, he was a homozygote for the Japanese founder-effect mutation (5, 6).

Patient, materials and methods

Patient

In Oct. 2009, a 73-year-old Japanese man with thrombocytopenia and purpura presented at our hospital (▶Figure 1). Fifteen years ago, he had undergone γ-interferon therapy for hepatitis C infection. The patient and his family did not have any history of bleeding tendency. At the age of 16 years, he had undergone left thoracic plastic surgery for pulmonary tuberculosis without any excessive bleeding. He started to experience repeated episodes of gingival bleedings at the age of 73 years, and he was tentatively diagnosed as immune thrombocytopenic purpura (ITP), because he showed purpura on the right hand, decreased platelet count (3.7 x 10^4/μl), increased immature platelet fraction (IPF, 19.8%), and increased levels of platelet-associated Immunoglobulin G (PAIgG; 146 ng/10^7 cells) in October 2009. Since then, he had been receiving treatment with prednisolone. When he started to manifest "delayed bleedings" after dental operations, he was administered an antifibrinolytic drug (tranexamic acid). The results of the coagulation tests were within normal ranges, except for the FXIII activity and antigen levels (measured by a commercial laboratory, unless stated specifically) that were 12% and 9% of the normal, respectively, in December 2009 (▶Table 1, left column). Therefore, the amount of FXIII was moderately reduced but its antigen and activity levels were proportional (12/9=1.33; normal specific activity), suggesting the absence of FXIII inhibitor. His FXIII activity remained as low as 5-10% for more than six months (▶Figure 1). He then manifested gingival bleeding and leg purpura, and was infused with plasma-derived FXIII concentrates (FXIII Conc.; Lyophilised Human Blood Coagulation Factor XIII Concentrate, Fibrogammin P I.V. Injection, CSL Behring K.K., Tokyo, Japan) at 960 units (4 vials)/53 kg/day twice for haemostasis because of the persistent moderate FXIII deficiency. Since the bleeding symptom was observed to be abnormally severe than that expected to result from his decreased platelet count alone, his plasma and serum samples were extensively examined in January and February 2011 during our nationwide survey on AH13 (▶Table 1, right column).

In addition to thrombocytopenia, the patient developed anemia (Hb 9.9 g/dl) in June 2011, and 19 months after the first visit to our hospital, cytological examination of his bone-marrow aspirate indicated the myelodysplastic syndrome (REAB-1). The patient developed a spontaneous intramuscular haematoma in his right thigh, and he received FXIII Conc. at 24-hour (h) intervals for five days (▶Figure 1). He experienced repeated bleeding symptoms such as gingival bleeding and haemorrhoidal bleeding more frequently, and was transfused with FXIII Conc. for four days along with 10 units of platelet concentrates for both the bleeding events. Although his bleeding episodes were treated using appropriate amounts of FXIII Conc. (960 U/53.3 kg body weight [BW] = 18.0 U/kg), his plasma FXIII activities were as low as 7-9% 24 h after previous infusions and just before next infusions (8, 7, and 9% during the second, third and fourth bleeding episodes, respectively), and remained at the levels observed in the asymptomatic/non-bleeding periods (5-12%). Fortunately, until December 2012 (the time of submission of this manuscript) he was not clinically 'refractory' to FXIII replacement therapy, because his bleeding symptoms have been well controlled by infusing FXIII Conc.

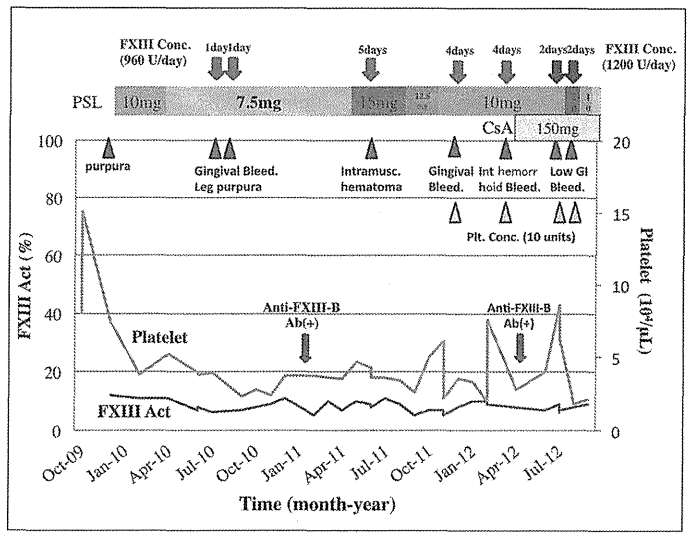

Figure 1: Clinical course of the present case (FXIII-B *Kurashiki*). *His FXIII* activity (FXIII Act, determined by a commercial ammonia release assay) and platelet count remained low for about two years. Each time the patient developed a bleeding symptom, he was infused with plasma-derived FXIII Conc. He had also been administered prednisolone (PSL) at the indicated dosages during the whole period. CsA, cyclosporine A.

Materials

Recombinant FXIII-A (rFXIII-A) was a kind gift from Dr. P. Bishop of Zymogenetics (Seattle, WA, USA). Recombinant FXIII-B (rFXIII-B) was expressed by baculovirus expression system and purified as previously described (14). Purified human plasma FXIII (A_2B_2 tetramer) was a generous gift from Dr. H. Kaetsu of Chaemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). An anti-FXIII-A monoclonal antibody was generously provided by Dr. G. Reed of Massachusetts General Hospital (Harvard Medical School, Boston, MA, USA). A rabbit anti-FXIII-A polyclonal antibody was generated in-house and affinity-purified using a rFXIII-A-coupled column. An anti-FXIII-B antiserum was purchased from Nordic Immunological Laboratories (AX Eindhoven, The Netherlands), and its IgG was purified using Protein A-Sepharose (GE Healthcare Bioscience AB, Uppsala, Sweden) and biotinylated using ECL Protein Biotinylation Module (GE Healthcare, Waukesha, WI, USA). A tetramethylbenzidine (TMB) peroxidase substrate kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Bovine thrombin, bovine serum albumin, *N,N*-dimethylcasein and monodansylcadaverine were purchased from Sigma-Aldrich (St. Louis, MO, USA). FXIII-deficient plasma was obtained from George King Bio-Medical (Overland Park, KS, USA).

Methods

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 including the present case and his family members.

Whole blood of the patients was collected into tubes containing 1/10 volume of 3.8% sodium citrate. Serum was separately prepared by using a tube containing glass microparticles to enhance coagulation. Plasma and serum samples were quick-frozen and sent to a commercial laboratory (SRL Ltd., Hachioji, Japan) for analysis of the plasma FXIII activity (measured by an ammonia release assay using a Berichrom FXIII kit of Siemens/Sysmex, Kobe, Japan; reference range, 70-140%) and FXIII antigen (measured by a latex agglutination assay using an NS auto FXIII type K kit of KAINOS Lab. Inc., Tokyo, Japan, with a rabbit anti-human FXIII [A_2B_2] polyclonal antibody; reference range, 70-140%) as well as the plasma and serum levels of α_2-plasmin inhibitor (α_2-PI), etc. (15). The amount of plasma minus serum α_2-PI and the ratio of plasma minus serum α_2-PI were calculated by previously described methods (13).

Cases of suspected AH13 were further examined for the presence of FXIII inhibitors in detail at our hands in the Yamagata University, as described below.

Amine-incorporation assay

According to the Lorand's method (16), 10 µl of plasma was incubated with 1 U bovine thrombin, 5 mM $CaCl_2$, 20 mM Tris-HCl (pH 7.5), 0.2% *N,N*-dimethylcasein, 2 mM monodansylcadaverine, and 2 mM dithiothreitol in a 0.1 ml mixture at 37°C for 60 minutes (min). The reaction was terminated by adding 0.1 ml of 10% trichloroacetic acid. The precipitate was collected by centrifugation, washed three times with 0.5 ml of ethanol-diethyl ether mixture (1: 1), and dissolved in 0.3 ml of 8 M urea, 1% sodium dodecyl sulfate (SDS) and 50 mM Tris-HCl (pH 8.0), which was abbreviated as UST buffer. The fluorescent intensity of emission at 520 nm with excitation at 360 nm was measured.

Table 1: Coagulation tests.

	Dec. 2009 in-hospital tests	Jan. 2011 commercial tests	Feb. 2011 our ELISA assays
Platelet count	3.5 x10⁴/µl		
Plasma α_2-PI	102%	107%	
Serum α_2-PI	—	118%	
XL-α_2-PI	—	10.4%	
XL-α_2-PI ratio	—	0.081	
	commercial tests		
FXIII activity	12%	10%	
1:1 cross-mixing	—	34% (control 128%)	
FXIII antigen	9%	45%	
FXIII-A antigen	—	—	<2%
FXIII-B antigen	—	—	<2%
A_2B_2 antigen	—	—	<2%

Abnormal values are underlined. XL, crosslinked.

Fibrin cross-linking test

Ten microliters of plasma was mixed with 5 mM $CaCl_2$ and 0.2 U bovine thrombin in a final volume of 20 µl, and incubated for the indicated time intervals. The reaction was terminated by adding 0.1 ml of 50 mM EDTA. The generated clot was collected by centrifugation at 10,000 *g* for 5 min, washed twice with 1 ml of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl (TBS), and dissolved in 40 µl of UST buffer, and then boiled with 40 µl of 2% SDS, 0.125 M Tris-HCl (pH 6.8), 15% glycerol, 5% β-mercaptoethanol, and 0.02% bromphenol blue (SDS-reducing buffer). A 10 µl sample was applied to SDS (0.1%)-polyacrylamide (10%) gel electrophoresis (PAGE) and proteins were stained with Coomassie brilliant blue R-250.

Western blotting

Plasma (1: 25, 1: 50, 1: 100 and 1: 200-dilutions with TBS) was boiled with 1% SDS, 50 mM Tris-HCl (pH 6.8), 7.5% glycerol, and 0.01% bromphenol blue in the presence (for FXIII-A) or absence (for FXIII-B) of 2.5% β-mercaptoethanol, and then applied to SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. The membrane was blocked, and then reacted with an affinity-purified rabbit anti-human FXIII-A or anti-human FXIII-B antibody. FXIII antigens were detected using a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and a chemi-luminescent substrate.

Enzyme-linked immunosorbent assay (ELISA)

An anti-human FXIII-A monoclonal antibody was coated in a microtitre plate for the measurement of FXIII-A and A_2B_2 complex, while anti-FXIII-B IgG was immobilised for measurement of FXIII-B. Plasma samples diluted 1/2,000 with TBS containing 2% bovine serum albumin were applied and incubated for 2 h at room temperature. After washing, for the determination of FXIII-A and A_2B_2 complex, an anti-human FXIII-A polyclonal antibody and an anti-human FXIII-B antiserum, respectively, were added and incubated for 60 min. After washing, HRP-conjugated anti-rabbit IgG was added and incubated for 60 min.

For the determination of FXIII-B, biotin-conjugated rabbit anti-human FXIII-B IgG was added and incubated for 60 min. After washing, HRP-conjugated streptavidin was added and incubated for 60 min. After final washing, TMB was added, and the reaction was stopped after 10 min by adding 0.5 M H_2SO_4. Absorbance at 450 nm was recorded by a microtitre plate reader Biolumin 960 (Molecular Dynamics, San Diego, CA, USA) and compared to standard curves, using purified FXIII-A, A_2B_2 complex, or FXIII-B.

Five-step cross-mixing test

In cross-mixing test, patient's plasma and normal control plasma were mixed at ratio of 0: 1, 1: 3, 1: 1, 3: 1, and 1: 0 and incubated at 37°C for 2 h, before amine-incorporation assay.

Dot blot assay

Fifty 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, MP Biomedicals, Solon, OH, USA) and a chemiluminescent substrate (GE Healthcare).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

A Japanese founder-effect mutation of the *F13B* gene, a deletion of adenosine [ag-(-)g] or IVS1-2delA at the boundary of intron A/exon II, resulting in a loss of the obligatory AG splicing sequence, was detected as described previously (5, 6). PCR was carried out using a pair of gene-specific primers for intron A/exon II, 5'-TGCAGACGGGATATTGGCAAGCT-3' & 5'-TCCATGTGTTCTCTTTCTTACCGAAG-3'. An amplified product of 500 bp was treated with 5 U of *TaqI* endonuclease (Toyobo, Tokyo, Japan) at 65°C for 90 min, then the sample was applied to a 2% agarose gel.

Results and discussion

Diagnosis of FXIII-B deficiency

Through our recent nationwide survey on the incidence of AH13 (12, 13), we identified a new case of severe FXIII-B deficiency. Since the results of all the coagulation tests were within normal ranges for this elderly Japanese male patient, various minute examinations related to FXIII were carried out. Patient's plasma had a moderately low level of FXIII activity; 10% of the normal by a commercial ammonia release assay (▶Table 1, middle column). The difference between α_2-PI activities in patient's plasma and serum (defined as XL-α_2-PI) was also observed to decrease by a commercial assay, suggesting a reduced FXIII activity against its physiological substrates as well (13). In the patient's plasma obtained about one month after the commercial tests, his FXIII activity was 5% of the normal by our amine-incorporation assay. Results of our fibrin cross-linking test showed delayed γ-dimerisation as well as delayed α-polymerisation (▶Figure 2A). In addition, no band of the FXIII-B protein was detected in our western blot analysis (▶Figure 2B), which is consistent with the findings of our ELISA (<2% of the normal; ▶Table 1, right column). These results clearly indicated the complete absence of FXIII-B antigen in the patient's plasma. He was diagnosed as congenital FXIII-B deficiency, as described later.

FXIII-A antigen was also barely detected by our western blotting analysis (▶Figure 2B) and <2% of the normal by our ELISA (▶Table 1, right column), indicating a secondary deficiency of FXIII-A due to the lack of protection by FXIII-B. In fact, FXIII-B prevents the rapid clearance of FXIII-A from the circulation in humans (11, 17) and mice (18).

Detection of anti-FXIII-B alloantibodies

Among our screening tests for detection of AH13 cases (13, 15), a cross-mixing test (1: 1) performed using the patient's plasma and normal control plasma with the commercial ammonia release assay showed moderate inhibition, because the resulting FXIII activity was 34% whereas the theoretical value was 69% [(10+128=138)/2] (▶Table 1, middle column). Moreover, his FXIII antigen level (45% by a commercial test) was 4.5 times higher than its activity (10%), suggesting the presence of inactivated or inhibited FXIII molecules (10/45=0.22; low specific activity). After about one month, our five-step dilution cross-mixing test performed using our amine-incorporation assay showed a straight "deficiency" pattern at large (▶Figure 2C), which indicated a negative result for anti-FXIII inhibitor. However, our dot blot assays performed using rFXIII-A, rFXIII-B, and their complex clearly demonstrated the presence of anti-FXIII-B antibodies (▶Figure 2D). The reason for the discrepancy between the result

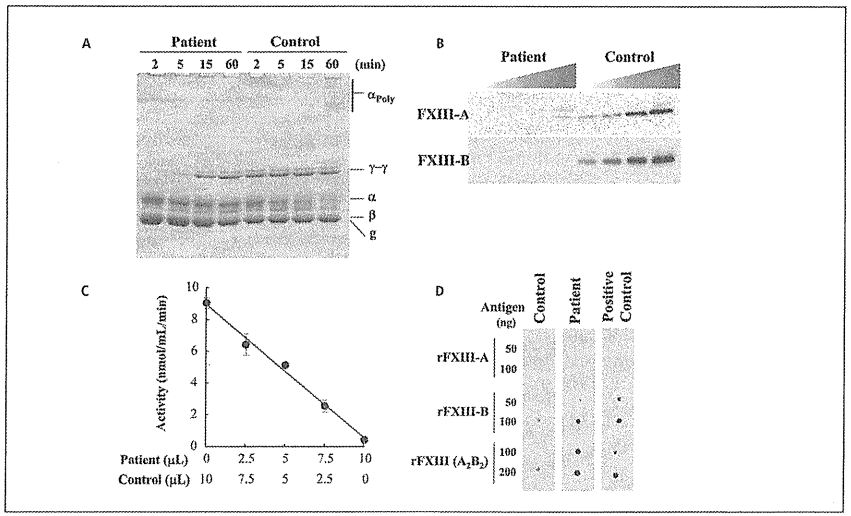

Figure 2: Analyses of FXIII in patient's plasma (Feb. 2011). Our fibrin cross-linking study was performed by adding 1 unit/ml thrombin and 5 mM $CaCl_2$ into the patient's plasma and normal control plasma. The clots were recovered at the indicated time intervals and subjected to SDS-PAGE analysis. Delay in both γ-dimerisation and α-polymerisation reactions were clearly shown (A). Our western blotting analysis was carried out using rabbit polyclonal anti-FXIII-A and FXIII-B antibodies. The results showed absence of FXIII-B protein and only a trace amount of FXIII-A protein when diluted patient's plasma was analysed (dilution: 1/25 to 1/200; from right to left; B). Our five-step dilution cross-mixing test of an amine-incorporation activity was performed using the patient's plasma at ratio of 0:1, 1:3, 1:1, 3:1, and 1:0 with normal plasma. Mixed samples between patient's plasma and normal plasma showed a deficiency pattern (C). Our dot blot assay was carried out using rFXIII-A, rFXIII-B and their complex at the indicated amounts shown as Antigen (ng) (D). The patient's plasma reacted with rFXIII-B as well as rFXIII complex (A_2B_2 tetramer), indicating the presence of anti-FXIII-B antibodies. The positive control stands for an AH13 patient's plasma identified previously (12).

of a commercial ammonia release assay and that of our amine-incorporation assay (about one month later) is unknown. It is possible that the majority of his anti-FXIII antibodies in January 2011 might be directed against A_2B_2 tetramer, leading to inhibition of FXIII activation/activity.

It is highly likely that the anti-FXIII-B antibodies were caused by therapeutic infusion of plasma-derived FXIII Conc. containing exogenous FXIII-B as A_2B_2 tetramer. The patient had been treated with prednisolone for suspected ITP. During the first period of the FXIII replacement therapy, the dose of prednisolone (7.5 mg/day) may not have been high enough to prevent the generation of alloantibodies against exogenous FXIII-B. As predicted, the patient's plasma clearly showed a drastic increase in anti-FXIII-B antibodies after three additional courses of the infusion with FXIII Conc. (▶ Figure 3A and B).

It is important to note that his anti-FXIII-B alloantibodies reacted with rFXIII (A_2B_2 tetramer) as well as rFXIII-B alone. His anti-FXIII-B antibodies also reacted with human plasma FXIII (A_2B_2 tetramer), as shown in ▶ Figure 3C.

The increase in anti-FXIII-B alloantibodies was confirmed by ELISA when FXIII Conc. was administered to the patient for arresting low gastrointestinal (GI) bleeding in July 2012 (▶ Figure 3D), although the antibodies had been reduced beforehand probably by the immunosuppressive treatment with cyclosporine A (CsA) (▶ Figure 1).

As of March 2012, only 11 cases (7 females and 4 males) of severe congenital FXIII-B deficiency have been identified on the basis of the absence of the FXIII-B antigen and the presence of defects in the *F13B* gene (9). Although seven cases with congenital FXIII-A deficiency were reported to have developed anti-FXIII-A alloantibodies (16, 19-25), no FXIII-B-deficient case with anti-FXIII-B alloantibodies has been described to date, to the best of our knowledge. Therefore, this is the first case of congenital FXIII-B deficiency in which the patient developed alloantibodies against exogenous FXIII-B.

'FXIII-resistance' to FXIII substitution by anti-FXIII-B alloantibodies

It is reported that a case with "acquired" FXIII-B deficiency due to anti-FXIII-B "autoantibodies" manifested severe bleeding symptoms (26). The presence of anti-FXIII-B 'alloantibodies' may also have clinical implications on the patient condition at least to some extent, every time when he undergoes FXIII replacement therapy. When the patient was infused with FXIII Conc. for arresting the bleeding, the antibodies may have bound to the exogenous FXIII and the resultant antigen-antibody complexes may have been rapidly removed from the circulation. This assumption was consistent with the fact that his FXIII activity increased only by 2-4% from the lowest basal level of 5%, even though the estimated increase in the activity after the infusion of FXIII Conc. was about 35% [960x2 U/53kg BW]. The patient's plasma FXIII activities remained at 7-9% 24 h after previous FXIII Conc. infusions. This state is, as it were, pharmacologically 'FXIII-resistance'.

To test this assumption further, we measured FXIII activity immediately after infusion of FXIII Conc. and estimated its half-life in the patient when he manifested GI bleeding in July 2012 (▶ Figure 4A). The increase in FXIII activity was only partial (10% measured vs. 1,200x2 U/50 kg BW = 48% calculated). Since the patient's FXIII activity returned to his baseline level after 12 h, the half-life was roughly estimated to be about 3 h. The half-life for the same plasma-derived FXIII Conc. (Fibrogammin P®) was reported as about 9 h in a Hispanic congenital FXIII-B deficient case (11), while it was 17 h in the Hungarian case with 'acquired' FXIII-B deficiency due to anti-FXIII-B autoantibodies (26). Thus, the extremely short half-life of the present case was very likely due to a combination of the complete absence of FXIII-B and the presence of anti-FXIII-B alloantibodies. The preceding infusion of FXIII concentrates may have boosted the production of anti-FXIII-B alloantibodies again, because essentially no increase in FXIII activity was observed after next infusion of 1,200 U FXIII Conc. for his gingival bleeding after three months (▶ Figure 4B).

The exogenous FXIII may have functioned transiently and/or locally at the sites of bleeding even though his systemic FXIII level was not confirmed to increase significantly. The alloantibodies against FXIII-B should be eradicated by immunosuppressive therapy (15) to improve his pharmacological FXIII-resistant state, if he continues to experience severe bleeding episodes, which may finally become clinically refractory to FXIII replacement therapy. Because he had already been treated with cyclosporine A since April 2012, other regimens such as cyclophosphamide and rituximab, as used for autoimmune FXIII deficiency (26), may be more successful in the eradication therapy. In addition, replacement therapy using a commercial recombinant FXIII-A preparation will be a useful and reasonable option to avoid further boosting the production of his anti-FXIII-B alloantibody, when it will be licensed in Japan in the near future.

Family study and identification of founder-effect mutation

The results indicating the patient's severe FXIII-B deficiency prompted us to carry out a family study as well as genetic analyses of his pedigree. In the two daughters of the proband (FXIII-B Kur-

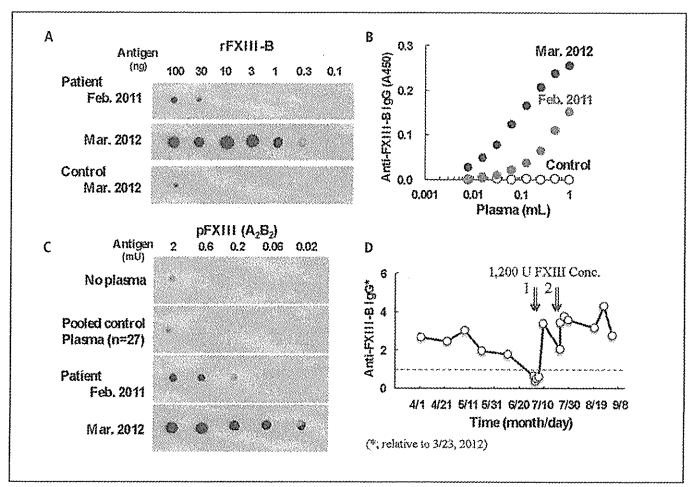

Figure 3: Changes in levels of anti-FXIII-B alloantibodies in patient's plasma. The minimal detectable antigen level (ng) for anti-FXIII-B alloantibodies was determined by our dot blot assay (A & C), while the relative amount of anti-FXIII-B IgG was measured by our ELISA (B & D). There was a drastic increase in anti-FXIII-B antibodies, likely boosted about 10 fold by three courses of FXIII Conc. infusion within 13 months. The boosted anti-FXIII-B alloantibodies were clearly shown twice (1 & 2 in D) after dosing 1,200 U FXIII Conc. for arresting low gastrointestinal bleeding in July 2012.

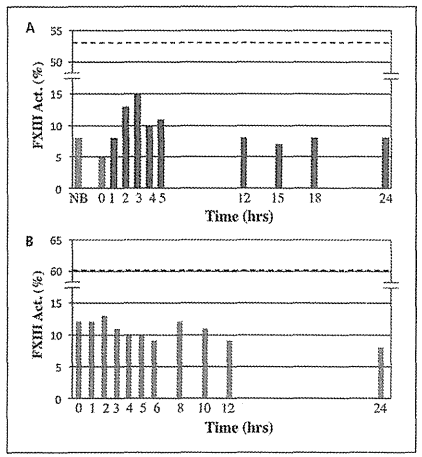

Figure 4: Time course of plasma FXIII activity after FXIII Conc. infusion. FXIII Conc. (1,200 U/50kg equivalent to a 48% increase in FXIII Act) was given to the patient for arresting low GI bleeding in July 2012, and plasma samples were collected at the indicated time intervals (A). After three months (Oct. 2012), the same amount of FXIII Conc. was administered for stopping gingival bleeding and plasma FXIII activity was measured by a commercial ammonia release assay at the indicated time intervals (B). The patient's FXIII activity increased only by 10% at 3 h from the lowest basal level of 5% at 0 h (pre-dose) after the infusion of FXIII Conc. in July 2012. Furthermore, there was essentially no increase, 1% if any, in the activity after infusing FXIII Conc. in Oct. 2012. NB stands for non-bleeding periods (two months before). Broken lines depict expected FXIII activities after FXIII Conc. infusion.

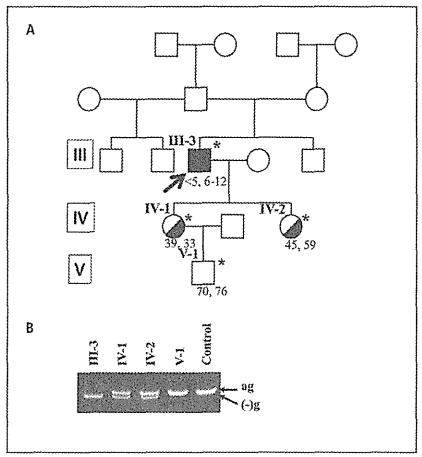

Figure 5: Family pedigree and FXIII levels and founder-effect mutation. In a family study, FXIII levels were examined by our ELISA, western blotting, and amine-incorporation assays, as described in *Methods*. Results of FXIII-B and FXIII-A antigen analysis by our ELISA for each family member are indicated at the right bottom corner in % of the normal (A). Case ID III-3 is the proband (arrow). PCR-RFLP analyses were performed using TaqI endonuclease in order to detect the Japanese founder-effect mutation: an IVS1-2delA or an ag->(-)g deletion at the intron A/exon II splicing site in the F13B gene (B).

ashiki), ELISA results clearly confirmed decreased amounts of both FXIII-B (39 and 45% of normal, respectively) and FXIII-A (33 and 59% of normal, respectively) proteins (▶Figure 5A), which was consistent with the results of western blotting analysis (data not shown). Both daughters showed FXIII amine-incorporation activity of 38 and 74% of the normal, respectively. A commercial ammonia release assay also demonstrated similar results (39 and 70% of the normal, respectively). It is interesting to note that about two thirds of 37 defined heterozygotes of congenital FXIII-B deficiency showed decreased FXIII activity and antigen levels (see Suppl. Table 1, available online at www.thrombosis-online.com), suggesting that more than a half of the normal FXIII-B level may be necessary to maintain/support normal FXIII/FXIII-A levels in the majority of these individuals. Since the concentration of free FXIII-B is constant regardless the concentration of FXIII-A or A_2B_2 tetramer (27), only a part of the reduced FXIII-B by half may complex with FXIII-A to stabilise it (10).

We previously identified a founder-effect mutation in the *F13B* gene among Japanese individuals (5, 6). This deletion mutation was detected by PCR-RFLP analyses in the homozygous and heterozygous states in the genomic DNA of the proband (III-3) and his daughters (IV-1 & -2), respectively (▶Figure 5B). Since the same mutation was observed in individuals from four geographically separated regions of Japan, Kanazawa, Fukuoka, Fukushima and Kurashiki (see Suppl. Table 1, available online at www.thrombosis-online.com), we believe that there would be many more patients with this type of congenital FXIII-B deficiency, at least in Japan. As recently addressed (9), bleeding symptoms of these Japanese patients seem to be mild; spontaneous bleedings rarely develop while provoked bleedings secondary to haemostatic challenges, such as surgery, trauma, and delivery in females are common.

In conclusion, congenital FXIII-B deficiency seems to be more common than what we perceived previously. Although the bleeding phenotype in congenital FXIII-B deficiency is naturally mild, severe bleeding episodes can occur when the condition is aggravated by additional haemostatic disorders, including the development of thrombocytopenia resulting from myelodysplastic syndrome and FXIII-resistance by anti-FXIII antibodies etc., like the present case. Thus, physicians need to be aware of congenital FXIII-B deficiency, and they must keep this disease in mind whenever they come across patients showing unexplained bleeding disorders.

What is known about this topic?

- A Hungarian case with "acquired" factor (F)XIII-B deficiency due to anti-FXIII-B "autoantibodies" manifested severe bleeding symptoms.
- Although seven cases with congenital FXIII-A deficiency were reported to have developed anti-FXIII-A alloantibodies, no FXIII-B deficient case with anti-FXIII-B alloantibodies has been described to date.

What does this paper add?

- Physicians need to be aware that patients with congenital FXIII-B deficiency may develop alloantibodies to exogenous FXIII-B contained in plasma-derived FXIII products.
- An originally mild bleeding phenotype of severe congenital FXIII-B deficiency can be exaggerated by additional acquired conditions.

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Conflicts of interest

None declared.

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VI. 参考資料

原因不明の出血！

出血症状があるのに、ハッキリした原因が分らない患者さんを診たら?

出血性後天性凝固異常症の可能性があります

症状

① 出血性素因の家族歴、既往歴がなく、抗凝固薬、抗血小板薬を服用中ではない患者さんで、

② a. 原因不明の皮下出血、筋肉内出血、あるいは(開放創の)後出血(いったん止血した12~36時間後に再び出血する)があるとき、血が滲みでるような、いわゆるウージング様の出血が見られるとき、

あるいは、

b. 原因不明の鼻出血、口腔内出血などの粘膜出血を繰り返すとき、

③ 通常の止血療法の効果が見られないとき、

a. 自己免疫性血友病(出血病)XIII/13や後天性FXIII/13欠乏症、後天性抗線溶因子(アルファ$_2$プラスミンインヒビターやプラスミノゲンアクチベーターインヒビター1など)欠乏症、

あるいは、

b. 後天性フォン・ヴィレブランド症候群(後天性フォン・ヴィレブランド因子欠乏症)などである可能性があります。

なお、血小板の減少や機能低下、PTやaPTTの延長を伴っている場合もあるので、御注意ください。

原因

第XIII/13因子や、抗線溶因子、フォン・ヴィレブランド因子の過剰な消費や産生減少による低下、あるいはそれらの因子に対する自己の抗体(インヒビター)による中和などが基盤となっています。

検査・診断

出血症状の原因が分からない症例で、第XIII/13因子、抗線溶因子、フォン・ヴィレブランド因子いずれかの活性が著しく低下していること。
(各因子抗原量、インヒビターの有無、力値を含め精密検査が必要です。)

治療

① 当面の止血療法:欠乏する各因子製剤の補充、DICがなければ抗線溶薬の投与
(後天性フォン・ヴィレブランド症候群では、デスモプレッシン投与も)

② インヒビターの産生阻止、除去:免疫抑制薬、血漿交換など(抗体確認後は可及的速やかに開始する必要があります。

全国調査中です。

出血性後天性凝固異常症疑いの患者さんに遭遇された場合は、研究班代表(山形大学・一瀬白帝)、あるいは最寄りの班員の方にご連絡/ご相談下さい。(裏面に班員のリストがあります。)
日本血栓止血学会のホームページもご覧下さい。(班研究の大まかな内容も記載されています。)

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出血性後天性凝固異常症研究班　研究代表者

一瀬 白帝

厚生労働科学研究費補助金　難治性疾患等克服研究事業（難治性疾患克服研究事業）

「診断困難な（原因不明の）出血性後天性凝固異常症の総合的診療指針の作成」研究班

（所在地によって北から南の順に記載した。）

研究代表者

一瀬　白帝	山形大学医学部　分子病態学	教　授	aichinos@med.id.yamagata-u.ac.jp

研究分担者

惣宇利 正善	山形大学医学部　分子病態学	准教授	msouri@med.id.yamagata-u.ac.jp
尾崎　司	山形大学医学部　分子病態学	助　教	tosaki@med.id.yamagata-u.ac.jp
浦野 哲盟	浜松医科大学　医生理学	教　授	uranot@hama-med.ac.jp
松下　正	名古屋大学医学部附属病院　輸血部	教　授	tmatsu@med.nagoya-u.ac.jp

研究協力者

家子 正裕	北海道医療大学歯学部　内科学	教　授	iekom@hoku-iryo-u.ac.jp
玉井 佳子	弘前大学医学部附属病院　輸血部	講　師	ytamai@cc.hirosaki-u.ac.jp
伊藤 俊広	国立病院機構　仙台医療センター　血液内科	医　長	toshi_ito_ito@yahoo.co.jp
川前 金幸	山形大学医学部　麻酔科学	主任教授	kkawamae@med.id.yamagata-u.ac.jp
山本 正雅	奥羽大学薬学部　生化学	准教授	n-yamamoto@pha.ohu-u.ac.jp
窓岩 清治	自治医科大学　分子病態治療研究センター 分子病態研究部	講　師	madochan@jichi.ac.jp
黒澤 秀光	獨協医科大学　小児科学	准教授	hidekuro@dokkyomed.ac.jp
小川 孔幸	群馬大学大学院医学系研究科 生体統御内科（血液内科）	助　教	yo-ogawa@med.gunma-u.ac.jp
矢冨　裕	東京大学医学部附属病院　検査部	教　授	yatoyuta-tky@umin.ac.jp
花房 規男	東京大学医学部附属病院　血液浄化療法部	講　師	hanafusa-tky@umin.ac.jp
川杉 和夫	帝京大学医学部　内科学	教　授	kaz@med.teikyo-u.ac.jp
石田 文宏	信州大学医学部　内科学第二	准教授	fumishi@shinshu-u.ac.jp
北島　勲	富山大学大学院医学薬学研究部 臨床分子病態検査学講座	教　授	kitajima@med.u-toyama.ac.jp
朝倉 英策	金沢大学附属病院　高密度無菌治療部	准教授	hasakura@staff.kanazawa-u.ac.jp
細野 奈穂子	福井大学医学部附属病院　血液腫瘍内科	助　教	hosono@u-fukui.ac.jp
毛利　博	藤枝市立総合病院	事業管理者（院長）	mohrih@hospital.fujieda.shizuoka.jp
小林 隆夫	浜松医療センター	院　長	tkoba@hmedc.or.jp
和田 英夫	三重大学大学院医学系研究科　検査医学	准教授	wadahide@clin.medic.mie-u.ac.jp
宮田 茂樹	国立循環器病研究センター　輸血管理室	医　長	smiyata@hsp.ncvc.go.jp
柏木 浩和	大阪大学大学院医学系研究科　血液・腫瘍内科	講　師	kashi@hp-blood.med.osaka-u.ac.jp
池田 正孝	国立病院機構　大阪医療センター　外科	医　長	mikeda@onh.go.jp
湯川 真生	近畿大学医学部奈良病院　外科	准教授	yukawa@nara.med.kindai.ac.jp
日笠　聡	兵庫医科大学　血液内科	講　師	parasol@mua.biglobe.ne.jp
羽藤 高明	愛媛大学医学部附属病院　輸血・細胞治療部	病院教授	takahato@m.ehime-u.ac.jp
岡本 好司	北九州市立八幡病院 消化器・肝臓病センター　外科	センター長、 外科主任部長	kohji.okamot@gmail.com
岡村　孝	久留米大学医学部　内科学講座　血液・腫瘍内科	教　授	okamura@med.kurume-u.ac.jp
内場 光浩	熊本大学医学部附属病院　輸血・細胞治療部	助　教	mituhiro-uchiba@fc.kuh.kumamoto-u.ac.jp
橋口 照人	鹿児島大学大学院医歯学総合研究科 血管代謝病態解析学	教　授	terutoha@m3.kufm.kagoshima-u.ac.jp
丸山 征郎	鹿児島大学大学院医歯学総合研究科 システム血栓制御学	特任教授	rinken@m3.kufm.kagoshima-u.ac.jp

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「診断困難な（原因不明の）出血性後天性凝固異常症の総合的診療指針の作成」班研究

臨床調査票（新規）

Ver.20131219

施設・診療科名		調査票記載医師名と記入年月日	医師名	記入年月日 西暦　年　月　日	
患者略名（匿名化）*2	登録番号*2	発症（出血）の年月日	西暦　年　月　日		
患者生年月（年齢）・性別	西暦　年　月生（　才）　男・女	出血の有無（過去）初発時・最悪時	出血の頻度（初発時・最悪時）：	出血部位（初発時・最悪時）：	
原（基礎）疾患名	有（　） 不明	出血の有無（現在）	出血の頻度：	出血部位：	
F13 低下に関する手術・輸血歴・薬剤歴等		貧血の有無（Hb 値）	有・無	Hb 値（　g/dL）	
現在の状況	入院・外来（西暦　年　月現在）	創傷治癒異常の有無	有（具体的に　） 無		
診断の年月日	西暦　年　月　日	出血初発時の第 13 因子（F13 投与前・後）	第13 因子活性（F13:C）：　%	第13 因子抗原量（F13:Ag）：　%	
診断／転帰	病名（　） 治癒・軽快・不変・悪化・死亡（死因　）	最悪時の第 13 因子（F13 投与前・後）	第13 因子活性（F13:C）：　%	第13 因子抗原量（F13:Ag）：　%	
止血の年月日	西暦　年　月　日	止血時の第 13 因子（F13 投与前・後）	第13 因子活性（F13:C）：　%	第13 因子抗原量（F13:Ag）：　%	
止血時の第13 因子製剤	名称（　） 量（　単位）　体重（　kg）	家族*5 の第 13 因子（続柄：　と）	第13 因子活性（F13:C）：　%と　%	第13 因子抗原量（F13:Ag）：　%と　%	
出血治療・予防の方法	F13 以外の血液製剤（　）・抗線溶薬（　）・その他（　）				
インヒビターの有無*3	現在 有・無・不明・過去に有	インヒビター確認年月日	西暦　年　月　日／不明		
インヒビターの治療方法	免疫抑制薬：　ステロイド（　）　リツキシマブ（　）　その他（　）		血漿交換：	その他：	
	治療効果：薬剤名（　）により　インヒビターが（消失・減少・不変・上昇）した				
直近のフィブリノゲン（Fbg）濃度	Fbg（　）mg/dL	その他*4			

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*4 HIV, HCV, HBV など陽性の場合は、各施設の取り決めに従って検体にその旨記載してください。
*5 家族は、なるべく多数の症例本人の両親、子供等の血縁関係者としてください。
記入上の不明な点については、出血性後天性凝固異常症研究班 事務局（山形大学医学部 分子病態学 Tel：023-628-5276）にお問い合わせください。
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「診断困難な（原因不明の）出血性後天性凝固異常症の総合的診療指針の作成」班研究

臨床調査票（追跡）

Ver.20131219

施設・診療科名		調査票記載医師名と記入年月日	医師名	記入年月日 西暦　年　月　日	
患者略名（匿名化）*2	登録番号*2	発症（出血）の年月日	西暦　年　月　日		
患者生年月（年齢）・性別	西暦　年　月生（　才）　男・女	出血の有無（過去）初発時・最悪時	出血の頻度（初発時・最悪時）：	出血部位（初発時・最悪時）：	
原（基礎）疾患名	有（　） 不明	出血の有無（現在）	出血の頻度：	出血部位：	
F13 低下に関する手術・輸血歴・薬剤歴等		貧血の有無（Hb 値）	有・無	Hb 値（　g/dL）	
現在の状況	入院・外来（西暦　年　月現在）	創傷治癒異常の有無	有（具体的に　） 無		
診断の年月日	西暦　年　月　日	出血初発時の第 13 因子（F13 投与前・後）	第13 因子活性（F13:C）：　%	第13 因子抗原量（F13:Ag）：　%	
診断／転帰	病名（　） 治癒・軽快・不変・悪化・死亡（死因　）	最悪時の第 13 因子（F13 投与前・後）	第13 因子活性（F13:C）：　%	第13 因子抗原量（F13:Ag）：　%	
止血の年月日	西暦　年　月　日	止血時の第 13 因子（F13 投与前・後）	第13 因子活性（F13:C）：　%	第13 因子抗原量（F13:Ag）：　%	
止血時の第13 因子製剤	名称（　） 量（　単位）　体重（　kg）	家族*4 の第 13 因子（続柄：　と）	第13 因子活性（F13:C）：　%と　%	第13 因子抗原量（F13:Ag）：　%と　%	
出血治療・予防の方法	F13 以外の血液製剤（　）・抗線溶薬（　）・その他（　）				
インヒビターの有無*3	現在 有・無・不明・過去に有	インヒビター確認年月日	西暦　年　月　日／不明		
インヒビターの治療方法	免疫抑制薬：　ステロイド（　）　リツキシマブ（　）　その他（　）		血漿交換：	その他：	
	治療効果：薬剤名（　）により　インヒビターが（消失・減少・不変・上昇）した				
臨床経過	（前回の回答からの変化を中心に、具体的に記述）				

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*4 家族は、なるべく多数の症例本人の両親、子供等の血縁関係者としてください。
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臨床調査票（新規；後天性 vWD 疑い用）

Ver.20131225

施設・診療科名			調査票記載医師名と記入年月日	医師名	記入年月日 西暦　年　月　日
患者略名（匿名化）*2		登録番号*2	発症（出血）の年月日	西暦　年　月　日	
患者生年月（年齢）・性別	西暦　年　月生（　才）　男・女		出血の有無（過去）初発時・最悪時	出血の頻度（初発時・最悪時）：	出血部位（初発時・最悪時）：
原（基礎）疾患名	有（　） 不明		出血の有無（現在）	出血の頻度：	出血部位：
vWF 低下に関する手術・輸血歴・薬剤歴			貧血の有無（Hb 値）	有・無	Hb 値（　g/dL）
現在の状況	入院・外来（西暦　年　月現在）		創傷治癒異常の有無	有（具体的に　） 無	
診断の年月日	西暦　年　月　日		出血初発時の vWF（vWF 投与前・後）	vWF 活性（vWF:C）：　%	vWF 抗原量（vWF:Ag）：　%
診断／転帰	病名（　） 治癒・軽快・不変・悪化・死亡（死因　）		最悪時の vWF（vWF 投与前・後）	vWF 活性（vWF:C）：　%	vWF 抗原量（vWF:Ag）：　%
止血の年月日	西暦　年　月　日		止血時の vWF（vWF 投与前・後）	vWF 活性（vWF:C）：　%	vWF 抗原量（vWF:Ag）：　%
止血時の vWF 製剤	名称（　） 量（　単位）　体重（　kg）		家族*5 の vWF（続柄：　と）	vWF 活性（vWF:C）：　%と　%	vWF 抗原量（vWF:Ag）：　%と　%
出血治療・予防の方法	vWF 以外の血液製剤（　）・抗線溶薬（　）・その他（　）				
インヒビターの有無*3	現在有・無・不明・過去に有		インヒビター確認年月日	西暦　年　月　日／不明	
インヒビターの治療方法	免疫抑制薬：　ステロイド（　）　リツキシマブ（　）　その他（　）			血漿交換：	その他：
	治療効果：薬剤名（　）により　インヒビターが（消失・減少・不変・上昇）した				
直近の血小板数	（　）×10⁴/μL	その他*4			

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*4 HIV, HCV, HBV など陽性の場合は、各施設の取り決めに従って検体にその旨記載してください。
*5 家族は、なるべく多数の症例本人の両親、子供等の血縁関係者としてください。
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臨床調査票（追跡；後天性 vWD 疑い用）

Ver.20131225

施設・診療科名			調査票記載医師名と記入年月日	医師名	記入年月日 西暦　年　月　日
患者略名（匿名化）*2		登録番号*2	発症（出血）の年月日	西暦　年　月　日	
患者生年月（年齢）・性別	西暦　年　月生（　才）　男・女		出血の有無（過去）初発時・最悪時	出血の頻度（初発時・最悪時）：	出血部位（初発時・最悪時）：
原（基礎）疾患名	有（　） 不明		出血の有無（現在）	出血の頻度：	出血部位：
vWF 低下に関する手術・輸血歴・薬剤歴			貧血の有無（Hb 値）	有・無	Hb 値（　g/dL）
現在の状況	入院・外来（西暦　年　月現在）		創傷治癒異常の有無	有（具体的に　） 無	
診断の年月日	西暦　年　月　日		出血初発時の vWF（vWF 投与前・後）	vWF 活性（vWF:C）：　%	vWF 抗原量（vWF:Ag）：　%
診断／転帰	病名（　） 治癒・軽快・不変・悪化・死亡（死因　）		最悪時の vWF（vWF 投与前・後）	vWF 活性（vWF:C）：　%	vWF 抗原量（vWF:Ag）：　%
止血の年月日	西暦　年　月　日		止血時の vWF（vWF 投与前・後）	vWF 活性（vWF:C）：　%	vWF 抗原量（vWF:Ag）：　%
止血時の vWF 製剤	名称（　） 量（　単位）　体重（　kg）		家族*4 の vWF（続柄：　と）	vWF 活性（vWF:C）：　%と　%	vWF 抗原量（vWF:Ag）：　%と　%
出血治療・予防の方法	vWF 以外の血液製剤（　）・抗線溶薬（　）・その他（　）				
インヒビターの有無*3	現在有・無・不明・過去に有		インヒビター確認年月日	西暦　年　月　日／不明	
インヒビターの治療方法	免疫抑制薬：　ステロイド（　）　リツキシマブ（　）　その他（　）			血漿交換：	その他：
	治療効果：薬剤名（　）により　インヒビターが（消失・減少・不変・上昇）した				
臨床経過	（前回の回答からの変化を中心に、具体的に記述）				

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ISTH/SSC 出 血 評 価 表 （日本語試用版*1）

症例の 匿名化暗号: 調査年月日: 性別: 生年月:

評価時（何れかに○） 最重症期・ 初診時 ・ 診断時・ 治療前 ・ 治療後 ・ 治癒後 ・寛解後 ・ 退院時／現在

症状	出 血 ス コ ア				
	0	1	2	3	4
鼻出血	無し か 軽微	・年5回以上 か ・10分間以上	診察／検査のみ	パッキング か 焼灼術 か 抗線溶薬	輸血 か 補充療法（止血因子、rFVIIaの使用）（か デスモプレッシン）*2
皮膚の（出血）	無し か 軽微	露出部に年5回以上の挫創（1cm以上）	診察／検査のみ	広範囲	自発性血腫で輸血が必要
軽度外傷からの出血	無し か 軽微	・年5回以上 か ・10分間以上	診察／検査のみ	手術による止血	輸血 か 補充療法（か デスモプレッシン）
口腔（内出血）	無し か 軽微	有り	診察／検査のみ	手術による止血 か 抗線溶薬	輸血 か 補充療法（か デスモプレッシン）
胃腸管出血	無し か 軽微	有り（潰瘍、門脈圧亢進症、痔、血管形成異常に伴わない）	診察／検査のみ	手術による止血 か 抗線溶薬	輸血 か 補充療法（か デスモプレッシン）
血尿	無し か 軽微	有り（肉眼的）	診察／検査のみ	手術による止血 か 鉄剤投与	輸血 か 補充療法（か デスモプレッシン）
抜歯（時出血）	無し か 軽微 か 未施術	全ての施術の25%以下で報告/申告、無介入	全ての施術の25%以上で報告/申告、無介入	再縫合 か パッキング	輸血 か 補充療法（か デスモプレッシン）
手術（関連出血）	無し か 軽微 か 未施術	全ての施術の25%以下で報告/申告、無介入	全ての施術の25%以上で報告/申告、無介入	手術による止血 か 抗線溶薬	輸血 か 補充療法（か デスモプレッシン）
過多月経	無し か 軽微	診察／検査のみ か ・2時間おき以上頻繁にパッド交換 か ・凝血塊を伴う多量出血 ・経血量図評価チャートスコア100以上	・年2回以上の欠勤／欠席 か ・抗線溶薬 か 女性ホルモン か 鉄剤投与必要	・抗線溶薬と女性ホルモン両方必要 か ・初経以来12ヶ月以上有り	・入院と緊急治療が必要な急性出血 か ・輸血 か 補充療法（か デスモプレッシン）必要 か ・子宮内容除去術 か 子宮内膜焼灼 か 子宮摘出術必要
産後出血	無し か 軽微 か 未分娩	診察／検査のみ か ・オキシトシン使用 か ・6週以上の悪露	・鉄剤投与 か ・抗線溶薬	・輸血 か 補充療法（か デスモプレッシン） か ・麻酔下の検査と子宮へのバルーン設置、タンポナーデ必要	緊急治療 か 手術介入（子宮摘出術、内腸骨動脈結紮、子宮動脈塞栓術か子宮ブレース縫合）必要
筋肉血腫	一度も無い	外傷後で無治療	自発性で無治療	自発性 か 外傷性で補充療法（か デスモプレッシン）必要	自発性 か 外傷性で手術介入 か 輸血必要
関節出血	一度も無い	外傷後で無治療	自発性で無治療	自発性 か 外傷性で補充療法（か デスモプレッシン）必要	自発性 か 外傷性で手術介入 か 輸血必要
中枢神経系出血	一度も無い	—	—	硬膜下出血で全ての介入	脳内出血で全ての介入
その他の出血＊3	無し か 軽微	有り	診察／検査のみ	手術による止血 か 抗線溶薬	輸血 か 補充療法（か デスモプレッシン）

*1 Vicenza groupの協力を得て翻訳、一部改訂した（赤字部分）

*2 （か デスモプレッシン）は後天性von Willebrand症候群用

*3 体腔内（胸腔内、腹腔内など）の出血を含めて評価し、自由記入欄に詳述する

合計点

自由記入欄:

作成者: 一瀬 白帝 2014/2/2

メールアンケート(診療方針について) 2014.01.17 実施

厚生労働科学研究費補助金 難治性疾患等克服研究事業（難治性疾患克服研究事業）
「診断困難な（原因不明の）出血性後天性凝固異常症の総合的診療指針の作成」研究班
症例の主治医　各位

前略ごめんください。
班研究につきましては、日頃より大変お世話になっております。

お忙しいところ恐れ入りますが、以下の３点について、
「症例の主治医としての御考え」をお知らせください。

１．止血治療について
（お知らせ頂く際は、活性の数字を記入したり、２つの薬品名、有・無のどちらかを消してください。）
(1) 出血する F13 活性レベル（　　）%、VWF 活性（　　）%、アルファ 2PI 活性（　　）%
(2) 止血する F13 活性レベル（　　）%、VWF 活性（　　）%、アルファ 2PI 活性（　　）%
(3) 抗線溶薬（トランサミンあるいはアプロチニン）が止血に効果があったか（ 有・無 ）
(4) 他の有効な止血治療があったか（ 有・無 ）（ある場合、具体的に；_____）
(5) その他（検査、診断、治療についての御考え、疑問等を具体的に；_____）

２．治療方法について
(1) 本疾患の止血治療における F13 濃縮製剤、あるいは VWF 含有 F8 製剤の投与の量と期間
（_____）出血の場合、（_____）単位／日
あるいは（_____）単位／kg／日　期間（_____）日
（_____）出血の場合、（_____）単位／日
あるいは（_____）単位／kg／日　期間（_____）日

(2) 本疾患の止血治療における抗線溶薬投与量と期間
（_____）出血の場合、（トランサミン；_____）mg／日
あるいは（_____）mg／kg／日　期間（_____）日
（_____）出血の場合、（その他(具体的に)_____；_____）mg／日
あるいは（_____）mg／kg／日　期間（_____）日

(3) 自己抗体に対する免疫抑制薬の投与量と期間
（プレドニンゾロン）の場合、（_____）mg／日
あるいは（_____）mg／kg／日　期間（_____）日
（エンドキサン）の場合、（_____）mg／日
あるいは（_____）mg／kg／日　期間（_____）日
（リツキサン）の場合、（_____）mg／m2／回
あるいは（_____）mg／m2／回（_____）回
その他（具体的に；_____）の場合、（_____）mg／日
あるいは（_____）mg／kg／日　期間（_____）日

３．班研究終了後の検査経費について
厚労科研費がなくなる平成２６年４月１日より（実際は３月から）本疾患診断のための検査は、例えば、
(1) 凝固第 XIII(13)因子活性測定（交叉混合試験）：定価 4500 円×３（本人、健常者、１：１混合血漿）
(2) アルファ 2PI 活性測定：定価 2500 円×２（本人の血漿と血清）
(3) 凝固第 XIII(13)因子抗原量測定：定価 4500 円×１（本人）
(4) VWF 活性測定：定価 2500 円×３（本人、健常者、１：１混合血漿）
(5) VWF 抗原量測定：定価 2800 円×１（本人）
の全額負担となるものと思われます。最終的な価格は SRL と貴院との直接交渉となり、班研究の割引価格は適用されません。
例えば、F13 インヒビター検出のスクリーニングには、項目(1)は不可欠ですが、経理処理が自己負担になる場合、検査の実施が可能か否か、費用を誰が負担するのか率直に御記入ください。
（ 実施する　・　実施しない；具体的なコメント_____）
（ 検査費用を誰が負担するのか；_____）

また、インヒビターの可能性がある場合、山形大学（抗 VWF 抗体は名古屋大学）で精査する必要がありますが、検体を冷凍で送付することが可能か否か、検査費用を誰が負担するのか率直に御記入ください（送料は発送者払い。配送費用は 1000～2000 円と予想される。）
（ 可能　・　不可能；具体的なコメント_____）
（ 検査費用を誰が負担するのか；_____）

平成２６年１月２２日（水）までお送り頂ければ幸いです。
それでは、何卒宜しくお願い致します。

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研究班代表　　一瀬　白帝　　拝