Fibrinogen controls human platelet fibronectin internalization and cell-surface retention¹

Z. ZHAI,*† J. WU,*† X. XU,* K. DING,*† R. NI,* W. HU,‡ Z. SUN*† and H. NI§

*The Center Laboratory; †Department of Hematology of Anhui Provincial Hospital, the Second Aliated Hospital of Anhui Medical University, Hefei, Anhui, China; ‡Department of Pathology, Anhui Provincial Hospital, Hefei, Anhui, China; and §Department of Laboratory Medicine and Pathobiology, Canadian Blood Services and Keenan Research Centre, Li Ka Shing Knowledge Institute, St. Michael's Hospital, University of Toronto, Toronto, Canada

To cite this article: Zhai Z, Wu J, Xu X, Ding K, Ni R, Hu W, Sun Z, Ni H. Fibrinogen controls human platelet fibronectin internalization and cell-surface retention. *J Thromb Haemost* 2007; 5: 1740–6.

Summary. *Background:* We recently demonstrated that platelet aggregation occurred in fibrinogen-deficient mice. In these animals, platelet fibronectin (Fn) content was increased 3–5 fold, suggesting that Fn may also be involved in platelet aggregation. *Methods and results:* We compared platelet Fn content from a severe hypofibrinogenemic patient (with approximately 0.5% of normal fibrinogen levels) with his parents (heterozygous) and healthy donors. A significant increase in the patient's platelet Fn content was detected by immunoblot, flow cytometry, and immunoelectron microscopy (IEM). To examine the possible contribution of platelet Fn to platelet aggregation, we examined cell-surface Fn expression after thrombin treatment. Unexpectedly, IEM detected only trace amounts of Fn retained on the patient's platelet surface, and flow cytometry indicated that surface Fn was approximately 6-fold lower than that of his parents and tenfold lower than that of healthy donors. An ELISA further confirmed that the patient's platelet Fn was primarily released into the extracellular medium. To test whether retention of surface Fn was due to fibrin formation on the platelet surface, an antifibrin antibody (T2 G1) was employed. Fibrin was detected on platelets from healthy donors and from the father, but was negligible on the patient's platelets. Consistent with these data, when gel-filtered platelets of healthy donors were treated with

thrombin receptor activation peptide (SFLLRN-NH₂; no conversion of fibrinogen to fibrin), little surface Fn was detected. *Conclusion:* Fibrinogen not only competitively inhibits human platelet Fn internalization but also controls platelet-surface Fn retention via fibrin formation. The Fn–fibrin interaction is one possible mechanism to promote Fn interaction with platelets.

Keywords: factor XIII, fibrin, fibrinogen, fibronectin, hemostasis, hypofibrinogenemic patient, platelet aggregation, thrombosis.

Introduction

Platelet adhesion and subsequent aggregation at the site of vascular injury is one of two important mechanisms required to stop bleeding; the other is the coagulation system, which generates thrombin, converts fibrinogen to fibrin, and subsequently forms the polymerized fibrin matrix. Thrombin generated from the coagulation system is also the most potent platelet agonist, which induces platelet activation and granule release. Although activation of platelets and the coagulation system is essential for the arrest of bleeding, the same hemostatic processes also contribute to the generation of inopportune thrombi within atherosclerotic arteries, which is the leading cause of morbidity and mortality worldwide [1].

It has been demonstrated that von Willebrand factor (VWF) and fibrinogen are two key molecules that mediate platelet adhesion and aggregation [2]. However, we recently demonstrated that thrombus formation and platelet aggregation still occurred in mice lacking both VWF and fibrinogen [3,4]. In fibrinogen-deficient and/or fibrinogen and VWF double-deficient mice, platelet fibronectin (Fn) was increased 3–5 fold [3]. This was due to enhanced internalization of plasma Fn by the platelet $\alpha_{IIb}\beta_3$ integrin (GPIIb/IIIa) after depletion of fibrinogen (a competitive ligand for the same $\alpha_{IIb}\beta_3$ integrin) [5]. We subsequently demonstrated that plasma Fn promotes thrombus growth and stability in injured arterioles [6]. These results are consistent with recent observations that Fn self-assembly may be involved in thrombosis and hemostasis [7–9].

Correspondence: Zhimin Zhai, The Center Laboratory of Anhui Provincial Hospital, Anhui Medical University, Hefei 230001, China. Tel.: 86 551 228 3574; fax: 86 551 228 3986; e-mail: zhi_min@mail.hf.ah.cn

Correspondence: Heyu Ni, Canadian Blood Services and St. Michael's Hospital, Department of Laboratory Medicine and Pathobiology, University of Toronto, 30 Bond St., Room 2-006, Bond Wing, Toronto, M5B 1W8, Canada. Tel.: +1 416 864 6060 ext. 6758; fax: +1 416 864 3060; e-mail: nih@smh.toronto.on.ca

¹Parts of this manuscript were orally presented at the 2003 ISTH meeting.

Received 10 January 2007, accepted 11 May 2007

However, it is unclear whether the supportive role of plasma Fn in thrombus formation was due to its contribution to platelet aggregation. There is also no report regarding fibrinogen control of human plasma Fn internalization.

In the present study, we examined platelet Fn content in a human patient with severe hypofibrinogenemia [10] and found that the patient's platelet Fn content was significantly increased. Unexpectedly, this excess Fn was not efficiently retained on the platelet surface after thrombin treatment. We demonstrated that fibrin converted from platelet-released fibrinogen is important for cell-surface Fn retention. The role of platelet-released Fn in thrombosis and hemostasis is discussed.

Materials and methods

Human subjects

The patient with severe hypofibrinogenemia (homozygous) was a 30-year-old male who was born from parents (heterozygous) of a consanguineous marriage. The patient had a plasma fibrinogen level of 1.7 mg dL^{-1} (approximately 200 times lower than normal) and a platelet aggregation ratio of 10–20% (about 4–6 times lower than the normal level). His father and mother (53 years old) had fibrinogen levels that were approximately 50% of normal. Their clinical manifestation and genetic diagnosis had been reported previously [10]. Three healthy blood donors (two males and one female, aged 30, 40 and 45 years, respectively) were used as controls. Informed consent was obtained for each subject in accordance with the Declaration of Helsinki. This study was approved by the institutional review board.

Platelet preparation and aggregation

Blood samples from individual subjects were collected into a tube containing 3.8% sodium citrate (1/9 v/v). Platelet-rich plasma (PRP) was obtained by centrifugation at $300 \times g$ for 7 min. Platelets were then isolated from the PRP by gel filtration on a Sepharose 2B column (Sigma, St Louis, MO, USA) in PIPES buffer (PIPES 5 mM, NaCl 1.37 mM, KCl 4 mM, glucose 0.1%, pH 7.0) as previously described [3,4,10,11].

Platelet aggregation assays in the patient's PRP before and after fibrinogen transfusion (0.06 g kg^{-1}) were performed as we previously described [10]. Briefly, 250 μL PRP was prewarmed at 37°C for 10 min. Platelet aggregation was then induced with 10 μM adenosine diphosphate (ADP; Sigma, St Louis, MO, USA) and recorded on a computerized aggregometer (Chrono-Log Corp., Havertown, PA, USA).

Platelet activation and permeabilization

Gel-filtered platelets were activated with 1.5U mL^{-1} thrombin (Diagnostica Stago, Asnières, France) or 100 μM thrombin receptor activation peptide (TRAP) (H-Ser-Phe-Leu-Leu-Arg-Asn- NH_2 ; Peptides International Inc., Louisville, KY, USA) in the presence of 1 mM Ca^{2+} for 3 min.

Subsequently, the platelets were fixed with 1% paraformaldehyde before examination. To detect intracellular antigens, gel-filtered platelets were fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) buffer at room temperature for 20 min, and permeabilized in 10% Triton-X 100 in PBS buffer for 15 min.

Immunofluorescence staining and flow cytometry

For the labeling of β_3 integrin, gel-filtered platelets were incubated at room temperature for 20 min with PE-anti-CD41 antibody (Clone P2, which reacts with the α_{IIb} subunit in the intact $\alpha_{\text{IIb}}\beta_3$ complex; Coulter-Immunotech, Marseille, France). For the labeling of platelet intracellular or activated platelet-surface antigens, $10^6 \times$ either permeabilized or paraformaldehyde-fixed activated platelets were incubated at 4°C overnight with 1:2000 mouse antihuman fibrinogen (Diagnostica Stago), thrombospondin-1 (TSP-1; Clone TSP-B7, Sigma), antifibrin (Clone T2 G1, Accurate Chemical & Scientific Corp., Westbury, NY, USA), rabbit antihuman Fn (Sigma), or vitronectin (Chemicon, Temecula, CA, USA) in 50 μL PIPES buffer. After washing twice with PIPES buffer, platelets were incubated with 1:2000 fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin G (IgG; Coulter-Immunotech) or mouse antirabbit IgG (Huamei Biotech, Beijing, China) for 1 h at room temperature before being analyzed by flow cytometry on a FACScan flow cytometer (Coulter EPICS XL-MCL).

Immunogold labeling and immunoelectron microscope

The permeabilized and the activated gel-filtered platelets were incubated with individual antibodies against Fn, fibrinogen, or fibrin (as above), followed by incubation with gold-conjugated goat antimouse IgG or gold-conjugated goat antirabbit IgG (Kehao Bioscience, Beijing, China) for 1 h. The immunogold-labeled platelets were centrifuged at $500 \times g$ for 15 min. The platelet pellets were fixed with 5% glutaraldehyde, embedded onto paraffin, and sliced using a super-microtome (LKD, Bremma, Sweden). Platelet samples were then studied with a JEM 1230 perspective electron microscope (JEOL Ltd, Tokyo, Japan).

Western blot

Gel-filtered platelets were centrifuged at $2000 \times g$ for 10 min at room temperature, and lysed in lysis buffer [0.2% sodium dodecyl sulfate (SDS), 10% glycerol, 100 mM Tris-HCl, 4 mM ethylenediamine tetraacetic acid, 4 mM phenylmethylsulfonyl fluoride, 100 mM dithiothreitol, and 0.002% bromophenol blue]. The samples were boiled for 10 min and subjected to 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to immobilon-P polyvinylidene fluoride (PVDF) membrane (Hybond-P, Amersham Pharmacia Biotech, Baie d'Urfe, Canada). After blocking with 20 mM Tris-Cl, pH 7.6, containing 137 mM NaCl, 0.1% Tween-20

and 5% non-fat milk, the membrane was incubated with a 1:2000 dilution of antifibrinogen, Fn, and TSP-1 antibodies (above) at room temperature for 2 h. A mouse antihuman vinculin antibody (Clone hVIN-1, Sigma) was used as an internal control for equal protein loading. The membrane was then incubated with alkaline-phosphatase-conjugated anti-mouse or antirabbit polyvalent immunoglobulins. Immuno-reactive bands were visualized by reaction with substrate (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, Sigma).

To detect factor (F) XIII levels, platelet and plasma samples were lysed in SDS sample loading buffer (as above), boiled for 10 min, and subjected to 8% SDS-PAGE. After transfer to PVDF membrane, FXIII was detected with a 1:500 dilution of goat antihuman FXIII α -chain (sc-18012, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), which detects both active and inactive FXIII.

FXIII activity assay

FXIII activity was determined using a Semi-Quantitative Activity Assay kit (Diagnostics Stago) according to the manufacturer's instructions. Briefly, patient or control plasma was serially diluted in 4 mg mL⁻¹ of human FXIII-free fibrinogen at 1:2, 1:4, 1:8, 1:16, 1:32, 1:50, 1:64, 1:75, 1:128, and 1:256. The diluted samples (100 μ L) were then incubated with suspended kaolin (100 μ L, 10 mg mL⁻¹) containing 10 U of thrombin at 37 °C for 30 min followed by incubation with 5% chloroacetic acid (1 mL) at 37 °C for 5 min. The highest dilution at which the clot remained insoluble was recorded. FXIII activity was determined using the following calculation: FXIII activity (%) = 100 $\times$ (maximum effective dilution factor of patient plasma/maximum effective dilution factor of control plasma).

ELISA

Platelet-released Fn in the extracellular medium was measured by an ELISA kit according to the manufacturer's instructions (Sun Biotech Corp., Shanghai, China). Briefly, 2×10^7 mL⁻¹ gel-filtered resting or thrombin-activated platelets (1.5 IU mL⁻¹ thrombin for 20 min) were centrifuged at $2000 \times g$ for 10 min. A total of 100 μ L supernatant samples were incubated with monoclonal anti-Fn antibody precoated wells at 37 °C for 90 min. After washing, alkaline-phosphatase-conjugated antihuman Fn antibody and the substrate were successively added. The standard curve was generated at the same time, and the Fn content of the different samples was acquired from the standard curve according to the absorbance measurements.

Analysis of data

Data are presented as mean $\pm$ SD. Statistical significance was assessed by unpaired Student's *t*-test with statistical software (spss version 11.0, SPSS Inc, Chicago, IL, USA).

Results

Platelet Fn content was significantly increased in hypofibrinogenemic platelets

We examined the platelet Fn content of the homozygous patient, his heterozygous parents, and healthy donors using flow cytometry, Western blot, and immunoelectron microscopy (IEM). A significant increase in the patient's platelet Fn content was detected by all three methods (Fig. 1A,B), in which the mean fluorescence intensity (MFI) of the patient's platelet Fn by flow cytometry was 3-fold higher than that of the healthy donors (Fig. 1A). A much higher level of Fn (immunogold particles) in platelet α -granules was also observed by IEM (data not shown). We did not detect an obvious increase in platelet Fn content in the heterozygous parents (data not shown). There was also no significant alteration of platelet TSP-1 (Fig. 1B) or vitronectin (data not shown). Because β_3 integrin is the receptor that mediates murine plasma Fn internalization [5], we examined whether $\alpha_{IIb}\beta_3$ integrin (CD41) expression was upregulated on the surface of the hypofibrinogenemic patient's platelets. No significant difference in expression of surface $\alpha_{IIb}\beta_3$ integrin was found among any of the subjects (Fig. 1C: the MFI values of homozygous, heterozygous, and healthy donors were 24.3 ± 6.6 , 24.5 ± 8.1 , and 26.9 ± 8.4 , respectively, $P > 0.7$). These data are consistent with our earlier reports in fibrinogen-deficient mice [3].

Increased Fn secretion but not cell-surface retention in thrombin-activated hypofibrinogenemic platelets

Platelet-surface Fn expression after thrombin stimulation was examined by flow cytometry and IEM. Surprisingly, while platelet Fn content was increased 3-fold in the homozygous patient, cell-surface Fn was not increased. The MFI of platelet-surface Fn was approximately 6-fold lower than that of the father and tenfold lower than that of healthy donors (Fig. 2). Similar results were also found with electron microscopy (data not shown). Using ELISA, we observed that the patient's platelet Fn was primarily released into the extracellular medium after thrombin treatment (Table 1). The platelet supernatant contained 2–3-fold higher levels of Fn compared with healthy donors (472.8 ± 13 ng mL⁻¹ vs. 213.5 ± 4.7 ng mL⁻¹, $P < 0.0005$).

Decreased Fn retention is due to fibrinogen deficiency but not FXIII or platelet abnormalities

In order to exclude other possible platelet abnormalities that may cause decreased Fn retention, we examined the patient's platelet aggregation before and after fibrinogen transfusion (0.06 g kg⁻¹). Twenty-four hours post-transfusion, plasma fibrinogen increased to 110 mg dL⁻¹ (approximately 40–60% of the normal level) and platelet aggregation recovered (Fig. 3A). Interestingly, fibrinogen and Fn expression on

Fig. 1. Significant increase of platelet fibronectin (Fn) content in hypofibrinogenemic platelets. (A) Platelet Fn content detected by flow cytometry. Platelets were first fixed with paraformaldehyde and permeabilized with 10% Triton-X 100. Intracellular Fn was then detected by anti-Fn antibody. The hypofibrinogenemic patient (homozygous) had 3-fold higher platelet Fn when compared with the healthy control (left line). The mean fluorescence intensity (MFI) values are 2.6 ± 0.59 and 0.7 ± 0.23 ($P < 0.05$). (B) Platelet Fn content detected by Western blot. Proteins from 2×10^7 gel-filtered platelets of individual subjects were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and analyzed by immunoblot with specific antibodies. Platelet vinculin and thrombospondin-1 levels were similar in all samples. The homozygous patient's platelets contained a significantly higher level of Fn, while the fibrinogen (Fg) level was nearly absent. In contrast, the platelet Fn and Fg contents from the parents and healthy donor were similar. (C) Surface expression of CD41 ($\alpha_{IIb}\beta_3$ integrin complex) analyzed by flow cytometry. There were no significant differences between the homozygous patient, the heterozygous father and the healthy control. The MFI values of homozygous, heterozygous and healthy donors were 24.3 ± 6.6 , 24.5 ± 8.1 and 26.9 ± 8.4 , respectively ($P > 0.7$).

Fig. 2. Decreased cell-surface fibronectin (Fn) retention in thrombin-activated hypofibrinogenemic platelets. Platelet-surface fibrinogen (Fg), fibrin and Fn expression on thrombin-stimulated platelets was measured by flow cytometry. The mean fluorescence intensity of detected Fn on the homozygous patient platelets (left curves) was approximately 6-fold lower than that of the heterozygous parent (middle curves; $P < 0.05$) and tenfold lower than healthy donors (right curves; $P < 0.05$). Fg and fibrin levels of the heterozygous parent were lower than those of the healthy control, but much higher than those of the homozygous patient ($P < 0.05$).

Table 1 Platelet fibronectin (Fn) from the hypofibrinogenemic patient is primarily released into the extracellular supernatant after thrombin treatment. Platelets (2×10^7 mL $^{-1}$) were stimulated with 1.5 IU mL $^{-1}$ of thrombin for 20 min and the level of platelet-released Fn in the supernatant was measured using ELISA. The level of Fn released into the supernatant from the hypofibrinogenemic patient was much higher than that of either the healthy control or the heterozygous father ($P < 0.0005$)

Subject	Fn (ng mL $^{-1}$) mean $\pm$ SD
Healthy donor	213.5 ± 4.7
Heterozygous	216.1 ± 8.4
Homozygous	472.8 ± 13.0

gel-filtered platelets after thrombin stimulation was also significantly increased (Fig. 3B).

Because FXIII may play a role in the formation of covalent bonds between macromolecules (e.g. Fn and fibrin) that may contribute to Fn retention, we further assayed platelet and plasma FXIII via Western blot. No significant difference was observed (Fig. 3C) and there was no significant alteration of FXIII function in the patient's plasma. The highest dilution of the patient's plasma at which the clot remained insoluble was similar to the normal control (1:75 vs. 1:64). Thus, the decreased Fn retention on the patient's platelets was very probably due to fibrinogen deficiency.

Fibrin is important for Fn platelet-surface retention

Because fibrin has been reported to act as a bridging molecule for Fn retention on the platelet surface [12,13], we tested the hypothesis that the high retention of platelet-surface Fn in healthy donors was due to fibrin formation on the platelet surface after thrombin treatment. Gel-filtered platelets from the patient, the patient's parents, and healthy donors were treated with thrombin, and fibrin was detected by a fibrin-specific monoclonal antibody (Clone T2 G1), which does not recognize fibrinogen. As shown in Fig. 2 (middle panel), flow cytometry revealed high fibrin levels on the surface of platelets from healthy donors, moderate

Fig. 3. Decreased fibronectin (Fn) retention is not due to factor (F) XIII or platelet abnormalities. (A) Platelet aggregation in the patient's platelet-rich plasma was induced by adenosine diphosphate. A platelet aggregation ratio of 10–20% was observed before fibrinogen (Fg) transfusion (left panel), which was significantly increased after transfusion with 3 g Fg (0.06 g kg^{-1}) (right panel). The platelet aggregation ratio of 70% post-transfusion is close to the normal level of 80–95% in our aggregometry assay. (B) Fg (left panel) and Fn (right panel) on thrombin-treated gel-filtered platelets were detected by flow cytometry. Both Fg and Fn on the patient's platelets were significantly increased after Fg transfusion (right curves indicated by arrows) as compared with untreated samples (left curves). (C) FXIII in platelets (left panel) and plasma (right panel) were detected by Western blot. No significant differences were found between the patient and the other subjects tested.

expression on platelets from the heterozygous parents, and negligible fibrin formation on the patient's platelets (MFIs were 10.9 ± 2.5 , 4.6 ± 0.9 , and 0.84 ± 0.12 , respectively, $P < 0.05$). Similar results were observed with IEM after immunogold staining (data not shown).

In order to further confirm the role of fibrin in Fn cell-surface retention, we directly stimulated the thrombin receptor protease activated receptor 1 with TRAP on healthy donor platelets. This peptide is able to induce platelet granule release, including release of fibrinogen and Fn, but is unable to convert fibrinogen to fibrin. As expected, TRAP stimulation resulted in cell-surface fibrinogen expression on healthy donor platelets

Fig. 4. Fibronectin (Fn) was not significantly expressed on the surface of healthy platelets after stimulation with thrombin receptor activation peptide (TRAP). (A) Fibrinogen (Fg) was detected (right curve) by flow cytometry on the platelet surface of gel-filtered healthy control platelets after stimulation with TRAP ($100 \mu\text{M}$). An irrelevant antibody isotype control is also shown (left curve). Fibrin was not detected on the platelet surface following TRAP stimulation and was indecipherable from the irrelevant antibody isotype control. Consistent with fibrin levels, only nominal Fn was detected on the platelet surface (the curve shifted slightly to the right of isotype controls, indicated by the arrow). (B,C) Consistent with flow cytometry, immunoelectron microscopy analysis revealed significant levels of Fn on the surface of healthy control platelets only after thrombin treatment (panel B, as indicated by arrows), while TRAP stimulation failed to generate a significant amount of platelet-surface retained Fn (panel C). Micrographs from (B) and (C) were taken at 15 000 $\times$ magnification (left panels) and 30 000 $\times$ magnification (right panels).

(Fig. 4A, left panel) similar to that on thrombin-treated platelets (Fig. 2, left panel). However, nominal fibrin was detected (Fig. 4A, middle panel), which was coincident with very low platelet-surface levels of Fn (i.e. significant Fn was not detected on the TRAP-treated healthy platelet membranes). The MFI value of Fn expression on thrombin-treated healthy donor platelets (Fig. 2, right panel; $\text{MFI} = 10.9 \pm 2.5$) was significantly higher than that of TRAP-treated platelets (Fig. 4A, right panel; $\text{MFI} = 0.93 \pm 0.23$, $P < 0.05$).

Using electron microscopy, we observed that thrombin stimulation resulted in significant fibrin retention on the platelet surface and was concentrated in the vicinity of platelets from the healthy donors (Fig. 4B) and the parents (data not shown). These fibrin molecules were positively stained by gold-conjugated anti-Fn antibodies (Fig. 4B). In contrast, TRAP treatment resulted in very poor retention of both fibrin and Fn on the platelet surface of healthy donors (Fig. 4C). These data clearly demonstrated that thrombin-converted fibrin plays an important role in cell-surface Fn retention.

Discussion

Fibrinogen is considered to be an essential bridging molecule required for platelet aggregation [14–17]. However, we found that platelet-rich thrombi formed in both fibrinogen-deficient and fibrinogen/VWF double-deficient mice [3] and determined that fibrinogen/VWF-independent platelet aggregation can be induced *in vitro* under more physiological conditions (i.e. non-anticoagulated blood) [4]. Under these conditions, Fn is thought to be the most likely candidate molecule that may mediate platelet aggregation, because platelet Fn content is increased 3–5-fold in these mice. In this study, we found that the human platelet Fn content of a hypofibrinogenemic patient was also increased. Surprisingly, platelet-surface Fn expression in these hypofibrinogenemic platelets was not enhanced, but rather dramatically decreased following thrombin stimulation. We further demonstrated that fibrin formation on the platelet surface plays an important role in the surface retention of Fn.

The role of Fn in thrombosis and hemostasis was recently highlighted [6–9,18,19]. Fn is a large glycoprotein with a molecular weight of approximately 230–250 kDa. It was first discovered as a contaminant of fibrinogen preparations more than half a century ago. Subsequent studies demonstrated that it was a substrate of activated blood coagulation FXIII, a transglutaminase that covalently crosslinks Fn to fibrin. Fn usually forms as a dimer in which each polypeptide chain is linked by a pair of disulfide bonds. The amino acid sequence Arg-Gly-Asp (RGD), the consensus integrin binding motif [20], exists in the middle portion of each polypeptide chain. This could allow Fn to possibly bridge adjacent platelets, although the distance between the two RGD sites may be too short to efficiently facilitate this process. Recent studies demonstrated that Fn self-assembly occurred on the activated platelet surface [7,8,18,19]. In addition, Fn contains protein disulfide isomerase activity [21], which may allow for non-self assembly with other thrombotic/hemostatic proteins [22] via disulfide bond exchange with proteins either on the cell surface or in the extracellular matrix. Thus, at least four possible mechanisms suggest that Fn may support thrombosis and hemostasis. These include initiating platelet adhesion via binding to Fn on the injured vessel wall, mediating platelet aggregation, contributing to the fibrin clot, and enhancing thrombus growth via Fn assembly on the platelet surface [7,8,18,19,23].

However, there are also significant data indicating that Fn may play an inhibitory role in platelet aggregation [24]. The inhibitory effect of Fn on platelet aggregation is probably via competition with either fibrinogen or other potent integrin ligands from the plasma or ligands released from activated platelets. In addition, plasma Fn-deficient mice failed to demonstrate any overt bleeding disorder [25]. Although it has long been suspected that Fn plays a supportive role in thrombosis and hemostasis, its role in platelet aggregation remains controversial.

Our earlier studies demonstrated that platelet Fn was increased in fibrinogen-deficient mice [3] and in fibrinogen

$\gamma\Delta 5$ mice, which lack the C-terminal 5 amino acids of the fibrinogen γ -chain, which contains the binding site for $\alpha_{IIb}\beta_3$ integrin [5]. After breeding fibrinogen $\gamma\Delta 5$ mice with β_3 integrin-deficient mice, we observed that platelet Fn was decreased. We concluded that $\alpha_{IIb}\beta_3$ integrin, but not $\alpha_5\beta_1$ integrin (the Fn receptor), was the key receptor mediating plasma Fn internalization, and fibrinogen was a competitive ligand for this process [5]. Thus, it is not surprising that platelet Fn was also increased in our hypofibrinogenemic patient (Fig. 1A,B). When this patient received exogenous fibrinogen transfusion, his platelet Fn content was reduced (data not shown).

It was not expected, however, that the patient's excess platelet Fn was unable to be retained on the platelet surface. Platelets have multiple receptors for Fn, which include $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, and $\alpha_5\beta_1$ integrins. $\alpha_{IIb}\beta_3$ is the most abundant platelet membrane protein, with $5\text{--}12 \times 10^4$ copies/platelet [24]. The patient and his parents had normal levels of $\alpha_{IIb}\beta_3$ integrins on their platelet surfaces (Fig. 1C), and these integrins were able to efficiently support platelet aggregation when fibrinogen was increased (Fig. 3A) or normalized [10]. It seems that the affinity between Fn and the integrin receptors is insufficient to maintain Fn surface retention at relatively low *in vitro* Fn concentrations (such as those used in the present study, even with supplemented divalent cations). The high 'off' ratio between Fn and integrins leads to a significant amount of Fn released into the extracellular medium after thrombin treatment (Table 1). Our results do not exclude the possibility that, at high Fn concentrations, such as in normal plasma or a medium containing a large amount of exogenous Fn, platelet integrins may be able to retain a certain level of Fn on the platelet surface [12,13]. However, whether these transient Fn–integrin interactions play a significant role in platelet aggregation is currently unclear. It seems that other integrin ligands such as fibrinogen (Fig. 4A) and fibrin (Figs 2 and 4) can be retained on the cell surface and may have more potential to engage integrins on adjacent activated platelets and lead to platelet aggregation.

We found that significant fibrin formed on the surface of platelets from healthy donors and the heterozygous parents but not on the surface of platelets from the hypofibrinogenemic patient (Fig. 2). To the best of our knowledge, this is the first report that thrombin can directly induce granule release and convert platelet-released fibrinogen to fibrin on the platelet surface. It is interesting that this newly formed fibrin (Figs 2 and 4), but not fibrinogen (Fig. 4A), plays an important role in Fn retention on the platelet surface. We also found a significant increase in Fn retention on thrombin-activated patient platelets after fibrinogen transfusion (Fig. 3B). It is very likely that the platelet-released transglutaminase, FXIII, may participate in fibrin–Fn complex formation on thrombin-treated platelets. This fibrin-linked Fn may play a significant role in thrombosis and hemostasis in the normal population, but may have little to no effect in afibrinogenemic/hypofibrinogenemic patients.

The most interesting and difficult question arising from the present study may be whether enhancement of platelet Fn

content is a compensatory mechanism for hemostasis in afibrinogenemic/hypofibrinogenemic patients. As discussed above, Fn may be able to form large integrin–ligand complexes by either self- or non-self-assembly. At the site of vascular injury, large amounts of plasma Fn and thrombin are available. Triggered by platelet–vessel interactions and platelet agonists such as thrombin, platelets will release their granule proteins. The newly released Fn may dramatically enhance the local Fn concentration, which not only may extend the Fn retention time on the platelet surface but also may facilitate the Fn–Fn interaction or Fn interaction with other extracellular matrix proteins (i.e. Fn assembly). This Fn-complex formation may overcome the limitation of the short distance between the two RGD sites in the Fn dimer and could therefore play a significant role in platelet aggregation.

In summary, we found that human platelet Fn content was increased in a hypofibrinogenemic patient, which is consistent with our earlier reports in fibrinogen-deficient mice. We demonstrated that thrombin was able to convert platelet-released fibrinogen to fibrin, which played a crucial role in Fn retention on the platelet surface. Whether the enhancement of platelet Fn internalization is a compensatory mechanism for hemostasis and how this is accomplished in afibrinogenemic/hypofibrinogenemic patients should be of interest in future studies.

Acknowledgements

This work was supported in part by the International Cooperation Research Fund of Anhui Provincial Scientific and Technologic Committee #05088016; an Anhui Provincial Nature Science Research Grant #01043301; the Canadian Blood Services start-up fund; and the Heart and Stroke Foundation of Canada (Ontario). The authors thank C. M. Spring for assistance with preparation of the manuscript, and H. Yang for her valuable comments and technical support.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

- Ruggeri ZM. Platelets in atherothrombosis. *Nat Med* 2002; **8**: 1227–34.
- Ruggeri ZM. Mechanisms initiating platelet thrombus formation. *Thromb Haemost* 1997; **78**: 611–6.
- Ni H, Denis CV, Subbarao S, Degen JL, Sato TN, Hynes RO, Wagner DD. Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. *J Clin Invest* 2000; **106**: 385–92.
- Yang H, Reheman A, Chen P, Zhu G, Hynes RO, Freedman J, Wagner DD, Ni H. Fibrinogen and von Willebrand factor-independent platelet aggregation in vitro and in vivo. *J Thromb Haemost* 2006; **4**: 2230–7.
- Ni H, Papalia JM, Degen JL, Wagner DD. Control of thrombus embolization and fibronectin internalization by integrin alpha IIb beta 3 engagement of the fibrinogen gamma chain. *Blood* 2003; **102**: 3609–14.
- Ni H, Yuen PS, Papalia JM, Trevithick JE, Sakai T, Fassler R, Hynes RO, Wagner DD. Plasma fibronectin promotes thrombus growth and stability in injured arterioles. *Proc Natl Acad Sci USA* 2003; **100**: 2415–9.
- Cho J, Mosher DF. Enhancement of thrombogenesis by plasma fibronectin cross-linked to fibrin and assembled in platelet thrombi. *Blood* 2006; **107**: 3555–63.
- Cho J, Degen JL, Collier BS, Mosher DF. Fibrin but not adsorbed fibrinogen supports fibronectin assembly by spread platelets. Effects of the interaction of alphaIIb beta3 with the C terminus of the fibrinogen gamma-chain. *J Biol Chem* 2005; **280**: 35490–8.
- Ni H. Unveiling the new face of fibronectin in thrombosis and hemostasis. *J Thromb Haemost* 2006; **4**: 940–2.
- Xu X, Wu J, Zhai Z, Zhou R, Wang X, Wang H, Ding K, Sun Z, Ni H. A novel fibrinogen Bbeta chain frameshift mutation in a patient with severe congenital hypofibrinogenemia. *Thromb Haemost* 2006; **95**: 931–5.
- Reheman A, Gross P, Yang H, Chen P, Allen D, Leytin V, Freedman J, Ni H. Vitronectin stabilizes thrombi and vessel occlusion but plays a dual role in platelet aggregation. *J Thromb Haemost* 2005; **3**: 875–83.
- Plow EF, McEver RP, Collier BS, Woods VL Jr, Marguerie GA and Ginsberg MH. Related binding mechanisms for fibrinogen, fibronectin, von Willebrand factor, and thrombospondin on thrombin-stimulated human platelets. *Blood* 1985; **66**: 724–7.
- Plow EF, Marguerie GA, Ginsberg MH. Fibronectin binding to thrombin-stimulated platelets: evidence for fibrin(ogen) independent and dependent pathways. *Blood* 1985; **66**: 26–32.
- Phillips DR, Charo IF, Scarborough RM. GPIIb-IIIa: the responsive integrin. *Cell* 1991; **65**: 359–62.
- Born GV, Cross MJ. Effects of inorganic ions and of plasma proteins on the aggregation of blood platelets by adenosine diphosphate. *J Physiol* 1964; **170**: 397–414.
- Suh TT, Holmback K, Jensen NJ, Daugherty CC, Small K, Simon DI, Potter S, Degen JL. Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. *Genes Dev* 1995; **9**: 2020–33.
- Collier BS, Peerschke EI, Scudder LE, Sullivan CA. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. *J Clin Invest* 1983; **72**: 325–38.
- Cho J, Mosher DF. Impact of fibronectin assembly on platelet thrombus formation in response to type I collagen and von Willebrand factor. *Blood* 2006; **108**: 2229–36.
- Cho J, Mosher DF. Role of fibronectin assembly in platelet thrombus formation. *J Thromb Haemost* 2006; **4**: 1461–9.
- Ruoslahti E. RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol* 1996; **12**: 697–715.
- Langenbach KJ, Sottile J. Identification of protein-disulfide isomerase activity in fibronectin. *J Biol Chem* 1999; **274**: 7032–8.
- Chen VM, Hogg PJ. Allosteric disulfide bonds in thrombosis and thrombolysis. *J Thromb Haemost* 2006; **4**: 2533–41.
- Olorundare OE, Peyruchaud O, Albrecht RM, Mosher DF. Assembly of a fibronectin matrix by adherent platelets stimulated by lysophosphatidic acid and other agonists. *Blood* 2001; **98**: 117–24.
- Hynes R. *Fibronectin*. New York: Springer Verlag, 1990.
- Sakai T, Johnson KJ, Murozono M, Sakai K, Magnuson MA, Wieloch T, Cronberg T, Isshiki A, Erickson HP, Fassler R. Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing and hemostasis. *Nat Med* 2001; **7**: 324–30.