THROMBOSIS AND HEMOSTASIS

Impaired clot retraction in factor XIII A subunit-deficient mice

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FXIII-A IS REQUIRED FOR CLOT RETRACTION

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This study was presented in part at the 31st Meeting of the Japanese Society of Thrombosis and Hemostasis in Osaka, Japan, in November 2008.

The online version of the article contains a data supplement.
Factor XIII (FXIII) is a plasma transglutaminase which cross-links fibrin monomers, α2-plasmin inhibitor, etc. Congenital FXIII deficiency causes life-long bleeding symptoms. To understand the molecular pathology of FXIII deficiency \textit{in vivo}, its knockout mice have been functionally analyzed. Since prolonged bleeding times, a sign of defective/abnormal primary hemostasis, were commonly observed in two separate lines of FXIII A subunit (FXIII-A)-knockout mice, a possible role(s) of FXIII in platelet-related function was investigated in the present study. While platelet aggregation induced by ADP or collagen was normal, clot retraction (CR) was lost in the platelet-rich-plasma (PRP) of FXIII-A-knockout mice. In contrast, there was no CR impairment in the PRP of tissue transglutaminase-knockout mice when compared to that of wild-type mice. Furthermore, a transglutaminase inhibitor, cystamine, halted CR in the PRP of wild-type mice. These results indicate that the enzymatic activity of FXIII is necessary for CR, at least, in mice.
Introduction
Coagulation factor XIII (FXIII) is a pro-enzyme of plasma transglutaminase (TGase) consisting of two enzymatic A subunits (FXIII-A) and two non-catalytic B subunits, and plays a critical role in the generation of a stable hemostatic plug.1-3 FXIII catalyzes intermolecular cross-linking reactions between fibrin monomers, α2-plasmin inhibitor, fibronectin, etc. These reactions increase the mechanical strength of the fibrin clot and its resistance to proteolytic degradation, and enhance the assembly of the extra-cellular matrix.

Congenital FXIII deficiency is a rare autosomal recessive disorder, most of which cases are caused by defects in the FXIII-A gene.3 A life-long bleeding tendency, abnormal wound healing, and recurrent spontaneous miscarriage are common symptoms of FXIII deficiency.1,4

FXIII-A exists extra-cellularly in plasma as well as intra-cellularly as a cytosolic protein in megakaryocytes/platelets and monocytes/macrophages, although the function(s) of intra-cellular FXIII-A remains unknown.5,6 In particular, platelets cause clot retraction (CR) by retracting extended filopodia along fibrin strands.7 There have been conflicting reports about the effects of FXIII deficiency on CR; investigators reported that the absence of FXIII either abolished,7-9 did not affect,10,11 or rather enhanced12 CR in patients with congenital FXIII deficiency. However, platelet aggregation induced by various agents is uniformly normal in patients with congenital FXIII deficiency.8,9,13,14

To understand the precise molecular pathology of FXIII deficiency in vivo, FXIII-A-knockout (KO) mice have been analyzed. FXIII-A-KO mice demonstrated a severe bleeding tendency.15 We also reported that FXIII-A-KO mice developed severe uterine bleeding, resulting in spontaneous miscarriage in females, and male-specific intra-thoracic hemorrhage as well as excessive cardiac fibrosis.16,17

Since bleeding times in FXIII-A-KO mice were longer than in wild-type,15,18 we hypothesized that FXIII-A-KO mice might suffer from a defective platelet-related function(s). Accordingly, we have explored the contribution of FXIII to the process of CR in the present study.
Study design

Animal

Wild-type C57BL/6J mice were obtained from Japan SLC Inc. (Shizuoka, Japan). Gene-targeted mice of FXIII-A were generated as previously reported\textsuperscript{18} and tissue TGase (tTG)-KO mice were generously provided by Prof. Melino of Rome Tor Vergata University.\textsuperscript{19} These KO mice were maintained on a C57BL/6 genetic background for more than 10 generations. The animals were given free access to a standard food and water supply under specific pathogen-free conditions throughout the period of this study. Experimental procedures were approved by the Animal Care and Use Committee of Yamagata University and were carried out in accordance with the guidelines of this committee and Japanese governmental law.

Clot retraction (CR)

Blood was collected from the jugular vein of mice and anticoagulated with 3.8% sodium citrate in a ratio of 1 part anticoagulant to 9 parts blood, and platelet-rich-plasma (PRP) was obtained by centrifugation at 800g for 5 min. These samples were then activated with 1 NIH U/mL thrombin (Sigma, St. Louis, MO) and 5 mM CaCl\textsubscript{2} under gentle shaking, and the reaction mixtures were left unstirred at 37°C in siliconized test tubes. The extent of CR was assessed 10 and 60 minutes later by measuring the weight of each sample’s fluid not incorporated into the clot, and was expressed as a percentage by calculating the ratio between clot weight and that of the whole reaction mixture (weight ratio).

Since the quantity of blood collectable from mice is limited, the time-course of CR was monitored by taking photographic images of clots during time intervals, and measurement of clot areas was performed using ImageJ (Wayne Rasband, National Institute of Health, Bethesda, MD). The CR was expressed as a percentage by calculating the ratio between a specific clot area and that of the whole reaction mixture (area ratio) at indicated time intervals. The ratio at 0 min was set to 100%. Cystamine (Wako Pure Chemical Ltd., Osaka, Japan) was added to PRP immediately before thrombin. Results are presented as mean +/- SD of three independent experiments, and were analyzed using an unpaired Student’s t-test.
Results and discussion

Impaired CR in FXIII-A KO mice
A possible role(s) for FXIII-A in the process of CR was investigated using PRP obtained from FXIII-A-KO mice. Wild-type PRP showed significant retraction at 1 hour after thrombin treatment, while FXIII-deficient PRP showed no indication of retraction even after treatment of 1 hour at 37°C (Figure 1A). FXIII-A-KO mice exhibited a significantly reduced rate of CR (a weight ratio in Figure 1B and an area ratio in Figure 1C), suggesting that FXIII-A is essential for the CR reaction.

Platelet aggregation induced by ADP or collagen was normal in our FXIII-A-KO mice (data not shown), as expected.8,9,13,14,15

A lack of FXIII-A but not tTG abolished the CR
Since platelets contain both FXIII-A and tTG,20 both of which are also present on the surface of activated platelets,21 we next examined whether the absence of tTG affected CR using tTG-KO mice.19 CR, however, was normal in tTG-deficient PRP, as shown by the weight ratio in Figure 1B. Clots of wild-type and tTG-deficient PRPs were equally retracted by 1 hour at 37°C (average percentage of clot weight: 8.2% and 12.7%, respectively). In addition, Figure 1C (the area ratio) clearly shows the time-dependent nature of CR in the wild-type and tTG-KO mice. CR was completely halted in FXIII-A-KO mice.

Enzymatic activity of FXIII-A required for CR
We also investigated the requirement of enzymatic activity of FXIII-A for CR. As shown in Fig. 2, a potent inhibitor of tTG and FXIII-A,22 cystamine, completely inhibited CR in wild-type PRP. CR of wild-type PRP was also totally halted by the treatment with batroxobin, which converts fibrinogen to fibrin without activating FXIII, and by adding EDTA in place of Ca2+ ions essential for TGase activity (Supplemental Figures 1A,B). These findings indicate that the TGase activity of FXIII-A is a prerequisite to CR.

The indispensable role of the extra-cellular/plasma FXIII-A in the development of Ca2+-dependent CR was previously demonstrated in humans:7 i.e., when normal washed platelets were re-suspended in FXIII-deficient plasma, the tension developed in the thrombin-generated clot was totally diminished as compared to the control containing washed platelets re-suspended in normal plasma. When purified FXIII was added to the FXIII deficient plasma, the tension was restored to normal.7 However,
replacement of extrinsic FXIIIs only partially restored impaired CR in the PRP of FXIII-A knockout mice in our system (Supplemental Figure 2), suggesting that both extra-cellular/plasma and intra-cellular/platelet FXIIIs are required for CR, at least in mice.

FXIII has been reported to cross-link fibrin, glycoprotein IIb/IIIa, actin and myosin, which are involved in CR. Both actin and myosin are located in the platelet cytosol and are cross-linked by Ca$^{2+}$-dependent TGase activity. The platelet FXIII-A can be activated by calpain, an endogenous intra-cellular protease. Thus, it is possible that intra-cellular/platelet FXIII-A may function for CR via the cross-linking of cytosolic actin and myosin. Recently, myosin IIA has been reported to mediate fibrin-independent platelet contraction. Moreover, extra-cellular/plasma fibrin cross-linked by FXIII-A must be indispensable for CR, because afibrinogenemia, i.e., fibrinogen deficiency in plasma, also causes impaired CR.

The in vivo significance of CR for primary hemostasis is less understood: impaired CR may be, to some extent, responsible for the bleeding symptoms in patients with congenital FXIII deficiency. The precise molecular mechanism of FXIII-mediated CR remains to be explored in the future.

Acknowledgements
This work was supported in part by a Global Center of Excellence project to Yamagata University, and Grant-in-Aid for Scientific Research. The authors are greatly indebted to Prof. G. Melino of Rome Tor Vergata University for providing tTG-KO mice, and Ms. L. Boba for assisting in preparation of the manuscript.

Contribution:
K.K. performed research, analyzed data, and wrote the paper. M.S., M.K., and T.M., performed research. N.Y. performed research and analyzed data. A.I. designed the study, analyzed data, and wrote the paper.
Conflict-of-interest disclosure: No authors have any conflict.
References


Figure Legends

Figure 1. Impaired CR in FXIII-A-deficient mice but normal CR in tTG-KO mice. (A) Photograph of CR in wild-type mice (left), tTG-KO mice (middle), FXIII-A-KO mice (right). PRP was incubated with 1 U/mL thrombin, 5 mM CaCl$_2$ at 37°C. The photograph was taken after 1 hour. (B) Quantitative analysis of CR by a weight ratio method. The extent of CR was assessed at 10 and 60 minutes by measuring the clot weight, for wild-type (open), tTG-KO (shaded), or FXIII-A-KO (filled) mice. Data are presented as the mean +/- SD of triplicates. (C) Time-dependent CR measured by an area ratio method. The extent of CR was assessed at the indicated times by measuring the clot size, for wild-type (circle), tTG-KO (square), or FXIII-A-KO (triangle) mice. Data are presented as the mean +/- SD of triplicates. Statistically significant differences (\(P < .001\) shown by *) were observed at 10 and 60 min in panel B and at 15, 30, 45, 60, 75, and 1440 min in panel C between FXIII-A-deficient versus wild-type mice.

Figure 2. Impaired CR by a potent TGase inhibitor cystamine. PRP of wild-type mice was incubated with 1 U/mL thrombin, 5 mM CaCl$_2$ at 37°C for 1 hour in the absence (left) or the presence of 1 mM (middle), or 10 mM (right) of cystamine. The extent of CR was assessed at 60 min by measuring the clot weight. Data are presented as the mean +/- SD of triplicates. A statistically significant difference (\(P < .001\) shown by *) was observed between the absence vs. the presence of cystamine.
Figure 1

A

Wild-type  tTG-KO  FXIII-A-KO

B

Clot weight (%)

Time (min)

C

Clot area (%)

Time (min)
Figure 2
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