Table 1
PAC-1, anti P-selectin antibody, and annexin V binding to platelets induced by H12-latex beads

	PAC-1 binding (%)		P-selectin expression (%)		Annexin V binding (%)	
Stirring time (min)	0	10	0	30	0	30
PBS	0.43 ± 0.12	1.75 ± 0.41	0.50 ± 0.01	0.70 ± 0.14	0.26 ± 0.13	0.68 ± 0.21
H12-latex beads	0.47 ± 0.20	1.49 ± 0.26	0.53 ± 0.04	0.75 ± 0.23	0.31 ± 0.13	0.60 ± 0.14
Positive control	55.1 ± 6.1[a]		31.6 ± 6.9[b]		20.7 ± 4.3[c]	

[a] *ADP 100* μM.
[b] ADP 20 μM.
[c] Thrombin 0.5 U/mL.

of PAC-1, antiP-selectin antibody, and annexin V to the platelets slightly increased after stirring, as shown in Table 1; however, this was independent of the presence of the H12-latex beads. Indeed, this phenomenon was induced by stirring alone and was within the error limit for the platelet activation ratio of the positive control. This indicated that H12-latex beads did not cause the activation of platelets and that H12 was suitable as one of the entities for recognition sites of the platelet substitutes.

In conclusion, the H12-latex beads were shown to preferentially interact with an activated platelet surface via GPIIb/IIIa receptors and to facilitate platelet accumulation at sites of hemostasis. Thus, such particles may be suitable candidates for alternative to human platelet concentrates transfused into thrombocytopenic patients. After evaluating the function of the H12-latex beads in vitro, we plan to investigate more biocompatible particles such as vesicles, polymerized albumin particles, or such like, for in vivo investigation.

Acknowledgments

The authors thank Dr. M. Murata and K. Yokoyama at Keio University for useful discussion about the functional evaluation of H12 or RGD peptide. This work was supported in part by Health and Labor Sciences Research Grants (Research on Pharmaceutical and Medical Safety), Ministry of Health, Labor and Welfare, Japan, and Grants-in-Aid from the Ministry of Education, Culture, Sport, Science and Technology, Japan (No. 11877172), 21COE "Practical Nano-Chemistry" from MEXT, Japan. Y.T. was the recipient of a Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists.

References

[1] S.S. Graham, N.J. Gonchoroff, J.L. Miller, Infusible platelet membranes retain partial functionality of the platelet GPIb/IX/V receptor complex, Am. J. Clin. Pathol. 115 (2001) 144–147.
[2] M. Rybak, L.A. Renzulli, A liposome based platelet substitutes, the plateletsome, with hemostatic efficacy, Biomater. Artif. Cells Immob. Biotechnol. 21 (1993) 108–118.
[3] G. Agam, A.A. Livine, Erythrocytes with covalently bound fibrinogen as a cellular replacement for the treatment of thrombocytopenia, Eur. J. Clin. Invest. 22 (1992) 105–112.
[4] M. Levi, P.W. Friedrich, S. Middleton, P.G. De Groot, Y.P. Wu, R. Harris, B.J. Biemond, F.G. Heijien, J. Levin, J.W. Ten Cate, Fibrinogen-coated albumin microcapsules reduce bleeding in severely thrombocytopenic rabbits, Nat. Med. 5 (1999) 107–111.
[5] B.S. Coller, K.T. Springer, J.H. Beer, N. Mohandas, L.E. Scudder, K.J. Norton, S.M. West, Thromboerythrocytes. In vitro studies of a potential autologous, semi-artificial alternative to platelet transfusion, J. Clin. Invest. 89 (1992) 546–555.
[6] Y. Ikeda, M. Handa, K. Kawano, T. Kamata, M. Murata, Y. Araki, H. Ando, Y. Kawai, K. Watanabe, I. Itagaki, K. Sakai, Z.M. Ruggeri, The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress, J. Clin. Invest. 87 (1991) 1234–1240.
[7] P. Marchese, E. Saldivar, J. Ware, Z.M. Ruggeri, Adhesive properties of the isolated amino-terminal domain of platelet glycoprotein Ibα in a flow field, Proc. Natl. Acad. Sci. USA 96 (1999) 7837–7842.
[8] S.A. Santro, M.M. Zutter, The α2β1 integrin: a collagen receptor on platelets and other cells, Thromb. Hemost. 74 (1995) 813–821.
[9] J.J. Sixma, G.H. van Zanten, E.G. Huizinga, R.M. van der Plas, M. Verkley, Y.P. Wu, P. Gros, P.G. de Groot, Platelet adhesion to collagen: an update, Thromb. Hemost. 78 (1997) 434–438.
[10] Y. Teramura, Y. Okamura, S. Takeoka, H. Tsuchiyama, H. Narumi, M. Kainoh, M. Handa, Y. Ikeda, E. Tsuchida, Hemostatic effects of polymerized albumin particles bearing rGPIa/IIa in thrombocytopenic mice, Biochem. Biophys. Res. Commun. 306 (2003) 256–260.
[11] S. Takeoka, Y. Teramura, Y. Okamura, E. Tsuchida, M. Handa, Y. Ikeda, Rolling properties of rGPIbα-conjugated phospholipid vesicles with different membrane flexibilities on vWf surface under flow conditions, Biochem. Biophys. Res. Commun. 296 (2002) 765–770.
[12] T. Kitaguchi, M. Murata, K. Iijima, K. Kamide, T. Imagawa, Y. Ikeda, Characterization of liposomes carrying von Willebrand factor-binding domain of platelet glycoprotein Ibα: a potential substitute for platelet transfusion, Biochem. Biophys. Res. Commun. 261 (1999) 784–789.
[13] T. Nishiya, M. Murata, M. Handa, Y. Ikeda, Targetting of liposomes carrying recombinant fragments of platelet membrane glycoprotein Ibα to immobilized von Willebrand factor under flow conditions, Biochem. Biophys. Res. Commun. 270 (2000) 755–760.
[14] T. Nishiya, M. Kainoh, M. Murata, M. Handa, Y. Ikeda, Reconstitution of adhesive properties of human platelets in liposomes carrying both recombinant glycoproteins Ia/IIa and Ibα under flow conditions: specific synergy of receptor–ligand interactions, Blood 100 (2002) 136–142.
[15] S. Takeoka, Y. Teramura, Y. Okamura, M. Handa, Y. Ikeda, E. Tsuchida, Fibrinogen-conjugated albumin polymers and their interaction with platelets under flow conditions, Biomacromolecules 2 (2001) 1192–1197.
[16] S. Takeoka, Y. Teramura, H. Ohkawa, Y. Ikeda, E. Tsuchida, Conjugation of von Willebrand factor-binding domain of platelet

glycoprotein Ibα to size-controlled albumin microsheres, Biomacromolecules 1 (2000) 290–295.
[17] J. Hawiger, M. Kloczewiak, M.A. Bednarek, S. Timmons, Platelet receptor recognition domains on the α chain of human fibrinogen: structure–function analysis, Biochemistry 28 (1989) 2909–2914.
[18] M. Kloczewiak, S. Timmons, J. Hawiger, Localization of a site interacting with human platelet receptor on carboxy-terminal segment of human fibrinogen γ chain, Biochem. Biophys. Res. Commun. 107 (1982) 181–187.
[19] M. Kloczewiak, S. Timmons, T.J. Lukas, J. Hawiger, Platelet receptor recognition site on human fibrinogen. Synthesis and structure–function relationship of peptides corresponding to the carboxy-terminal segment of the γ chain, Biochemistry 23 (1982) 1767–1774.
[20] D.R. Phillips, I.F. Charo, R.M. Scarborough, GPIIb-IIIa: the responsive integrin, Cell 65 (1991) 359–362.
[21] S.C. Lam, E.F. Plow, M.A. Smith, A. Andrieux, J.J. Ryckwaert, G. Marguerie, M.H. Ginsberg, Evidence that arginyl-glycyl-aspartate peptides and γ chain peptides share a common binding site on platelets, J. Biol. Chem. 262 (1987) 110–115.
[22] D.E. Hallahan, L. Geng, A.J. Cmelak, A.B. Chakravathy, W. Martin, C. Scarfone, A. Gonzalez, Targeting drug delivery to radiation-induced neoantigens in tumor microvasculature, J. Control. Release 74 (2001) 183–191.
[23] A. Bonnefoy, Q. Liu, C. Legrand, M.M. Frojmovic, Efficiency of platelet adhesion to fibrinogen depends on both cell activation and flow, Biophys. J. 78 (2000) 2834–2843.
[24] J.H. Beer, K.T. Springer, B.S. Coller, Immobilized Arg-Gly-Asp (RGD) peptides of varying lengths as structural probes of the platelet glycoprotein IIb/IIIa receptor, Blood 79 (1992) 117–128.
[25] S.E. D'Souza, M.H. Ginsberg, T.A. Burke, E.F. Plow, The ligand binding site of the platelet integrin receptor GPIIb-IIIa is proximal to the second calcium binding domain of its subunit, J. Biol. Chem. 265 (1990) 3440–3446.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ACADEMIC PRESS
Biochemical and Biophysical Research Communications 306 (2003) 256–260
BBRC
www.elsevier.com/locate/ybbrc

Hemostatic effects of polymerized albumin particles bearing rGPIa/IIa in thrombocytopenic mice

Yuji Teramura,[a] Yosuke Okamura,[a] Shinji Takeoka,[a] Hiromi Tsuchiyama,[b] Hideki Narumi,[b] Mie Kainoh,[b] Makoto Handa,[c] Yasuo Ikeda,[c] and Eishun Tsuchida[a,*]

[a] *Advanced Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan*
[b] *Pharmaceutical Research Laboratories, Toray Industries, Inc., Kanagawa 248-8555, Japan*
[c] *Department of Internal Medicine and Blood Center, School of Medicine, Keio University, Tokyo 160-8582, Japan*

Received 6 May 2003

Abstract

The recombinant fragment of the platelet membrane glycoprotein Ia/IIa (rGPIa/IIa) was conjugated to the polymerized albumin particles (polyAlb) with the average diameter of 180 nm. The intravenous administration of rGPIa/IIa–polyAlb to thrombocytopenic mice ([platelet] = $2.1 \pm 0.3 \times 10^5$ particles/μL) with three doses of ca. 2.4×10^{10}, 7.2×10^{10}, and 2.4×10^{11} particles/kg, respectively, significantly reduced their bleeding time to 426 ± 71, 378 ± 101, and 337 ± 46 s, respectively, whereas that of the control groups (PBS) was 730 ± 198 s. The injection of rGPIa/IIa–polyAlb (2.4×10^{11} particles/kg) was approximately equal to the effect of the injection of the mouse platelets at a dose of 2.0×10^{10} particles/kg. It was confirmed that rGPIa/IIa–polyAlb had a recognition ability against collagen and could contribute to the hemostasis in the thrombocytopenic mice as a platelet substitute.

Keywords: Platelet substitutes; Polymerized albumin particles; Platelets; GPIa/IIa; Collagen; Thrombocytopenic mice; Bleeding time

In the platelet transfusion therapy for bleeding thrombocytopenic patients, platelet concentrates derived from donated human blood have been used, and the number of patients is increasing due to the development of tumor therapy and chemotherapy [1]. However, due to the short-term storage of platelet concentrates (3 days in Japan), the shortage of platelets has always been a serious concern [2]. Furthermore, the risk of viral and bacterial infections during transfusion is another significant issue. Several platelet substitutes have been developed for over two decades [3,4], such as fibrinogen-bound red blood cells [5], arginine–glycine–aspartic acid (RGD) peptide-bound red blood cells [6], solubilized platelet membrane protein-conjugated liposomes [7], and infusible platelet membranes (IPMs) prepared from the fractions of outdated platelets by a freeze–thawing, heat, and drying process [8]. Levi et al. [9] succeeded in reducing the bleeding time using fibrinogen-coated albumin microcapsules in thrombocytopenic rabbits. However, these platelet substitutes depend on human blood as sources and could not be accumulated at the site of the vascular injury. It is because they do not have the recognition ability for collagen at the injury site but for GPIIb/IIIa that emerged on the activated platelets in order to promote the platelet aggregation. However, because fibrinogen is not very stable in the solution state, it is difficult to use in practice [10].

We have been developing platelet substitutes using polymerized albumin particles (polyAlb) [10,11] and phospholipid vesicles (liposomes) [12–15] to study the hemostatic ability in vitro. In our strategy, the carriers having the recognition ability for collagen exposed at the site of the vascular injury or von Willebrand factor (vWf) attached to the collagen can accumulate at the bleeding site and take part in the hemostasis by collaborating with the platelets remaining in the thrombocytopenic patients. Platelet membrane glycoprotein (GP) Ia/IIa (integrin α2β1, VLA2, and CD49b/29) [16–19]

* Corresponding author. Fax: +81-3-3205-4740.
E-mail address: eishun@waseda.jp (E. Tsuchida).

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00943-4

and GPIbα [20,21] are the receptors for the collagen and vWf, respectively. We confirmed that recombinant GPIa/IIa (rGPIa/IIa)- and recombinant GPIbα (rGPIbα)-conjugated liposomes could attach to the collagen-immobilized surface under flow conditions [15]. We also reported that rGPIbα-conjugated polyAlb (rGPIbα–polyAlb) accumulated on the vWf-immobilized surface [11], whereas fibrinogen-conjugated polyAlb (fibrinogen–polyAlb) aggregated platelets after their attachment to the activated platelets-immobilized surface in vitro [10].

It is well known that GPIa/IIa as a member of the integrin family of heterodimeric molecules and GPVI as a member of immunoglobulin superfamily [22,23] are the major collagen receptor in platelets. It is well recognized that the adhesion of these receptors-mediated platelets plays an important role in the hemostasis process. Recently, we succeeded in developing the recombinant products of a soluble GPIa/IIa heterodimer (MW: 320 kDa) [24]. In this study, we prepared rGPIa/IIa–polyAlb and intravenously administered rGPIa/IIa–polyAlb to the thrombocytopenic mice to evaluate them as platelet substitutes from the bleeding time as one of the parameters of their hemostatic abilities.

Materials and methods

Preparation of rGPIa/IIa–polyAlb [10,11]. A solution of recombinant human serum albumin (rHSA, 250 mg/mL) was kindly donated by Mitsubishi Pharma (Osaka) and dialyzed against pure water for 12 h at 4 °C to remove the stabilizers such as *N*-acetyl D,L-tryptophan and sodium caprate. After dilution with saline to 10 mg/mL, a 0.1 N NaOH solution (800 μL) was added to the rHSA solution (25 mL) until the pH became 10.7 at room temperature (r.t.). After being heated at 80 °C for 20 min, the solution was cooled down to r.t. with an ice bath for ca. 10 min. After the solution was stirred at r.t. for 10 min, the pH became 10.5. Then, 0.1 N HCl solution (900 μL) was dropwise added until the pH of the solution became 6.1 and a white transparent rHSA solution was obtained. During stirring the solution for 90 min at 40 °C, the solution gradually became turbid. After the addition of excess iodoacetamide (25 mg) as a terminant of the polymerization at r.t., the solution was dialyzed against a phosphate-buffered saline (pH 7.4, PBS) for 20 h at 5 °C and a 25-mL dispersion of polyAlb ([HSA] = 9.0 mg/mL, pH 7.4) was thus prepared. The average diameter was determined by a dynamic scattering method (Coulter particle analyzer, model N4SD, Coulter, Fullerton) and scanning electron microscopy (SEM, Hitachi, Tokyo). The method to conjugate rGPIa/IIa to the surface of polyAlb with *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, Pierce Chemical, Rockford) was followed by our previous method [9]. To the polyAlb dispersion (17 mL, 10 mg/mL) was added a 4 mM SPDP ethanol solution (10 μL) and stirred for 30 min at r.t. After separation by gel permeation chromatography (GPC, 10 mm o.d. × 70 mm h, Sephadex G-25, Amersham Bioscience Co., Uppsala), the pyridyldithio-bound polyAlb (PD–polyAlb) was obtained. To a rGPIa/IIa solution (10.8 mg/mL, 180 μL) was added a 4-mM SPDP ethanol solution (3 μL) and incubated for 20 min at r.t. After the addition of a dithiothreitol solution (final concentration 10 mM) at r.t., the SH-rGPIa/IIa was obtained by the separation with GPC (Sephadex G25). The PD–polyAlb dispersion was mixed with the SH-rGPIa/IIa solution at 25 °C for 12 h and the rGPIa/IIa–polyAlb was thus prepared after the removal of unreacted rGPIa/IIa by GPC (Sephacryl S400). For the perfusion studies, FITC-labelled rGPIa/IIa–polyAlb was used. After a FITC solution was mixed with the polyAlb dispersion and washed with PBS with centrifugation (6000*g*, 20 min) for three times, rGPIa/IIa was conjugated to the FITC-labelled polyAlb as mentioned previously. The concentration of rGPIa/IIa conjugated to the surface of the polyAlb was determined with a sandwich enzyme-linked immunosorbent assay (ELISA) with using two kinds of antibodies; anti-GPIa monoclonal antibody and biotin-labelled anti-GPIa polyclonal antibody.

Preparation of reconstituted blood and collagen-immobilized surface [10]. Blood withdrawn from healthy volunteers was mixed with a 10:1 volume of acid–citrate–dextrose composed of 2.2% sodium citrate, 0.8% citric acid, and 2.2% glucose (ACD). The blood was centrifuged (100*g*, 15 min, r.t.) and the platelet-rich plasma (PRP) was replaced with an equal volume of a 0.9% NaCl solution (saline) containing 10% ACD (10% ACD–saline). After red blood cells were resuspended in 10% ACD–saline, they were centrifuged (2200*g*, 10 min, r.t.) and replaced with the saline again for the complete removal of the buffy coat. In perfusion studies, the red blood cells were reconstituted to 40% of hematocrit (Hct) in a Hepes–Tyrode buffer (pH 7.4) containing 1 mM $CaCl_2$. The residual platelet concentration was measured to be 1.2×10^4 particles/μL with an automated hematology analyzer (K-4500, SYSMEX, Kobe).

Collagen I-A (3.0 mg/mL, Cellmatrix, Nitta Gelatin, Osaka, Japan) was dispersed in PBS (pH 7.4) at 4 °C to give a final concentration of 30 μg/mL. A glass plate (diameter, 24 mm; thickness, 0.5 mm) was immersed into the collagen dispersion at 4 °C for 12 h.

Measurement of the interaction of rGPIa/IIa–polyAlb with collagen surface. The interaction of the FITC-labelled rGPIa/IIa–polyAlb with the collagen-immobilized surface was analyzed using a recirculating chamber mounted on an epifluorescence microscope (ECLIPS TE300, Nikon, Tokyo) equipped with a CCD camera [25]. All the perfusion studies were performed at 37 °C. The images of rGPIa/IIa–polyAlb on the collagen surface were obtained using an image processor, ARGUS-50 (Hamamatsu Photonics, Hamamatsu).

Measurement of PT and APTT of the mouse blood. Blood withdrawn from mice (BALB/c, 8-week-old, female) was mixed with a sodium citric solution (volume ratio 10:1) and the plasma was collected after the centrifugation (2200*g*, 10 min). The polyAlb or rGPIa/IIa–polyAlb dispersions were mixed with plasma ([HSA] = 0.8 mg/mL), and the prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured by the automated coagulation analyzer (Amelung KC-4A, Amelung, Lemgo).

Measurement of tail bleeding time of the thrombocytopenic mice [24]. Mice (BALB/c, 8-week-old, female) were exposed to total-body irradiation at a dose of 600 rad using X-ray apparatus (MBR-1520, Hitachi, Japan) to prepare thrombocytopenic mice. The rGPIa/IIa–polyAlb suspension was administered from the tail vein at a dose of 10 mL/kg and the tail was cut at the position of 0.2 cm from the tail end 10 min after the administration. The bleeding site was warmed at 37 °C in the saline and the bleeding time was measured until the bleeding was stopped. All animal studies were approved by the Animal Subject Committee of Toray Industries and performed according to NIH guidelines for the care and use of laboratory animals (NIH publication 85–23 Rev. 1985).

Results and discussion

Albumin (Alb) was polymerized with disulfide bonding to form particles by changing the pH and temperature of the Alb solution and the particle diameter could be controlled to 180 ± 35 nm. rGPIa/IIa was conjugated on the surface of the polyAlb with SPDP as a smart crosslinker [11]. The amount of rGPIa/IIa

conjugated on the surface of the polyAlb was determined by a sandwich ELISA method and about 60 molecules of rGPIa/IIa existed on one polyAlb particle. The LPS concentration in the rGPIa/IIa–polyAlb dispersion ([HSA] = 10 mg/mL) was determined after the solubilization of polyAlb with a 0.1 N NaOH solution, followed by the adjustment of the solution pH to 7.0 using a 0.1 N HCl solution. The LPS concentration was below 0.1 EU/mL and acceptable for the in vivo study.

To study the interaction between the rGPIa/IIa–polyAlb and collagen, FITC-labelled rGPIa/IIa–polyAlb was mixed with reconstituted blood and allowed to flow onto the collagen-immobilized surface at a shear rate of $350\,s^{-1}$. The interaction of rGPIa/IIa–polyAlb with the collagen-immobilized surface was observed using fluorescence microscopy. In this study, the diameter of the polyAlb was adjusted to ca. 1 μm in order to observe the rGPIa/IIa–polyAlb particles with fluorescence microscopy. As shown in Fig. 1, rGPIa/IIa–polyAlb became immediately attached and accumulated on the collagen surface. The attachment rate was about 2.5×10^2 particles/mm^2/s and the surface coverage of rGPIa/IIa–polyAlb was 22% at 180 s. The adhesion was suppressed in the presence of an antiGPIa/IIa monoclonal antibody, 7E10B [26], indicating the specific interaction between the rGPIa/IIa and collagen.

The prothrombin time (PT) and activated partial thromboplastin time (APTT) of the mouse blood containing the polyAlb or the rGPIa/IIa–polyAlb ([rHSA] = 0.8 mg/mL) was listed in Table 1 with those of the blood containing PBS. No significant difference between the polyAlb sample groups and the PBS group indicates no interference of the polyAlb particles on the inhibition and promotion of endogenous and exogenous coagulation activities.

The tail bleeding times of the normal mice ([platelet] = $9.9 \pm 2.0 \times 10^5$ particles/μL) and thrombocytopenic mice ([platelet] = $2.4 \pm 1.6 \times 10^5$ particles/μL) were 117 ± 14 and 660 ± 150 s, respectively (Fig. 2). The bleeding time of the thrombocytopenic mice was about 6.5 times longer than that of the normal mice. The intravenous injection of the mouse platelets to the thrombocytopenic mice at doses of 1.0×10^{10} and 3.0×10^{10} particles/kg reduced their tail bleeding times to 477 ± 133 and 203 ± 57 s, respectively, indicating that the bleeding time of the thrombocytopenic mouse correlated with the administered amount of platelets. Therefore, we confirmed that the tail bleeding time was one of the effective evaluation parameters of the hemostatic ability of the rGPIa/IIa–polyAlb as a platelet substitute.

We preliminarily studied the blood circulation clearance of the rGPIa/IIa–polyAlb (data not shown). The amount of rGPIa/IIa–polyAlb at a dose of 10 mg/kg (as an albumin concentration, 3.0×10^{10} particles/kg) in the circulation rapidly declined and the half-life of the injected rGPIa/IIa–polyAlb was about 13 min. We wanted to measure the tail bleeding time just after the administration of rGPIa/IIa–polyAlb. However, we measured the bleeding time starting 10 min after the administration due to a technical reason. The intravenous administration of rGPIa/IIa–polyAlb at a dose of 8 mg/kg (2.4×10^{10} particles/kg) reduced the bleeding time to 426 ± 71 s, whereas the bleeding times of the control PBS and polyAlb groups were 730 ± 198 and 871 ± 138 s, respectively. At doses of 24 and 80 mg/kg rGPIa/IIa–polyAlb (7.2×10^{10} and 2.4×10^{11} particles/kg, respectively), the bleeding times were reduced to 378 ± 101 and 337 ± 46 s, respectively, whereas the bleeding times of polyAlb groups (24 and 80 mg/kg)

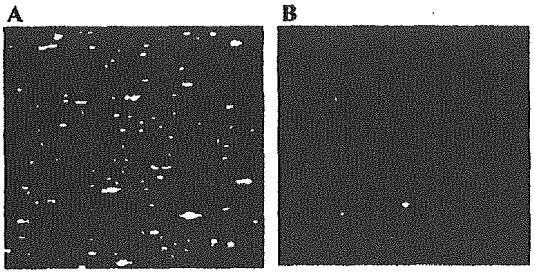

Fig. 1. Observation of the attachment of FITC-labelled rGPIa/IIa–polyAlb to the collagen surface at the shear rate of $350\,s^{-1}$ with a fluorescence microscopy, (A) in the absence of antiGPIa/IIa monoclonal antibody (7E10B) and (B) in the presence of 7E10B.

Table 1
Effects of polyAlb on blood coagulation time in vitro

Samples	PT (s)	APTT (s)
PBS	9.4 + 0.0	29.0 + 0.2
PolyAlb	9.4 + 0.1	30.3 + 0.2
rGPIa/IIa–polyAlb	9.4 + 0.1	27.9 + 0.0

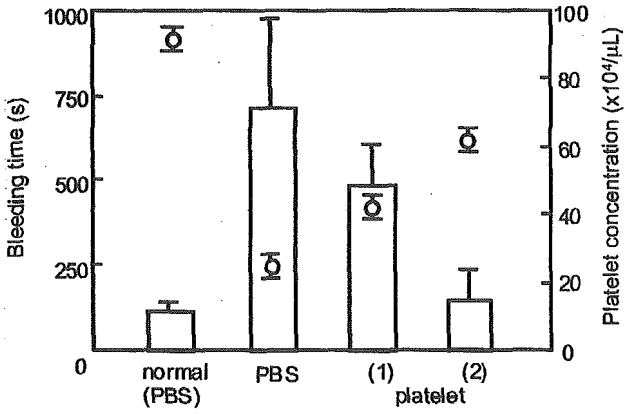

Fig. 2. Effect of the administration of the PBS and the mouse platelets on the tail bleeding time (white bar) in thrombocytopenic mice. The administered amounts of platelets are: (1) 1.0×10^{10} and (2) 3.0×10^{10} particles/kg. ○, platelet concentration in the mouse.

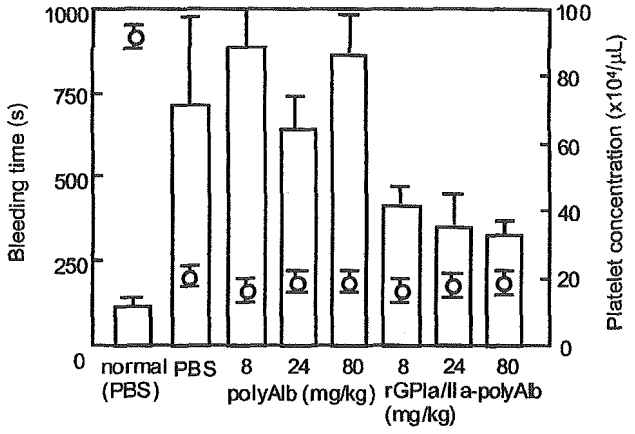

Fig. 3. Effect of the administration of rGPIa/IIa–polyAlb on the tail bleeding time (white bar). The administered amounts of rGPIa/IIa–polyAlb are 8, 24, and 80 mg/kg. ○, platelet concentration in the mouse.

were 634 ± 108 and 832 ± 147 s, respectively (Fig. 3). We estimated that the administration of 80 mg/kg rGPIa/IIa–polyAlb (2.4×10^{11} particles/kg) would be approximately equal to the administration of the mouse platelets at a dose of 2.0×10^{10} particles/kg.

Even though the bleeding time had a large deviation for the administration of PBS and polyAlb as the control groups as shown in Fig. 3, it was confirmed that the administration of rGPIa/IIa–polyAlb significantly caused the dose-dependent reduction in the bleeding time. These results suggested that the circulating rGPIa/IIa–polyAlb should recognize and accumulate on the collagen exposed at the bleeding site of the tail and help the hemostasis without showing any inhibition by covering the binding sites of the collagen surface for the platelets. The hemostatic ability of the rGPIa/IIa–polyAlb was about one-tenth that of the platelets, which was based on the bleeding time and administration concentration (Fig. 3). It is because platelets should have other receptors such as GPIbα and GPVI for the bleeding sites and also have various functions such as aggregation and the release of granules, whereas the rGPIa/IIa–polyAlb has the only function of recognition for the collagen.

In this study, we have succeeded in reducing the bleeding time of the thrombocytopenic mouse by injecting rGPIa/IIa–polyAlb having the recognition ability for collagen exposed at the bleeding site. rGPIa/IIa–polyAlb was confirmed to have the potential to be a platelet substitute.

Acknowledgments

The authors thank Mr. S. Hara at Toray Industries, Inc., for in vivo evaluation of rGPIa/IIa–polyAlb. This work was supported by Health and Labour Sciences Research Grants, Research on Pharmaceutical and Medical Safety, Ministry of Health, Labor and Welfare, Japan, Grants-in-Aid (No. 11877172), and 21COE "Practical Nano-Chemistry" from MEXT, Japan. One of the authors (Y.T.) thanks Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

References

[1] P. Rebulla, G. Finazzi, F. Marangoni, G. Avvisati, L. Gugliotta, G. Tognoni, T. Barbui, F. Mandelli, G. Sirchia, The threshold for prophylactic platelet transfusions in adults with acute myeloid leukemia, N. Engl. J. Med. 337 (1997) 1870–1875.

[2] T.S. Kickler, Improving the quality of stored platelets, Transfusion 31 (1991) 1–3.

[3] M.A. Blajchman, Platelet substitutes, Vox Sang. 78 (2000) 183–186.

[4] M.A. Blajchman, Novel platelet products, substitutes and alternatives, Transfus. Clin. Biol. 8 (2001) 267–271.

[5] G. Agam, A.A. Livne, Erythrocytes with covalently bound fibrinogen as a cellular replacement for the treatment of thrombocytopenia, Eur. J. Clin. Invest. 22 (1992) 105–112.

[6] B.S. Coller, K.T. Springer, J.H. Beer, N. Mohandas, L.E. Scudder, K.J. Norton, S.M. West, Thromboerythrocytes. In vitro studies of a potential autologous, semi-artificial alternative to platelet transfusion, J. Clin. Invest. 89 (1992) 546–555.

[7] M.E. Rybak, L.A. Renzulli, A liposome based platelet substitute, the plateletsome, with hemostatic efficacy, Biomater. Artif. Cells Immobilization Biotechnol. 21 (1993) 101–118.

[8] S.S. Graham, N.J. Gonchoroff, J.L. Miller, Infusible platelet membranes retain partial functionality of the platelet GPIb/IX/V receptor complex, Am. J. Clin. Pathol. 115 (2001) 144–147.

[9] M. Levi, P.W. Friederich, S. Middleton, P.G. De Groot, Y.P. Wu, R. Harris, B.J. Biemond, F.G. Heijien, J. Levin, J.W. Ten Cate, Fibrinogen-coated albumin microcapsules reduce bleeding in severely thrombocytopenic rabbits, Nat. Med. 5 (1999) 107–111.

[10] S. Takeoka, Y. Teramura, Y. Okamura, M. Handa, Y. Ikeda, E. Tsuchida, Fibrinogen-conjugated albumin polymers and their interaction with platelets under flow conditions, Biomacromolecules 2 (2001) 1192–1197.

[11] S. Takeoka, Y. Teramura, H. Ohkawa, Y. Ikeda, E. Tsuchida, Conjugation of von Willebrand factor-binding domain of platelet glycoprotein Ibα to size-controlled albumin microspheres, Biomacromolecules 1 (2000) 290–295.

[12] S. Takeoka, Y. Teramura, Y. Okamura, E. Tsuchida, M. Handa, Y. Ikeda, Rolling properties of rGPIbα-conjugated phospholipid vesicles with different membrane flexibilities on vWf surface under flow conditions, Biochem. Biophys. Res. Commun. 296 (2002) 765–770.

[13] T. Kitaguchi, M. Murata, K. Iijima, K. Kamide, T. Imagawa, Y. Ikeda, Characterization of liposomes carrying von Willebrand factor-binding domain of platelet glycoprotein Ibα: a potential substitute for platelet transfusion, Biochem. Biophys. Res. Commun. 261 (1999) 784–789.

[14] T. Nishiya, M. Murata, M. Handa, Y. Ikeda, Targetting of liposomes carrying recombinant fragments of platelet membrane glycoprotein Ibα to immobilized von Willebrand factor under flow conditions, Biochem. Biophys. Res. Commun. 270 (2000) 755–760.

[15] T. Nishiya, M. Kainoh, M. Murata, M. Handa, Y. Ikeda, Reconstitution of adhesive properties of human platelets in liposomes carrying both recombinant glycoproteins Ia/IIa and Ibα under flow conditions: specific synergy of receptor–ligand interactions, Blood 100 (2002) 136–142.

[16] S.A. Santoro, M.M. Zutter, The α2β1 integrin: a collagen receptor on platelets and other cells, Thromb. Haemost. 74 (1995) 813–821.
[17] H.K. Nieuwenhuis, K.S. Sakariassen, W.P.M. Houdijk, P.F.E.M. Nievelstein, J.J. Sixma, Deficiency of platelet membrane glycoproteins Ia associated with a decreased platelet adhesion to subendothelium: a defect in platelet spreading, Blood 68 (1986) 692–695.
[18] J.J. Sixma, G.H. van Zanten, E.G. Huizinga, R.M. van der Plas, M. Verkley, Y.P. Wu, P. Gros, P.G. de Groot, Platelet adhesion to collagen: an update, Thromb. Haemost. 78 (1997) 434–438.
[19] M. Kainoh, Y. Ikeda, S. Nishio, T. Nakadake, Glycoprotein Ia/IIa-mediated activation-dependent platelet adhesion to collagen, Thromb. Res. 65 (1992) 165–176.
[20] Y. Ikeda, M. Handa, K. Kawano, T. Kamata, M. Murata, Y. Araki, H. Ando, Y. Kawai, K. Watanabe, I. Itagaki, K. Sakai, Z.M. Ruggeri, The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress, J. Clin. Invest. 87 (1991) 1234–1240.
[21] P. Marchese, E. Saldivar, J. Ware, Z.M. Ruggeri, Ahesive properties of the isolated amino-terminal domain of platelet glycoprotein Ibα in a flow field, Proc. Natl. Acad. Sci. USA 96 (1999) 7837–7842.
[22] B. Nieswandt, C. Brakebusch, W. Bergmeier, V. Schulte, D. Bouvard, R. Mokhtari-Nejad, T. Lindhout, J.W. Heemskerk, H. Zirngibl, R. Fassler, Glycoprotein VI but not α2β1 integrin is essential for platelet interaction with collagen, EMBO J. 20 (2001) 2120–2130.
[23] S. Goto, N. Tamura, S. Handa, M. Arai, K. Kodama, H. Takayama, Involvement of glycoprotein VI in platelet thrombus formation on both collagen and von Willebrand factor surfaces under flow conditions, Circulation 106 (2002) 266–272.
[24] M. Kainoh, T. Tanaka, Production of soluble integrin α2β1 heterodimer complex functionally active in vitro and in vivo, Biochem. Biophys. Res. Commun. 290 (2002) 765–770.
[25] K. Kawakami, Y. Harada, M. Sakasita, H. Nagai, M. Handa, Y. Ikeda, A new method for continuous measurement of platelet adhesion under flow conditions, ASAIO J. 39 (1993) M558–M560.
[26] K. Suzuki-Inoue, Y. Ozaki, M. Kainoh, T. Shin, Y. Yatomi, T. Ohmori, T. Tanaka, K. Satoh, T. Morita, Rhodocytin induces platelet aggregation by interacting with glycoprotein Ia/IIa (GPIa/IIa, integrin α2β1), J. Biol. Chem. 276 (2001) 1643–1652.

Functional phenotype of phosphoinositide 3–kinase p85α–null platelets characterized by an impaired response to GP VI stimulation

Naohide Watanabe, Hideaki Nakajima, Hidenori Suzuki, Atsushi Oda, Yumiko Matsubara, Masaaki Moroi, Yasuo Terauchi, Takashi Kadowaki, Harumi Suzuki, Shigeo Koyasu, Yasuo Ikeda, and Makoto Handa

Phosphoinositide 3–kinases (PI3Ks), a family of lipid kinases comprising 3 classes with multiple isoforms, have been shown to participate in different phases of platelet signaling. To investigate the roles that enzymes play in platelet function in vivo and determine which isoforms are important for particular signaling events, we analyzed platelet function of gene knockout mice deficient in the p85α regulatory subunit of heterodimeric class IA PI3K. The kinase activity of p85α−/− platelets was only 5% of the activity of platelets from wild-type littermates. Platelet aggregation induced by adenosine diphosphate (ADP), thrombin, U46619, phorbol 12-myristate 13-acetate (PMA), or botrocetin was not defective in p85α−/− mice, compared with wild-type animals. In contrast, aggregation induced by collagen and collagen-related peptide (CRP) was partially but readily impaired in p85α−/− mice. Both P-selectin expression and fibrinogen binding in response to CRP were also decreased to a similar extent in p85α−/− platelets. Platelets from p85α−/− mice appeared to spread poorly over a CRP-coated surface with intact filopodial protrusions. Significant attenuation of CRP-induced tyrosine phosphorylation in known PI3K effectors such as Btk, Tec, PKB/Akt, and phospholipase Cγ2 were observed with p85α−/− platelets, whereas no alteration was noted in upstream molecules of Syk, LAT, and SLP-76. Considered as a whole, these results provide the first genetic evidence that PI3K p85α plays a significant role in platelet function, almost exclusively in the glycoprotein (GP) VI/Fc receptor γ chain complex–mediated signaling pathway. (Blood. 2003;102:541-548)

Introduction

Phosphoinositide 3–kinases (PI3Ks) constitute a family of lipid kinases that are ubiquitously expressed in many cell types. These enzymes play a key role in the regulation of a variety of cellular processes: proliferation, survival, glucose metabolism, cytoskeletal remodeling, and vesicular trafficking.[1] PI3Ks phosphorylate an inositol ring of membrane-embedded inositol phospholipid at the 3′ position and generate D3 phosphoinositides (PtdIns-3-P; PtdIns-3,4-P2; PtdIns-3,5-P2; and PtdIns-3,4,5-P3), which then function as potent second messengers to relay signals by recruiting downstream molecules to the vicinity of the cellular membrane.[2,3] The most favored targets of the enzyme via its phosphoinositide products are the pleckstrin homology (PH) domain–containing effector molecules. These include Tec family tyrosine kinases, serine/threonine kinases such as Akt/protein kinase B (PKB), guanosine diphosphate/guanosine triphosphate (GDP/GTP) exchange factor (GEF) families such as Vav, and phospholipase (PLC)γ subtypes.[1-3] Of the 3 classes of PI3K (classes I-III), class IA and IB enzymes have been extensively studied in platelets[4-6] and are positioned downstream of membrane receptor stimulation and preferentially catalyze phosphorylation of PtdIns-4,5-P2 in vivo. The class IA subclass enzyme is a heterodimer comprising p110 catalytic and regulatory subunits, and to date 3 p110 catalytic subunits (α, β, and δ) and 3 regulatory subunits (85α, 85β, and 55γ) derived from different genes have been reported.[1] In addition, the 85α protein possesses 2 alternatively spliced variants, p55α and p50α.[7] Regulatory subunits act as adaptor molecules to activate the p110 catalytic subunit via interactions between their Src homology 2 (SH2) domains and specific phosphorylated tyrosine residues (Y-X-X-M motif) of upstream signaling molecules. In addition, class IA enzymes are known to be activated by G proteins.[8,9] A class IB subclass enzyme, PI3Kγ, is also abundant in platelets; it comprises a heterodimer complex with a p110γ catalytic subunit and a unique p101 regulatory subunit. The enzyme has been shown to be specific for G protein–coupled receptor (GPCR) activation through G protein βγ subunits.[4,10] Class IA PI3K utilizes a series of nonreceptor tyrosine kinases and associated adaptor molecules when it is activated downstream of adhesion receptor engagement to transduce signals. In fact, the collagen-induced platelet

From the Blood Center, Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, Tokyo; the Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo; the Division of Cellular Therapy, Institute of Medical Science, University of Tokyo; the Department of Cardiovascular Research, Tokyo Metropolitan Institute of Medical Science; the Laboratory of Environmental Biology, Department of Preventive Medicine, Hokkaido University School of Medicine, Sapporo; the Institute of Life Science, Kurume University; the Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo; Core Research for Evolutional Science and Technology Corporation, Kawaguchi; and the Department of Microbiology and Immunology, Yamaguchi University School of Medicine, Ube, Japan.

Submitted November 25, 2002; accepted March 10, 2003. Prepublished online as *Blood* First Edition Paper, March 20, 2003; DOI 10.1182/blood-2002-11-3327.

Supported in part by grants from Keio Gijuku Academic Department Funds and Keio University Grant-in-Aid for Encouragement of Young Medical Scientists (N.W.) and by Health Science Research Grants for Pharmaceutical and Medical Safety (Y.I. and M.H.) from the Ministry of Health, Labor and Welfare, Tokyo, Japan.

Reprints: Makoto Handa, Blood Center, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; e-mail: mhanda@sc.itc.keio.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

activation pathway via the glycoprotein (GP) VI/Fc receptor γ chain (FcRγ) complex evokes significant lipid kinase activity.[11] Upon GP VI cross-linking by collagen, immunoreceptor tyrosine-based activation motifs (ITAMs) displayed on the FcRγ subunit are phosphorylated by the protein tyrosine kinases Lyn and Fyn, leading to binding and activation of the Syk tyrosine kinase. Following assembly with adaptor molecules such as LAT and SLP-76, PI3K is activated via its p85 adaptor subunit.[12,13] With GP VI stimulation, platelet activation induced by aggregated immunoglobulin G (IgG) also involves class IA PI3K as a major signaling element associated with another ITAM-containing receptor, FcγR IIA.[14,15] Class IA enzymes are also implicated in processes downstream of GP Ib/IX/V complex–mediated signaling[16] and upstream of integrin α_{IIb}/β_3 (GP IIb/IIIa) complex activation (inside-out signaling),[4] and in postintegrin cellular responses (outside-in signaling), including conformational changes induced by actin rearrangement.[17,18] Platelet activation stimulated by GPCRs is physiologically important for platelet thrombus formation and is closely associated with PI3K activity.[4] Since PI3Kγ is activated only by G protein βγ subunits, the enzyme may be responsible for causing the observed increase in PI3K lipid products in response to stimulation with thrombin, adenosine diphosphate (ADP), and the thromboxane A_2 analog U46619.[4,19,20] However, class IA PI3K is also activated by GPCRs, and this type of activation may involve either a nonreceptor tyrosine-phosphorylated intermediate or the direct engagement of G protein βγ subunits.[9,21] Although a large body of evidence has clearly demonstrated that PI3K is intimately associated with different phases of platelet activation, the exact roles the enzyme plays in platelet functions in vivo and which isoforms are important for particular signaling events are yet to be determined. Platelets are terminally differentiated anucleate cells, and conclusions from most studies predominantly rely on indirect measurement of PI3K activity using structurally distinct inhibitors of PI3K (wortmannin and LY294002). This means that data analyses may be inherently limited in terms of specificity. To directly address these issues, we analyzed the function of platelets deficient in class IA PI3K p85α proteins in gene knockout mice.

Materials and methods

Materials and antibodies

Adenosine 5′-triphosphate (ATP), apyrase, phorbol 12-myristate 13-acetate (PMA), Arg-Gly-Asp-Ser (RGDS) peptide, acetylsalicylic acid (ASA), prostaglandin E_1 (PGE_1), bovine serum albumin (BSA), and poly-L-lysine were all purchased from Sigma-Aldrich (Tokyo, Japan). Phosphatidylinositol was obtained from Funakoshi (Tokyo, Japan), U46619 and A23187 from Merck KGaA (Darmstadt, Germany), and adenosine diphosphate (ADP) from Biopool (Ventura, CA). Collagen was supplied by Nycomed (Munchen, Germany). Collagen-related peptide (CRP) was prepared as previously described.[22] Botrocetin was a generous gift from Dr Yoshihiro Fujimura (Nara Medical College, Kashiwara, Japan). Human thrombin was provided by Welfide (Osaka, Japan). Alexa Fluor 488–conjugated human fibrinogen was from Molecular Probes (Eugene, OR). Fluorescein isothiocyanate (FITC)–conjugated antimouse P-selectin antibody was purchased from BD Pharmingen (San Diego, CA). Polyclonal anti-Btk antibody was kindly provided by Dr Owen Witte (University of California, Los Angeles). Anti-Syk, PLCγ2, SLP-76, p110α, p110β, p110δ and Akt/PKB polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antiphosphotyrosine monoclonal antibody (clone 4G10) and anti-PI3K $p85^{PAN}$, LAT, and Tec polyclonal antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Anti–phospho-Akt antibody was obtained from New England Biolabs (Beverly, MA). A specific antibody against p85β was prepared as described.[23] Thin-layer chromatography (TLC) plates were purchased from Whatman International (Maidstone, United Kingdom).

Mice

PI3K p85α−/− mice[24,25] were backcrossed to C57BL/6 mice for more than 7 generations before intercrossing heterozygous mice. The targeting strategy allowed selective disruption of p85α expression, while leaving gene products for p55α and p50α isoforms intact.[25] All mice were maintained under strict pathogen-free conditions. All experiments were performed in accordance with Keio University Institutional Guidelines.

Bleeding time

At 2 to 3 months of age, mice were anesthetized using diethylether. An incision was made 1 cm from the tip of the tail, and the emerging blood was blotted onto Whatman 3M paper (Whatman International) every 15 seconds. Bleeding times were defined as the time required for all visible signs of bleeding to stop.[26]

Blood collection and preparation of platelets

Whole murine blood was collected in syringes containing 100 μL acid citrate dextrose (ACD; 120 mM sodium citrate, 110 mM glucose, and 80 mM citric acid) by cardiac puncture under diethylether anesthesia. Blood cell counts were determined using an automated blood cell counter. Blood from 2 to 6 mice (2 to 4 months old, both sexes) was pooled for preparing platelet samples. Platelet-rich plasma (PRP) was obtained by centrifuging whole blood at 660*g* for 1 minute at 22°C. The residual blood sample was diluted using 400 μL RCD solution (36 mM citric acid, 5 mM glucose, 5 mM KCl, 103 mM NaCl, and 2 μM PGE_1, pH 6.5) containing 10% (volume/volume) ACD and 0.4 U/mL apyrase, then centrifuged at 660*g* for 1 minute to recover diluted PRP. To prepare washed platelets, PRP was combined with diluted PRP pretreated with 0.2 mM ASA at 37°C for 15 minutes and diluted using an equal volume of RCD solution containing 10% (vol/vol) ACD and 0.4 U/mL apyrase. Washed platelets were then obtained by centrifuging at 1000*g* for 7 minutes. Isolated platelets were resuspended at a final concentration of 5×10^5/μL in modified Tyrode-HEPES buffer (134 mM NaCl, 0.34 mM NaH_2PO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid], and 5 mM glucose, pH 7.3) containing apyrase.

Aggregation studies

Platelet concentrations of PRP were adjusted to 3×10^5/μL using platelet-poor plasma (PPP). A total of 125 μL PRP was placed in siliconized glass tubes and incubated at 37°C for 10 minutes before stimulation. Aggregation was optically monitored using a platelet aggregometer (Hema Tracer TM Model 601; Niko Bioscience, Tokyo, Japan). PPP was used as a reference to indicate 100% aggregation. For studies utilizing thrombin, washed platelets without pretreatment under ASA were suspended in modified Tyrode-HEPES buffer before use.

Flow cytometry

Washed platelets suspended in modified Tyrode-HEPES buffer containing 0.4 U/mL apyrase were labeled using FITC-conjugated antimouse P-selectin antibody (with 1 mM RGDS peptide and 1 mM $CaCl_2$) or Alexa Fluor 488–conjugated human fibrinogen (1 mM $CaCl_2$). Following stimulation with the appropriate concentrations of CRP, labeling of murine platelets was performed for 30 minutes. Samples were then analyzed using a FACSCalibur flow cytometer (Nippon Becton Dickinson, Tokyo, Japan).

Morphologic analysis of adherent platelets under electron microscopy

Control discoid platelets were obtained as follows: platelets in modified Tyrode-HEPES buffer containing 2 mM $MgCl_2$ were fixed in 1% glutaraldehyde, placed on poly-L-lysine–coated coverslips, and allowed to adhere to the surface for 60 minutes. Collagen and CRP were diluted to 10 μg/mL and

6 μg/mL, respectively, with modified Tyrode-HEPES buffer and immobilized on coverslips in culture dishes overnight at 4°C. Coverslips were washed 3 times with modified Tyrode-HEPES buffer and incubated with 2% BSA for 2 hours at room temperature for blocking. Platelets suspended in modified Tyrode-HEPES buffer containing 2 mM $MgCl_2$ (1×10^5 platelets/μL, 100 μL) were brought into contact with collagen- or CRP-coated coverslips in culture dishes and platelets were allowed to adhere at 37°C for 10 minutes. After rinsing 3 times to remove nonadherent platelets, coverslips were further incubated at 37°C for 20, 50, and 80 minutes. Adherent platelets were fixed using 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 minutes. Fixed platelets adhering to coverslips were washed 5 times in 0.1 M phosphate buffer (pH 7.4), postfixed using 1% osmium tetroxide in the same buffer for 15 minutes, dehydrated in a graded ethanol series, and then dried using a Hitachi ES-2020 freeze dryer (Hitachi, Tokyo, Japan) and *t*-butyl alcohol. Specimens were coated with an amorphous and continuous layer of sublimated osmium tetroxide (approximately 10 nm) using an NL-OPC80 osmium plasma coater (Nippon Laser & Electronics Lab, Nagoya, Japan). Slides were then examined under a Hitachi S-4500 field emission scanning electron microscope (Hitachi) at an accelerating voltage of 10 kV. Lengths of filopodia, defined as protrusions from the platelet body less than 130 nm in width, were measured using NIH Image software (National Institutes of Health; http://rsb.info.nih.gov/nih-image/). The area of the CRP-coated slides covered by platelets was analyzed using the same software.

Immunoprecipitation and Western blotting

Platelets in modified Tyrode-HEPES buffer containing 0.4 U/mL apyrase, 1 mM RGDS peptide, and 1 mM EGTA (ethylene glycol tetraacetic acid) were stimulated using the appropriate concentrations of collagen or CRP. Reactions were terminated with the addition of an equal volume of ice-cold lysis buffer (2% Nonidet P40 [NP-40], 20 mM Tris [tris(hydroxymethyl)aminomethane], 300 mM NaCl, 10 mM EDTA [ethylenediaminetetraacetic acid], 2 mM Na_3VO_4, 10 mg/mL aprotinin, 1 mg/mL pepstatin, 10 mg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.3). Debris was removed by centrifugation at 20 000*g* for 10 minutes. Platelet lysates were incubated with antibodies for various proteins and protein A–sepharose beads (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) for 2 hours at 4°C under continuous rotation. The beads were washed extensively in 2-fold diluted lysis buffer. Precipitated proteins were extracted in Laemmli sample buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Separated proteins were transferred to Hybond-ECL membrane (Amersham Pharmacia), which was then blocked in 10% BSA in TBS-T (20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20) and hybridized sequentially using primary antibodies and horseradish peroxidase–conjugated secondary antibody (Amersham Pharmacia). Bound antibodies were detected using an enhanced chemiluminescence (ECL) Western blotting kit (Amersham Pharmacia).

PI3K assay

Platelets were lysed and immunoprecipitated using anti-$p85^{PAN}$ antibody (Upstate Biotechnology), which reacts with all 3 p85α isoforms (p85α, p55α, and p50α) in addition to p85β and p55γ subunits. Beads were washed 3 times in PI3K buffer (25 mM Tris, 0.5 mM EGTA, and 100 mM NaCl, pH 7.4) and suspended in PI3K buffer containing 200 μg/mL phosphatidylinositol. Following preincubation at 37°C for 10 minutes, the reaction was initiated with the addition of 2.5 μL start solution (200 mM $MgCl_2$, 200 μM ATP, 3 μCi [0.111 MBq] γ-^{32}P-ATP; Amersham Pharmacia). After incubation for 10 minutes, the reaction was terminated with the addition of 100 μL stop solution (chloroform, methanol, and 11.6 N HCl in the ratio of 50:100:1). Labeled phosphoinositides were extracted using chloroform, washed 3 times in 100 μL wash solution (mixture of 50 μL methanol and 50 μL 1 N HCl), separated by thin-layer chromatography, and analyzed using a BAS 2000 bioimaging analyzer (Fuji, Tokyo, Japan).

Results

The p85α protein is the major class IA PI3K isoform expressed in platelets

To explore the relative contributions of the various isoforms of class IA PI3K, we first examined the expression of various isoforms in murine platelets by Western blotting using anti-$p85^{PAN}$ antiserum, which has been shown to recognize all variants of p85α (p85α, p55α, and p50α), p85β, and p55γ.[23-25] Among the variety of hematopoietic tissues examined, expression levels of p85α isoform far exceeded those of p55α and p50α in platelets, spleen, thymus, and fetal liver, whereas bone marrow expressed similar amounts of all 3 isoforms (Figure 1A). In platelets from p85α−/− mice, selective disruption of the p85α isoform did not result in significant up-regulation of p55α, p50α, and p85β proteins to compensate for the lack of p85α protein (Figure 1B).[24,25] In addition, expression patterns of p85α, p55α, and p50α isoforms in p85α+/− platelets did not significantly differ from those of p85α+/+ platelets (Figure 1B). The p85β protein was detected in p85α−/− platelets only when the film was overexposed (data not shown). When expression of the p85β protein in platelets was examined using a specific antibody, the levels of p85β in p85α−/− and p85β+/+ platelets were found to be not significantly different (Figure 1C). It was also found that the $p85^{PAN}$ antiserum equally and efficiently captured p85β proteins in platelet lysates from either p85α+/+ or

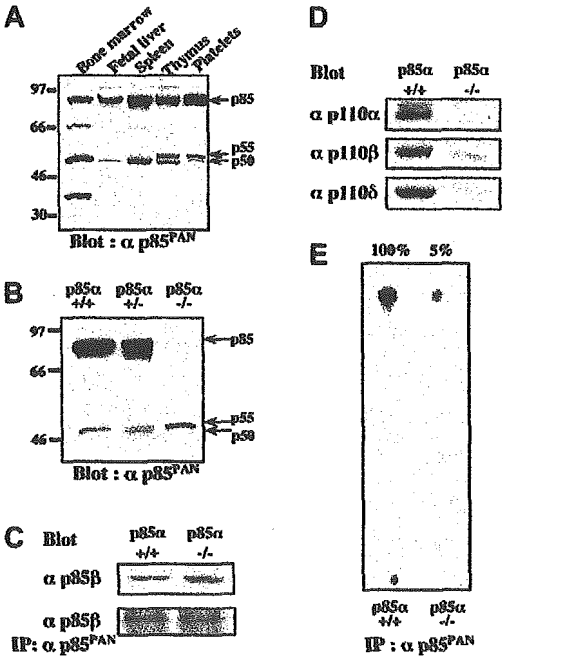

Figure 1. Differential expression patterns of regulatory and catalytic subunits of PI3K class IA and their kinase activities in platelets from p85α−/− and wild-type mice. (A-B) Expression patterns of regulatory subunits of PI3K class IA in wild-type hematopoietic organs (A) and in platelets from wild-type p85α+/+, p85α+/−, and p85α−/− mice (B), shown by Western blot with $\alpha p85^{PAN}$. (C-D) The amount of p85β protein in platelet lysate and its immunoprecipitate with anti-$p85^{PAN}$ antiserum (C) and the amount of p110 catalytic subunits (p110α, p110β, and p110δ) in platelet lysate from p85α+/+ and p85α−/− mice (D), shown by Western blot with p85β-specific antiserum and anti-p110 polyclonal antibodies, respectively. Whole-cell lysate (50 μg per lane) or its immunoprecipitate was resolved using SDS-PAGE. (E) PI3K activity of wild-type and p85α−/− platelets measured using anti-$p85^{PAN}$ antiserum and resolved by thin-layer chromatography. The wild-type response was set to 100% and the activity of p85α−/− platelets was estimated to be 5% of the wild-type response.

p85α−/− mice (Figure 1C). In addition, the expression of catalytic subunit p110α in p85α−/− compared with p85α+/+ platelets was almost undetectable, while expression of p110β and p110δ subunits were greatly reduced in amount, consistent with the instability of the p110 proteins in the absence of sufficient adaptor subunit concentrations (Figure 1D).[27] Consistent with these observations, remaining class IA PI3K activity in p85α−/− platelets was only 5% of the activity in wild-type littermates (Figure 1E).

PI3K p85α-deficient mice displayed perturbation of platelet aggregation response to collagen and CRP, but no bleeding disorders

In contrast to Syk-, SLP-76–, or PLCγ2-deficient mice, PI3K p85α−/− mice were born intact, with no bleeding disorders.[28-30] Bleeding times for PI3K p85α−/− mice (178 ± 89 seconds, n = 5) were not significantly prolonged compared with littermate controls (216 ± 97 seconds, n = 5), and peripheral blood cell counts were indistinguishable among wild-type, p85α+/−, and p85α−/− mice (data not shown). These results suggest that p85α deficiency does not cause profound defects in platelet production or function in vivo. These observations prompted us to examine the effect of PI3K p85α deficiency on platelet function in vitro.

Since many studies have suggested that the involvement of PI3K in signaling cascades in platelets is stimulated by various types of agonists, we investigated the platelet aggregation response in knockout mice. As shown in Figure 2, p85α deficiency led to an approximate 40% to 60% reduction of platelet aggregation in response to suboptimal or optimal concentrations of collagen (10 and 20 μg/mL, respectively) or the GP VI–specific agonist CRP (2.5 and 5 μg/mL). In particular, responses to lower dose stimuli, such as 5 μg/mL collagen or 1 μg/mL CRP, which induced 60% to 80% aggregation in wild-type platelets, were completely abrogated in p85α−/− mice. In contrast, responses to ADP, the thromboxane A_2 analog U46619, thrombin, PMA, A23187, or botrocetin were all intact in p85α−/− mice compared with littermate controls, even at subthreshold concentrations. The same responses to collagen and CRP were observed in both p85α+/− mice and wild-type littermates (data not shown). These results are consistent with previous in vitro observations in humans that class IA PI3K plays an important role in collagen-induced platelet signaling through GP VI engagement.[12,13]

PI3K p85α deficiency led to impaired P-selectin expression or fibrinogen binding in response to CRP

Next we analyzed the impact of p85α deficiency on platelet signaling events induced by GP VI activation. We first investigated whether p85α deficiency affects P-selectin expression or fibrinogen binding in response to CRP stimulation. As shown in Figure 3, panels A and B, 5 μg/mL CRP induced an approximately 10-fold increase in P-selectin expression in wild-type platelets, while expression in p85α−/− platelets was approximately 50% of the wild-type level. The impaired P-selectin expression in p85α−/− platelets was observed at CRP concentrations ranging from 0.01 to 5 μg/mL. Similarly, CRP-induced fibrinogen binding to p85α−/− platelets was significantly impaired (Figure 3C-D).

PI3K p85α-deficient platelets displayed impaired spreading over collagen- or CRP-coated surfaces

In order to further elucidate the role of p85α in collagen-induced platelet signaling, the adhesive response of platelets from knockout

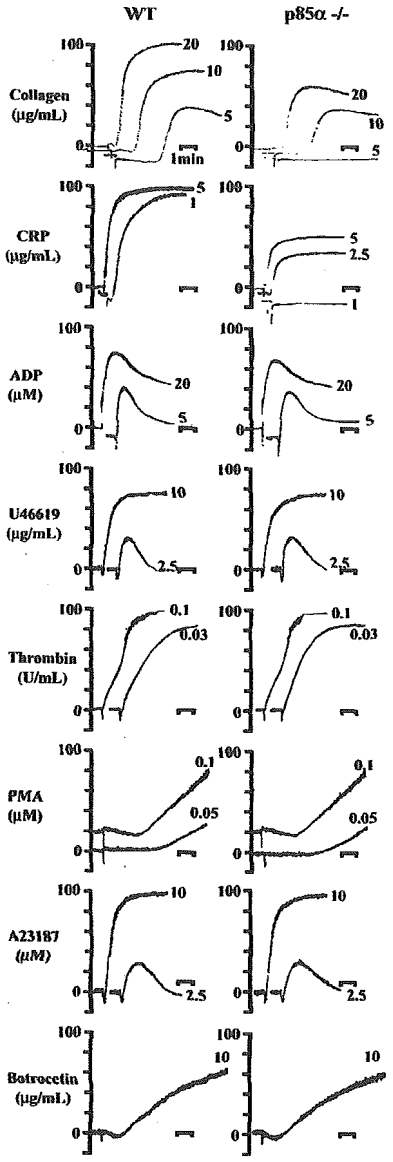

Figure 2. Platelet aggregation responses to various kinds of agonists. PRPs (3×10^5/μL) were incubated at 37°C for 10 minutes prior to stimulation. Changes to morphology and aggregation of platelets from wild-type (WT, left column) and p85α−/− (right column) were measured using an aggregometer after stimulation with collagen, CRP, ADP, U46619, thrombin, PMA, A23187, and botrocetin at the indicated concentrations. Bars indicate 1 minute. Results are from 1 experiment but are representative of at least 4 separate experiments.

mice was investigated. Platelets were placed on collagen- or CRP-coated plates and allowed to adhere to the surface; then morphology was analyzed under scanning electron microscopy. As shown in Figure 4A, wild-type platelets adhered and spread over collagen- or CRP-coated surfaces after 90 minutes of incubation. In contrast, platelets from p85α−/− mice demonstrated reduced spreading, although filopodial protrusions were relatively intact (Figure 4A). Compared with the collagen-coated plates, poor lamellae formation in p85α−/− platelets was pronounced on the CRP-coated plates, consistent with the relative specificity of this isozyme to GP VI pathways among the multiple signaling cascades stimulated by collagen. Indeed, compared with wild-type platelets, filopodia on adhered platelets from p85α−/− mice had increased numbers and length (Figure 4B). These data suggest that p85α

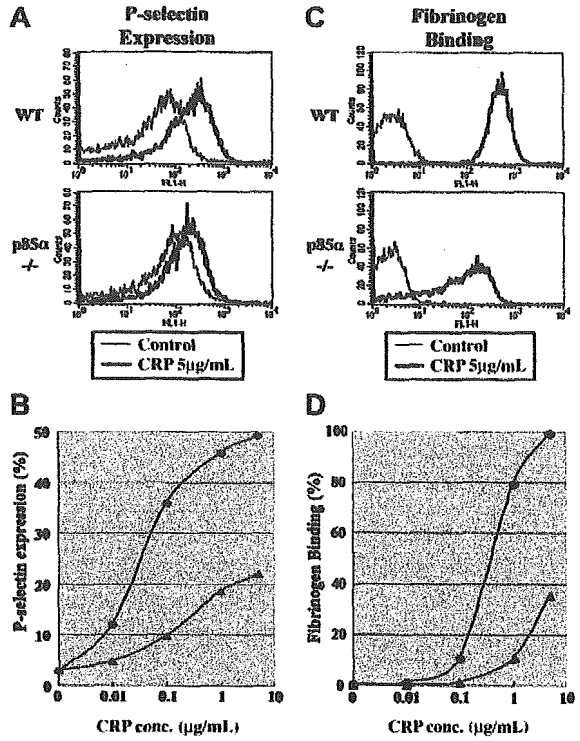

Figure 3. Surface expression of P-selectin on platelets and fibrinogen binding to platelets induced by GP VI stimulation. (A-B) P-selectin expression was detected using FITC-conjugated antimouse P-selectin antibody (A) and analyzed by flow cytometry (B). (C-D) Fibrinogen binding was detected using activated integrin $\alpha_{IIb}\beta_3$ and binding of Alexa Fluor 488–conjugated human fibrinogen (C) and analyzed by flow cytometry (D). Washed wild-type (WT) and p85α−/− platelets suspended in modified Tyrode-HEPES buffer containing apyrase and RGDS peptide with 1 mM $CaCl_2$ (A-B) or modified Tyrode-HEPES buffer containing apyrase with 1 mM $CaCl_2$ (C-D) were stimulated by CRP. Data are from 1 experiment but are representative of 3 independent experiments. In panels B and D, ● indicates WT platelets and ▲ indicates p85α−/− platelets.

plays an essential role in platelet lamellipodia formation during adhesive responses triggered by GP VI engagement.

Collagen and CRP induce a reduction in tyrosine phosphorylation of PLCγ2 and several other downstream molecules in PI3K p85α-deficient platelets

Collagen and CRP induced tyrosine phosphorylation of various signaling molecules, including Syk, LAT, SLP-76, Btk, Tec, Akt/protein kinase B (PKB), and PLCγ2 in platelets.[11,13,28-34] To investigate the underlying molecular mechanisms of impaired platelet function mediated by the GP VI/FcRγ complex in PI3K p85α–deficient mice, we analyzed tyrosine phosphorylation of these signaling molecules in response to collagen and CRP. Key molecules, Syk, LAT, and SLP-76, initially recruited and activated in the vicinity of the GP VI/FcRγ complex, were equally phosphorylated by CRP in both wild-type and p85α−/− platelets (Figure 5A-C). In contrast, phosphorylation of Btk, Tec, or Akt in downstream PH domain–containing effectors for PI3K in response to CRP was partially abrogated in p85α−/− mice (Figure 5D-F). Similar results were also obtained using collagen as the GP VI stimulator (data not shown). It is well known that PLCγ2 is one of the critical targets of collagen- and CRP-induced signaling in platelets. As shown in Figure 5G, phosphorylation of PLCγ2 was also clearly decreased in p85α−/− platelets.

These results suggest that the impaired response to collagen and CRP seen in p85α-deficient platelets is, at least partially, based on reduced phosphorylation and/or activation of PLCγ2.

Discussion

This report provides the first direct evidence of the effect of PI3K p85α deficiency on platelet function in vivo, demonstrating that class IA PI3K functions exclusively as a major element in the GP VI/FcRγ complex–mediated signaling cascade in mice. In contrast, the fact that this enzyme subclass did not exert any significant effect on other relevant platelet signaling pathways, such as those triggered by thrombin, ADP, U46619, and botrocetin, was rather unexpected.

GP VI ligation by collagen or CRP initiates intracellular signals through phosphorylated ITAMs on the FcRγ chain, leading to activation of serially connected downstream molecules in a phosphotyrosine-dependent manner.[13] At the end of this signaling cascade, the fully activated PLCγ2 is assembled at or in the vicinity of membrane rafts with signaling complexes such as tyrosine kinases Lyn/Fyn, Syk, Btk/Tec, and adaptors of LAT and SLP-76.[35-37] Evidence of the participation of class IA PI3K in this particular signaling cascade is that in the absence of PI3K p85α, platelets become defective in the following GP VI–induced signaling events: platelet aggregation, degranulation of α granules, integrin activation, lamellipodia formation, and tyrosine phosphorylation of putative effector molecules. Consistent with this conclusion, Btk mutations in humans[32,33] and Syk, LAT, SLP-76, and

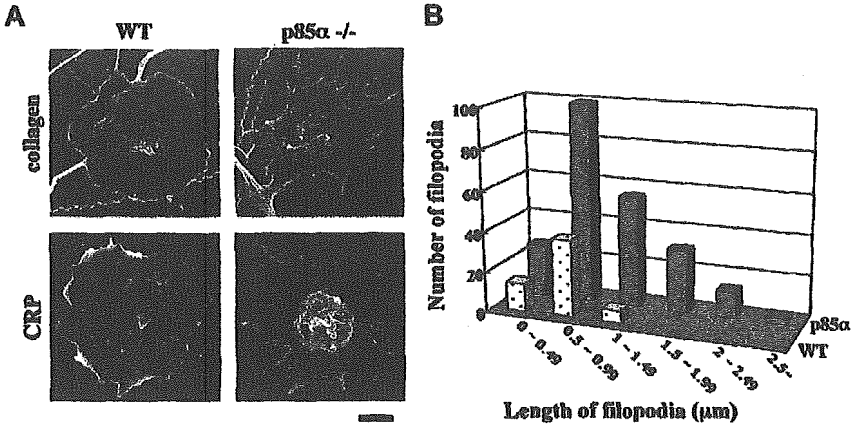

Figure 4. Morphologic examination of platelets adhering to collagen- or CRP-coated surfaces. (A) Washed platelets suspended in modified Tyrode-HEPES buffer containing apyrase and 2 mM $MgCl_2$ were exposed to surfaces coated with collagen (top row) or CRP (bottom row). Scanning electron images show wild-type (WT; left panels) and 85α−/− (right panels) platelets after 90 minutes of incubation. Scale bar indicates 1 μm. (B) Frequency analysis of the length of remnant filopodia from wild-type (dotted columns) and p85α−/− (solid columns) platelets (n = 50) adhering to CRP-coated surface after 60 minutes. Analysis was performed using NIH Image software.

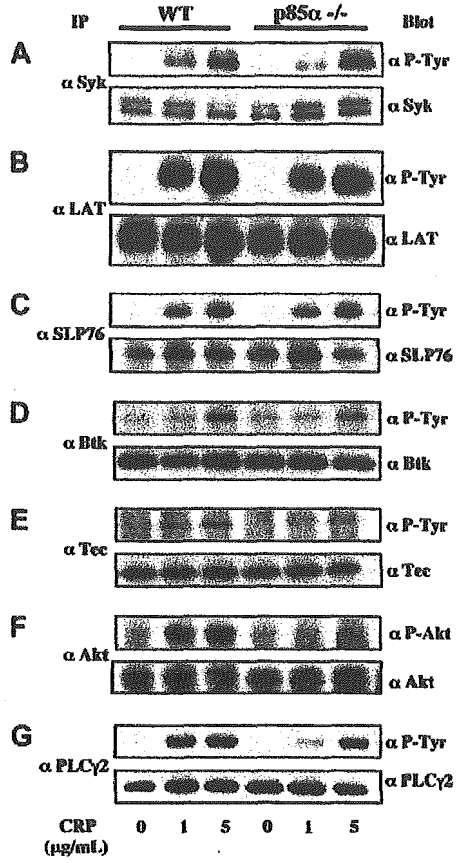

Figure 5. Protein phosphorylation in GP VI–stimulated wild-type and PI3K p85α−/− platelets. Murine platelets were treated using 0.2 mM acetylsalicylic acid and suspended in modified Tyrode-HEPES buffer containing 0.4 U/mL apyrase, 1 mM RGDS peptide, and 1 mM EGTA. The platelets were stimulated with CRP at 0, 1, and 5 μg/mL on an aggregometer with constant stirring and were lysed 90 seconds after stimulation. Then they were subjected to immunoprecipitation using anti-Syk (A), anti-LAT (B), anti–SLP-76 (C), anti-Btk (D), anti-Tec (E), anti-Akt (F), and anti-PLCγ2 (G) antibodies. Proteins were resolved using SDS-PAGE, transferred to nitrocellulose membrane, immunoblotted with antiphosphotyrosine (P-Tyr) antibody 4G10 or anti–phospho-Akt–specific antibody, and reprobed with the antibodies used for immunoprecipitation to demonstrate equal amounts of immunoprecipitated proteins in each lane. Identical results were obtained in at least 4 separate experiments.

PLCγ2 mutations in mice[28-31] all produce platelet phenotypes similar to those seen in p85α-deficient mice.

The finding that GP VI–induced tyrosine phosphorylation of Syk, LAT, and SLP-76 is not defective in p85α-deficient platelets might indicate that these molecules are active upstream or are independent of the PI3K-pathway. In fact, the p85 subunit of class IA PI3K has been shown to associate with tyrosine-phosphorylated LAT and tyrosine-phosphorylated ITAM of the FcRγ chain through each of the tandem SH2 domains following GP VI stimulation.[12] This association then triggers PI3K-pathway activation, resulting in the liberation of D3 phosphoinositides. LAT is tyrosine-phosphorylated by Syk following binding of the kinase to the phosphorylated ITAM of the FcRγ chain. LAT then forms a complex with SLP-76 via the adaptor protein Gads, becoming a scaffold for PLCγ2 recruitment.[13,31] The PI3K lipid products target the Btk PH domain and the PLCγ2 SH2 or PH domains to induce subcellular localization.[13,38] Therefore, 2 independent pathways exist—one mediated by membrane protein LAT and the other by PI3K membrane lipid products—for the full activation of PLCγ2 at specific membrane microdomains. Keeping this model in mind, the finding that the loss of the p85α protein does not exert a profound effect on platelet function is not particularly surprising. Although PLCγ2 activity in p85α-deficient platelets was not measured in this study, we identified partial but substantial reduction in GP VI–induced tyrosine phosphorylation of PLCγ2 in p85α-deficient platelets, which may be due to the incomplete defect in platelet cellular responses following GP VI stimulation. Other groups have reported that treatment with pharmacologic PI3K inhibitors strongly suppresses GP VI–induced PLCγ2 activation but has a minimal effect on tyrosine phosphorylation in human platelets.[11,17] This discrepancy might be attributed to species differences or the methods of inducing PI3K disruption. The lipid kinase activity of class IA PI3K is almost completely deleted in p85α-deficient platelets. Only trace amounts of activity may be attributed to the very low levels of the other kinase isoforms associated with the p85α splice variants p55 and p50, p85β, or p55γ. Nevertheless, complete loss of p85α activity does not result in the severe platelet phenotype seen in response to GP VI stimulation, supporting the hypothesis that full activation of PLCγ2 may involve a PI3K-independent pathway, possibly via the scaffold complex formed by LAT, Gads, and SLP-76.[13,39] However, this concept is challenged by the finding that the extent and spectrum of immune deficiency resulting from the loss of p85α in mice B lymphocytes closely approximates those seen in PLCγ2-deficient mice.[25,30,40] In addition, mice with the Btk deficiency Xid (a naturally occurring point mutation in the PH domain of the kinase) are unable to bind PI3K products PtdIns-3,4,5-P3 or SLP-65/BLNK (an adaptor connecting Btk and PLCγ2 and thought to be a counterpart of the LAT/SLP-76 adaptor complex in T lymphocytes) and also display remarkably similar immune phenotypes. This indicates that p85α and PLCγ2 are serially connected through Btk and SLP-65/BLNK.[41-43] Although this discrepancy may simply be due to differences in cell type, careful comparisons of the platelet phenotypes of p85α-deficient mice and PLCγ2-deficient mice of identical genetic backgrounds are required to address the issue.

Partial reduction of GP VI–induced tyrosine phosphorylation of Btk and Tec in p85α-deficient platelets supports the previously proposed hypothesis that 2 PH domains containing tyrosine kinases are located proximally to class IA PI3K and function as redundant PI3K effectors in human platelets.[33] B lymphocytes from Tec-deficient mice exhibit no apparent immune phenotype, but the double deficiency of Btk and Tec results in a more severe disorder than the Btk deficiency alone.[44] Whether this is also true for murine platelets is yet to be determined. Since the immune phenotype of Xid mice (with normal levels of protein expression) is comparable to that of Btk-deficient animals, Btk activation should be virtually PI3K-dependent.[45] Despite nearly total loss of PI3K activity and expression in p85α-deficient platelets, minimal tyrosine phosphorylation of both kinases remains inducible by GP VI stimulation. This implies the existence of an alternate pathway for Btk/Tec activation that is independent of PI3K.[46,47]

The defective spreading of p85α-deficient platelets over collagen- or CRP-coated surfaces is most likely caused by the loss of PI3K products. Lamellipodia formation in adherent cells is mediated by Rac-1, a member of the Rho family of small guanosine triphosphatases (GTPases). This protein is regulated by a PH domain–containing GEF Vav family.[48,49] In fact, Vav-1, a member of this family, is present in high quantities in platelets.[48] In contrast, filopodial protrusion is mediated through the RhoA small GTPase with the help of cdc42 GEF, which is regulated in a PI3K-independent manner.[49] The observation that p85α-deficient platelets show defective lamellipodia formation while retaining intact filopodial protrusions is therefore unsurprising. In agreement with

these observations, treatment of human platelets with wortmannin and LY294002 followed by contact with a collagen- or CRP-coated surface reportedly results in similar phenotypic changes to platelet morphology.[17]

In p85α-deficient mice, the apparent lack of alteration in platelet aggregation response to other important platelet stimulators (thrombin, ADP, U46619, PMA, A23187, and botrocetin) was unexpected, since class IA PI3K involvement has been proposed to activate $\alpha_{IIb}\beta_3$ integrin (inside-out signaling) or assist in the postaggregation response through integrin engagement (outside-in signaling)[4,50] in the final common pathways. For example, thrombin- or PMA-induced accumulation of PtdIns-3,4-P2 or PtdIns-3,4,5-P3 and the concomitant up-regulation of $\alpha_{IIb}\beta_3$ integrin receptor function in human platelets are effectively inhibited by low nanomolar ranges of wortmannin, indicating that class IA PI3K is downstream of protein kinase C in the induction of integrin activation.[4,51,52] Following thrombin stimulation, the p85α subunit is reportedly translocated into the focal contact area of human platelets by association with the SH3 domain and the proline-rich region of the focal adhesion kinase p125FAK.[18] However, these observations disagree with our finding that p85α-deficient platelets display intact aggregation responses to thrombin and PMA, even at suboptimal concentrations. One possible explanation for the discrepancy may be the existence of a class II PI3K, which is also sensitive to wortmannin, downstream of $\alpha_{IIb}\beta_3$ integrin.[53] Activation of GPCRs triggered by weak agonists such as ADP and U46619 readily induces PI3K activity in platelets.[4,19,20] G protein βγ–specific PI3Kγ is the most plausible candidate for this particular pathway, and class IA PI3K is also postulated to be activated directly by G protein βγ subunits or tyrosine-phosphorylated intermediates following GPCR engagement.[9,21] Indeed, a recent study has shown that PI3K 110γ–deficient platelets exhibit impaired response to ADP, and the defect is limited to the Gi-coupled ADP receptor (P2Y12).[54] This finding, together with the observations of p85α-deficient platelets described herein, suggests that other PI3K species with involvement in the signaling pathway elicited by thrombin and U46619 or with redundant functions may exist.

The snake venom–derived botrocetin induces binding of von Willebrand factor via the receptor GP Ib/IX/V complex and mediates platelet-platelet interactions that may be accompanied by activation of class IA PI3K through either the cytoplasmic tail of the complex or ITAM-containing FcR (FcRγ in humans and mice and FcγRIIA in humans) colocalized with the complex.[16,55,56] However, we were unable to observe any perturbed aggregatory response to botrocetin, even at suboptimal concentrations. The role of p85α in the GP Ib/IX/V pathway thus requires further study.

In conclusion, absence of PI3K p85α in mice leads to compromised platelet responses to GP VI stimulation in vitro, but no significant bleeding disorder. Although class IA PI3K is reportedly involved in multiple signaling pathways or different stages of cellular process in platelets, the p85α isoform functions exclusively as a major component of the ITAM-mediated signaling pathway. Platelets lack a nucleus but retain a similar set of intracellular machinery for immune receptor signaling. The cells therefore represent appropriate targets for research to elucidate the mechanisms of immediate immune responses.

Acknowledgments

We wish to thank Dr Owen Witte and Dr Yoshihiro Fujimura for providing anti-Btk antibody and botrocetin, respectively. We would also like to thank Mari Fujiwara for technical assistance in the PI3K assay.

References

1. Katso R, Okkenhaug K, Ahmadi K, White S, Timms J, Waterfield MD. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annu Rev Cell Dev Biol. 2001;17:615-675.
2. Vanhaesebroeck B, Leevers SJ, Ahmadi K, et al. Synthesis and function of 3-phosphorylated inositol lipids. Annu Rev Biochem. 2001;70:535-602.
3. Rameh LE, Cantley LC. The role of phosphoinositide 3-kinase lipid products in cell function. J Biol Chem. 1999;274:8347-8350.
4. Rittenhouse SE. Phosphoinositide 3-kinase activation and platelet function. Blood. 1996;88:4401-4414.
5. Zhang J, Shattil SJ, Cunningham MC, Rittenhouse SE. Phosphoinositide 3-kinase gamma and p85/phosphoinositide 3-kinase in platelets. Relative activation by thrombin receptor or beta-phorbol myristate acetate and roles in promoting the ligand-binding function of alphaIIbbeta3 integrin. J Biol Chem. 1996;271:6265-6272.
6. Zhang J, Vanhaesebroeck B. Human platelets contain p110delta phosphoinositide 3-kinase. Biochem Biophys Res Commun. 2002;296:178-181.
7. Fruman DA, Cantley LC, Carpenter CL. Structural organization and alternative splicing of the murine phosphoinositide 3-kinase p85 alpha gene. Genomics. 1996;37:113-121.
8. Chan TO, Rodeck U, Chan AM, et al. Small GTPases and tyrosine kinases coregulate a molecular switch in the phosphoinositide 3-kinase regulatory subunit. Cancer Cell. 2002;1:181-191.
9. Kurosu H, Maehama T, Okada T, et al. Heterodimeric phosphoinositide 3-kinase consisting of p85 and p110beta is synergistically activated by the betagamma subunits of G proteins and phosphotyrosyl peptide. J Biol Chem. 1997;272:24252-24256.
10. Thomason PA, James SR, Casey PJ, Downes CP. A G-protein beta gamma-subunit-responsive phosphoinositide 3-kinase activity in human platelet cytosol. J Biol Chem. 1994;269:16525-16528.
11. Pasquet JM, Bobe R, Gross B, et al. A collagen-related peptide regulates phospholipase Cgamma2 via phosphatidylinositol 3-kinase in human platelets. Biochem J. 1999;342:171-177.
12. Gibbins JM, Briddon S, Shutes A, et al. The p85 subunit of phosphatidylinositol 3-kinase associates with the Fc receptor gamma-chain and linker for activitor of T cells (LAT) in platelets stimulated by collagen and convulxin. J Biol Chem. 1998;273:34437-34443.
13. Watson SP, Asazuma N, Atkinson B, et al. The role of ITAM- and ITIM-coupled receptors in platelet activation by collagen. Thromb Haemost. 2001;86:276-288.
14. Gratacap MP, Herault JP, Viala C, et al. FcgammaRIIA requires a Gi-dependent pathway for an efficient stimulation of phosphoinositide 3-kinase, calcium mobilization, and platelet aggregation. Blood. 2000;96:3439-3446.
15. Saci A, Pain S, Rendu F, Bachelot-Loza C. Fc receptor-mediated platelet activation is dependent on phosphatidylinositol 3-kinase activation and involves p120(Cbl). J Biol Chem. 1999;274:1898-1904.
16. Munday AD, Mitchell CA. Phosphoinositide 3-kinase forms a complex with platelet membrane glycoprotein Ib-IX-V complex and 14-3-3zeta. Blood. 2000;96:577-584.
17. Falet H, Barkalow KL, Barnes MJ, Geha RS, Hartwig JH. Roles of SLP-76, phosphoinositide 3-kinase, and gelsolin in the platelet shape changes initiated by the collagen receptor GPVI/FcR gamma-chain complex. Blood. 2000;96:3786-3792.
18. Guinebault C, Payrastre B, Racaud-Sultan C, Breton M, Mauco G, Chap H. Integrin-dependent translocation of phosphoinositide 3-kinase to the cytoskeleton of thrombin-activated platelets involves specific interactions of p85 alpha with actin filaments and focal adhesion kinase. J Cell Biol. 1995;129:831-842.
19. Kauffenstein G, Bergmeier W, Eckly A, et al. The P2Y(12) receptor induces platelet aggregation through weak activation of the alpha(IIb)beta(3) integrin—a phosphoinositide 3-kinase-dependent mechanism. FEBS Lett. 2001;505:281-290.
20. Lauener RW, Stevens CM, Sayed MR, Salari H, Duronio V. A role for phosphatidylinositol 3-kinase in platelet aggregation in response to low, but not high, concentrations of PAF or thrombin. Biochim Biophys Acta. 1999;1452:197-208.
21. Ptasznik A, Traynor-Kaplan A, Bokoch GM. G protein-coupled chemoattractant receptors regulate Lyn tyrosine kinase Shc adapter protein signaling complexes. J Biol Chem. 1995;270:19969-19973.
22. Jung SM, Moroi M. Platelets interact with soluble and insoluble collagens through characteristically different reactions. J Biol Chem. 1998;273:14827-14837.

23. Inukai K, Funaki M, Ogihara T, et al. p85 gene generates three isoforms of regulatory subunit for phosphatidylinositol 3-kinase (PI3-Kinase), p50α, p55α, and p85α, with different PI3-Kinase activity elevating responses to insulin. J Biol Chem. 1997;272:7873-7882.
24. Terauchi Y, Tsuji Y, Satoh S, et al. Increased insulin sensitivity and hypoglycaemia in mice lacking the p85 alpha subunit of phosphoinositide 3-kinase. Nat Genet. 1999;21:230-235.
25. Suzuki H, Terauchi Y, Fujiwara M, et al. Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. Science. 1999;283:390-392.
26. Offermanns S, Toombs CF, Hu Y, Simon MI. Defective platelet activation in Gαq-deficient mice. Nature. 1997;389:183-186.
27. Fruman DA, Mauvais-Jarvis F, Pollard DA, et al. Hypoglycaemia, liver necrosis and perinatal death in mice lacking all isoforms of phosphoinositide 3-kinase p85α. Nat Genet. 2000;26:379-382.
28. Poole A, Gibbins JM, Turner M, et al. The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. EMBO J. 1997;16:2333-2341.
29. Clements JL, Lee JR, Gross B, et al. Fetal hemorrhage and platelet dysfunction in SLP-76-deficient mice. J Clin Invest. 1999;103:19-25.
30. Wang D, Feng J, Wen R, et al. Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity. 2000;13:25-35.
31. Pasquet JM, Gross B, Quek L, et al. LAT is required for tyrosine phosphorylation of phospholipase cgamma2 and platelet activation by the collagen receptor GPVI. Mol Cell Biol. 1999;19:8326-8334.
32. Quek LS, Bolen J, Watson SP. A role for Bruton's tyrosine kinase (Btk) in platelet activation by collagen. Curr Biol. 1998;8:1137-1140.
33. Oda A, Ikeda Y, Ochs HD, et al. Rapid tyrosine phosphorylation and activation of Bruton's tyrosine/Tec kinases in platelets induced by collagen binding or CD32 cross-linking. Blood. 2000;95:1663-1670.
34. Barry FA, Gibbins JM. Protein kinase B is regulated in platelets by the collagen receptor glycoprotein VI. J Biol Chem. 2002;277:12874-12878.
35. Locke D, Chen H, Liu Y, Liu C, Kahn ML. Lipid rafts orchestrate signaling by the platelet receptor glycoprotein VI. J Biol Chem. 2002;277:18801-18809.
36. Ezumi Y, Kodama K, Uchiyama T, Takayama H. Constitutive and functional association of the platelet collagen receptor glycoprotein VI-Fc receptor gamma-chain complex with membrane rafts. Blood. 2002;99:3250-3255.
37. Bodin S, Giuriato S, Ragab J, et al. Production of phosphatidylinositol 3,4,5-trisphosphate and phosphatidic acid in platelet rafts: evidence for a critical role of cholesterol- enriched domains in human platelet activation. Biochemistry. 2001;40:15290-15299.
38. Bobe R, Wilde JI, Maschberger P, et al. Phosphatidylinositol 3-kinase-dependent translocation of phospholipase Cgamma2 in mouse megakaryocytes is independent of Bruton tyrosine kinase translocation. Blood. 2001;97:678-684.
39. Judd BA, Myung PS, Obergfell A, et al. Differential requirement for LAT and SLP-76 in GPVI versus T cell receptor signaling. J Exp Med. 2002;195:705-717.
40. Fruman DA, Snapper SB, Yballe CM, et al. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. Science. 1999;283:393-397.
41. Fruman DA, Cantley LC. Phosphoinositide 3-kinase in immunological systems. Semin Immunol. 2002;14:7-18.
42. Leo A, Schraven B. Adapters in lymphocyte signalling. Curr Opin Immunol. 2001;13:307-316.
43. Wong J, Ishiai M, Kurosaki T, Chan AC. Functional complementation of BLNK by SLP-76 and LAT linker proteins. J Biol Chem. 2000;275:33116-33122.
44. Ellmeier W, Jung S, Sunshine MJ, et al. Severe B cell deficiency in mice lacking the tec kinase family members Tec and Btk. J Exp Med. 2000;192:1611-1624.
45. Satterthwaite AB, Witte ON. The role of Bruton's tyrosine kinase in B-cell development and function: a genetic perspective. Immunol Rev. 2000;175:120-127.
46. Fruman DA, Ferl GZ, An SS, Donahue AC, Satterthwaite AB, Witte ON. Phosphoinositide 3-kinase and Bruton's tyrosine kinase regulate overlapping sets of genes in B lymphocytes. Proc Natl Acad Sci U S A. 2002;99:359-364.
47. Satterthwaite AB, Willis F, Kanchanastit P, et al. A sensitized genetic system for the analysis of murine B lymphocyte signal transduction pathways dependent on Bruton's tyrosine kinase. Proc Natl Acad Sci U S A. 2000;97:6687-6692.
48. Nobes CD, Hall A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell. 1995;81:53-62.
49. Soulet C, Gendreau S, Missy K, Benard V, Plantavid M, Payrastre B. Characterisation of Rac activation in thrombin- and collagen-stimulated human blood platelets. FEBS Lett. 2001;507:253-258.
50. Miyakawa Y, Oda A, Druker BJ, et al. Thrombopoietin and thrombin induce tyrosine phosphorylation of Vav in human blood platelets. Blood. 1997;89:2789-2798.
51. Kovacsovics TJ, Bachelot C, Toker A, et al. Phosphoinositide 3-kinase inhibition spares actin assembly in activating platelets but reverses platelet aggregation. J Biol Chem. 1995;270:11358-11366.
52. Toker A, Bachelot C, Chen CS, et al. Phosphorylation of the platelet p47 phosphoprotein is mediated by the lipid products of phosphoinositide 3-kinase. J Biol Chem. 1995;270:29525-29531.
53. Zhang J, Banfic H, Straforini F, Tosi L, Volinia S, Rittenhouse SE. A type II phosphoinositide 3-kinase is stimulated via activated integrin in platelets. A source of phosphatidylinositol 3-phosphate. J Biol Chem. 1998;273:14081-14084.
54. Hirsch E, Bosco O, Laffargue M, Altruda F, Montrucchio G. Resistance to thromboembolism in PI3Kgamma-deficient mice. FASEB J. 2001;15:2019-2021.
55. Sullam PM, Hyun WC, Szollosi J, Dong J, Foss WM, Lopez JA. Physical proximity and functional interplay of the glycoprotein Ib-IX-V complex and the Fc receptor FcgammaRIIA on the platelet plasma membrane. J Biol Chem. 1998;273:5331-5336.
56. Wu Y, Suzuki-Inoue K, Satoh K, et al. Role of Fc receptor gamma-chain in platelet glycoprotein Ib-mediated signaling. Blood. 2001;97:3836-3845.

原著

フィブリノーゲンγ鎖ドデカペプチド結合粒子の血小板代替物の展開

Development of fibrinogen γ chain dodecapeptide-conjugated particles

岡村陽介[1)]，寺村裕治[1)]，武岡真司[1)]，土田英俊[1)]，鈴木英紀[2)]，渡辺直英[3)]，半田　誠[3)]，池田康夫[3)]

Yosuke Okamura[1)], Yuji Teramura[1)], Shinji Takeoka[1)], Eishun Tsuchida[1)], Hidenori Suzuki[2)], Naohide Watanabe[3)], Makoto Handa[3)], Yasuo Ikeda[3)]

和文抄録

血小板代替物としての基礎知見を得るために，フィブリノーゲンγ鎖C末端のアミノ酸配列（400-411，CHHLGGAKQAGDV，H12）を担持したラテックスビーズ（H12-ラテックスビーズ）を用い，活性化した血小板のみを認識して結合する系の構築を検討した．フローサイトメトリーによるPAC-1結合，抗P-セレクチン抗体結合，アネキシンV結合では，正常血小板に対する相互作用はほとんど見られなかった．更に，H12-ラテックスビーズは，血小板数減少症（正常値の10分の1）を再現した血液流動下において，出血部位モデル基板であるコラーゲン基板に対して血小板の凝集を促進した．このことから，H12を担持した微粒子では，二次凝集を引き起こすことが期待でき，血小板代替物の認識部位として有効であると考えられた．

Abstract

In order to perform a fundamental study of platelet substitutes, novel particles that bound to activated platelets were prepared by conjugating a oligopeptide, CHHLGGAKQAGDV (H12), which is a fibrinogen γ-chain carboxy-terminal sequence (γ 400-411). Based on the result of flow cytometric analyses of agglutination, PAC-1 binding, anti P-selectin antibody binding, and annexin V binding, the H12-conjugated latex beads showed minimal interaction with non-activated platelets. Furthermore, H12-conjugated latex beads enhanced the *in vitro* platelets thrombus formation on collagen-immobilized plates under flowing thrombocytopenic-imitation blood. These results indicate the excellent potential of H12-conjugated particles as a candidate for a platelet substitute.

Keywords

platelet substitutes, dodecapeptide (H12), fibrinogen, latex beads, flow cytometry

1．はじめに

血小板輸血は，外科手術，悪性腫瘍に対する化学療法，あるいは骨髄移植時において必要不可欠な補助療法であり，医療技術の進歩と共にその使用量も年々増大している．しかしながら，血小板製剤はその短い保存期間（72時間）のために慢性的な供給不足の状態にあるので災害など緊急時の対応は困難になるものと予想されている．核酸増幅検査（NAT）の導入により血液製剤の安全性は著しく向上したものの，未だにウィルス感染などのリスクは否定できず社会的な問題となっており，ヒト由来に依存しない血小板代替物（人工血小板）の開発が急がれている．最近では，遺伝子組換え技術の向上により，血小板機能異常症ならびに血小板輸血代替療法として，胚性幹細胞からの血小板産生技術の開発も期待されている[1)]．

血小板代替物の開発は国内外問わずまだ緒についたところで

1) 早稲田大学 理工学総合研究センター　〒169-8555 東京都新宿区大久保3-4-1 Advanced Research Institute for Science and Engineering, Waseda University, 3-4-1 Ohkubo, Shinjuku-ku, Tokyo 169-8555, Japan. 2) 東京都臨床医学総合研究所循環器病研究部門　〒113-8613 東京都文京区本駒込3-18-22 Medical Research and Development Center, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Motokomagome, Bunkyo-ku Tokyo, 113-8613, Japan. 3) 慶応義塾大学 医学部内科　〒169-8582 東京都新宿区信濃町 35 Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 169-8582, Japan.

論文受付 2004年1月15日　受理 2004年1月30日

あり，研究段階にとどまっている[2-6]．我々のグループでは，血小板膜蛋白質GPIbαあるいはGPIa/IIa複合体の遺伝子組換え体（rGPIbα、rGPIa/IIa複合体）を遺伝子組換えヒト血清アルブミンから調製した微粒子（アルブミン重合体）[7]やリン脂質小胞体[8]の担体に担持させて，出血部位に特異的に集積する微粒子を作製，*in vitro*系あるいは*in vivo*系における止血能評価を行なっている[7-14]．例えば，血小板数が正常の5分の1に減少した血小板数減少症マウスを用いた動物実験では，rGPIa/IIa複合体を担持させたアルブミン重合体にて出血時間の大幅短縮に成功した（切創モデル）[11]．ところが，rGPIbαあるいはrGPIa/IIa複合体を認識部位として利用する微粒子は，出血部位への特異的集積はみられるものの，残存血小板を巻き込んだ血小板凝集（二次凝集）を期待するには更なる工夫が必要である．

本論文では，出血部位へ粘着後，残存している血小板の凝集を促進する微粒子を構築するため，フィブリノーゲンγ鎖C末端のアミノ酸配列（400-411，H12[15-17]）を認識部位として着目した．フィブリノーゲンは，溶液状態では熱変性のために不安定であり，製剤としての利用が困難である[10]．従って，活性化血小板に発現するGPIIb/IIIaを認識するH12やRGDを含むペプチドを選択し，これを*in vitro*用評価に有効であるラテックスビーズに担持させて，流動下において流動血小板の粘着挙動を解析した．

2．実験方法

2.1 試薬，試料

フィブリノーゲンγ鎖 ドデカペプチド（HHLGGAKQAGDV，H12）のN末端にシステインを結合したH12とRGDを有するオリゴペプチド（C-GGRGDF，RGD）は，固相合成法により合成した（ベックス（株））．FITC標識ラテックスビーズ（Fluoresbrite™ plain，200 nm，1 μm）はPolysciences社，*N*-succinimidyl 3-(2-pyridyldithio) propionate（SPDP）はPierce Chemical 社から購入した．3,3'-Dihexyloxacarbocyanine iodide（$DiOC_6$）はMolecular Probes社，D-Phe-Pro-Arg-chloromethylketone（PPACK）はCalbiochem社，FITC標識PAC-1，FITC標識抗P-セレクチン（CD62P）抗体は，それぞれBecton Dickinson社，BD-Pharmingen社から購入した．遺伝子組換えヒト血清アルブミン（rHSA）は，三菱ウェルファーマ社（株）より提供頂いた．

2.2 H12-，RGD-ラテックスビーズの調製

rHSA溶液（50 mg/mL）にラテックスビーズを混合、振とうさせた（20℃，2 hr）．遠心分離（13000g，5分，4℃，3回）にて未吸着rHSAを除去後，リン酸緩衝液（PBS，pH7.4，500 μL）にて再分散させた．rHSA吸着ラテックスビーズ分散液（rHSA-ラテックスビーズ，4.0x10⁶/μL，500 μL）にSPDPエタノール溶液（5 mM，5 μL）を添加，振とうさせた（20℃，30 min）．未反応SPDP，副生成物を遠心分離（13000g，5分，4℃，3回）にて除去後，ピリジルジスルフィド基結合rHSA-ラテックスビーズ（PD-ラテックスビーズ）を得た．

PD-ラテックスビーズ（4.0x10⁶/μL，500 μL）にH12（10 mM，8 μL）あるいはRGD（10 mM，8 μL）を混合，振とうさせた（20℃，12時間）．遠心分離（13000g，5分，4℃，3回）にて上清を除去後，H12-ラテックスビーズあるいはRGD-ラテックスビーズ（2.0x10⁶/μL，1 mL）を得た．ラテックスビーズ表面のH12あるいはRGD結合量は，チオール・ジスルフィド交換反応で化学量論的に遊離する2-チオピリドンの343 nmにおける吸光度からHPLCを用いて決定した（TSK-GEL G3000SW$_{XL}$ column，7.8mm o.d. x 300mm h，1 mL/min；PBS）[7]．

2.3 凝集計を用いた機能評価

健常人より採取した全血に3.8%（w/v）クエン酸ナトリウムを添加して（1/10（v/v）），遠心分離後（100g，15分，22℃），多血小板血漿（PRP）を得た．遠心分離（2200g，10分，22 ℃）にて得た乏血小板血漿（PPP）をPRPに添加して血小板濃度2.0×10⁵/μLに調整した．血小板濃度は自動血球計測計（K-4500，シスメックス社（株））を用いて測定した．

H12あるいはRGDを混合したPRPに，終濃度20 μMとなるようにADPを添加し，血小板凝集計（HEMA TRACER T-638，日光バイオサイエンス社）を用いて血小板分散液の透過光変化を連続的に計測した．同様に，PRP（160 μL）にH12-ラテックスビーズ（20 μL，各1.0，2.0 x 10²，2.0 x 10³/μL）を添加し攪拌後，ADP（20 μL，3 μM）にて血小板を活性化させ，分散液の透過光変化を計測した．

2.4 フローサイトメトリーを用いた血小板解析

血小板分画からの散乱光あるいは蛍光と区別するために，粒径200 nmのラテックスビーズを用いて，H12-あるいはRGD-ラテックスビーズを調製した（2.2参照）．

H12-あるいはRGD-ラテックスビーズ（1.0×10⁵/μL）と，PRP（2.0×10⁴/μL）を混合し，バイオシェイカー（BR-13UM，タイテック（株））を用いて攪拌後（200rpm，37℃，30分），ホルムアルデヒド（1.5 wt%）にて固定した．フローサイトメーター（FACSCalibur，日本ベクトン・ディッキンソン社（株））を用いて側方散乱光，前方散乱光にて血小板分画を選択し，全血小板（20000 counts）に対してFITC由来の蛍光が検出される血小板の割合を算出した．

PRP（1.0×10⁵/μL）とH12-ラテックスビーズ（1.0×10⁵/μL）を混合し，37℃にて30分間攪拌，FITC標識抗P-セレクチン抗体（0.5 μg）を添加後，37℃にて10分間振とうした．続いてホルムアルデヒド（1.5 wt%）にて固定した．また，PRP（1.0×10⁵/μL）とH12-ラテックスビーズ（1.0×10⁵/μL）を混合液にFITC標識PAC-1（0.5 μg）を添加後，37℃にて10分間振とう，ホルムアルデヒド（1.5 wt%）にて固定した．ポジティブコントロール群として，10分間ADP刺激（20 μM，100 μM）させた血小板を用いた．フローサイトメーターにより，血小板分画のFITC蛍光からP-セレクチン抗体あるいはPAC-1の結合率を算出した．

2.5 流動下におけるH12-ラテックスビーズの機能評価

1）コラーゲン固定化基板の調製[10]

コラーゲンI-A（3.0 mg/mL，Cellmatrix，新田ゼラチン（株））

を終濃度30μg/mLとなるようにPBS（4℃）に分散し，ガラス基板（直径24 mm，厚み0.5 mm）を浸漬させた（4℃，8時間）．PBSにて洗浄後，ウシ血清アルブミン溶液中（BSA，20 mg/mL）に浸漬させた（20℃，2時間）．

2）血小板固定化基板の調製法[10]

PRPにACD溶液を混合しプロスタグランジン E_1（PGE_1，終濃度1μM）を添加後2200gで7分間遠心し，上清を除去後RCD溶液（含1μM PGE_1）に再分散させた．同操作を再度行なった後，Hepes-Tyrode緩衝液で再分散して洗浄血小板溶液（2.0x10^5/μL）を調製した．コラーゲン基板上に洗浄血小板溶液を展開し，37℃にて1時間静置し，Hepes-Tyrode緩衝液で洗浄してから使用した．

3）再構成血液の調製法[12]

血液をACD溶液と体積比10:1で混合，100gで15分間遠心分離して，PRPを除去し，同量のACD混合生理食塩水（10 g/dL）に分散させた．同操作を再度行なった後，2200gで10分間遠心してACD混合生理食塩水に再分散後，再度遠心分離してバフィーコートを完全に除去した．得られた再構成血液（Hct 70.7%，血小板数1.7x10^4/μL）はHct 50%に調整して使用した．

4）血小板数減少血液の調製法

全血（含PPACK 40μM）を白血球除去フィルター（NEO1J，日本ポール製）に重力落差にて通過させ，回収した血液の血球を計測した．別に用意したPRPを添加することにより，血小板濃度2.0x10^4/μLに調整し，血小板減少モデル血液とした．

5）フローチャンバーを用いたH12-ラテックスビーズの機能評価[10]

再構成血液5 mL（Hct 50%，[PLT]=4.0x10^3/μL）にH12-ラテックスビーズ（1.0×10^5/μL）を混合後（37℃，10分），血小板固定化基板上を流動（37℃）させ，蛍光顕微鏡（ECLIPS TE-300、ニコン（株））で観察し，CCDカメラにて収録した．また，血小板減少モデル血液5 mL にH12-ラテックスビーズ（10×10^4/μL）を混合後（37℃，10min），コラーゲン基板上を流動させ，同装置にて観察した．

2.6 走査型電子顕微鏡による基板観察

流動実験後のガラス基板をHepes-Tyrode緩衝液で洗浄し，1%グルタルアルデヒドで30分間，続いて1%四酸化オスミウムにて30分間試料固定を行った．エタノール処理による脱水後，*t*-ブチルアルコールから凍結乾燥した．オスミウムプラズマコーター（NL-OPC80，日本電子データム（株））にて試料を四酸化オスミウムで表面処理後，走査型電子顕微鏡（SEM，S-4500，日立製作所）にて観察した．

3．結果及び考察

3.1 H12-，RGD-ラテックスビーズ

ラテックスビーズ表面に吸着させたrHSAのアミノ残基に対し，架橋剤SPDPを用いてピリジルジスルフィド基を導入後，H12あるいはRGDをそれぞれ混合した．両者のラテックスビーズ分散液を遠心分離後，その上清の2-チオピリドンをHPLCを用いて343nmの吸光度より定量したところ，それぞれ13.5，12.1μMであり，これはH12あるいはRGDがラテックスビーズ1粒子あたりそれぞれ約2.0×10^5，1.8×10^{5}分子結合した計算となり，ほぼ同じ結合密度であった．2-チオピリドンが検出されたことから，H12あるいはRGDは含有ペプチド配列にあるシステイン残基のチオール基を介してラテックスビーズに結合したことを確認した．

3.2 凝集計を用いた機能評価

Fig.1に示したとおり，血小板凝集は、H12あるいはRGDの添加により濃度依存的に抑制が認められ，H12では4mM，RGDでは1mMでほぼ完全に抑制された．従って，H12あるいはRGDは，そのN末端にシステインが結合していても従来の報告[15-17]と同様な凝集抑制機能を有するアミノ酸配列であり，GPIIb/IIIaへの認識能が低下しないこと確認した．また，H12の血小板凝集抑制能は，RGDよりおよそ4倍低いが，これはGPIIb/IIIaに対する結合部位が異なるためと考えられている[15]．

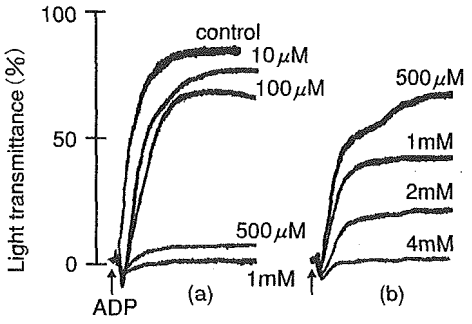

Figure 1. ADP-induced platelet aggregation in PRP ([ADP]=20μM, [platelet]=2.0 x 10^5/μL) in the presence of (a) RGD (10μM to 1 mM) or (b) H12 (500μM to 4 mM).

次に，H12-ラテックスビーズをPRPに添加し15分間攪拌後，血球カウンターにて血小板を計測したところ，H12-ラテックスビーズの濃度によらずほぼ一定であった．これは，H12-ラテックスビーズは未活性血小板には結合しないことを示している（Fig. 2 △）．H12-ラテックスビーズを混合したPRPに対してADPを添加すると，直ちに透過光の増加がみられ，活性化血

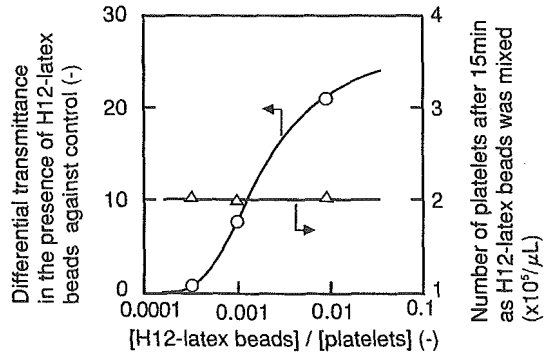

Figure 2. Number of platelets (2.0 x 10^5/μL) in the presence of H12-latex beads (1.0 x 10^2 to 2 x 10^3/μL) after 15 min (△, [ADP]=0μM), and facilitation of platelet aggregation involving H12-latex beads (○, [ADP]=20μM).

小板の凝集が認められた．対照群として同濃度のラテックスビーズを添加した血小板凝集の透過光に対するH12-ラテックスビーズを添加した場合の透過光との差は，H12-ラテックスビーズ濃度の増加とともに増大した（Fig. 2 ○）．これは，H12結合させた微粒子が血小板膜表面に発現したGPIIb/IIIaと多点結合したために，凝集促進が生じたものと考えられた．

3.3 フローサイトメトリーを用いた血小板解析

血小板（2.0x10⁴/μL）に対するラテックスビーズ，H12-あるいはRGD-ラテックスビーズ（1.0x10⁵/μL）の非特異的粘着率をフローサイトメトリーにて算出した（Fig. 3）．ラテックスビーズあるいはH12-ラテックスビーズでは，血小板に対する非特異的粘着率に変化はみられず，血小板とほとんど相互作用しないと考えられた．他方，RGD-ラテックスビーズでは血小板に対する非特異的粘着率が2.9±1.3%上昇した（Fig. 3）．このことから，RGD-ラテックスビーズは正常血小板と相互作用して，血流中で血栓形成を引き起こしやすいことが示唆された．

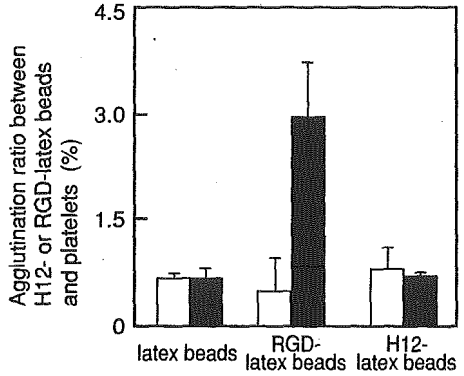

Figure 3. Agglutination ratios of RGD-latex beads or H12-latex beads to platelets by flow cytometry. Zero minute (open bar) and 30 min (closed bar), after the mixture of RGD-latex beads or H12-latex beads with platelets.

さらにフローサイトメトリーを用いて、H12-ラテックスビーズによる血小板の膜表面状態変化の有無について検討した．GPIIb/IIIaあるいはP-セレクチンは，活性化した血小板に特異的に発現される膜蛋白質であるために，それぞれに対する抗体，PAC-1と抗P-セレクチン抗体にて血小板の表面状態を解析でき，活性状態に関する知見を得ることができる[21-24]．例えば，Table 1に示すように，ADP刺激した血小板では，PAC-1結合

Table 1. The bonding ratios of PAC-1 and anti P-selectin antibody to platelets in the presence of H12-latex beads.

	PAC-1 binding (%)		P-selectin expression (%)	
Stirring time (min)	0	10	0	30
PBS	0.43±0.12	1.75±0.41	0.50±0.01	0.70±0.14
H12-latex beads	0.47±0.20	1.49±0.26	0.53±0.04	0.75±0.23
Positive control	55.1±6.1[1)]		31.6±6.9[2)]	

1) ADP 100 μM, 2) ADP 20 μM

率が55.1±6.1%と，未刺激（PBS群）の血小板のそれ（0.4±0.1%）と比較すると明らかなようにGPIIb/IIIaの発現を認める．また，同様にADP刺激した血小板では，P-セレクチンの発現率も著しく上昇し31.6±6.9%と非常に高くなっている．しかしながら，H12-ラテックスビーズと混合した血小板では，GPIIb/IIIaあるいはP-セレクチンの発現はいずれも認められず，PBS群とほとんど差はみられなかった．このことから，H12-ラテックスビーズは正常血小板を活性化することなく，血小板に対して不活性であることを証明した．

3.4 流動下におけるH12-ラテックスビーズの機能評価

流動下におけるH12-ラテックスビーズのGPIIb/IIIaに対する認識能の評価を行うために，まず基板に固定化した活性化血小板に対する粘着挙動を観察した．H12-ラテックスビーズは流動直後から血小板固定化基板上に一様に粘着し，その粘着数は時間の経過とともに増加した（Fig. 4）．また，未結合H12を添加した場合にはH12-ラテックスビーズの粘着は抑制され，またラテックスビーズでも同様に粘着がみられないことから，H12-ラテックスビーズの粘着はH12と活性化血小板上に発現するGPIIb/IIIaとの特異的相互作用に起因するものであることが確認できた（Fig. 4）．また，流動血液のずり速度の上昇と共に血小板固定化基板に対するH12-ラテックスビーズの粘着数は減少した（Fig. 5）．これはGPIIb/IIIaのようなインテグリンとの相互作用に見られる現象[12]であり，H12-ラテックスビーズは，ず

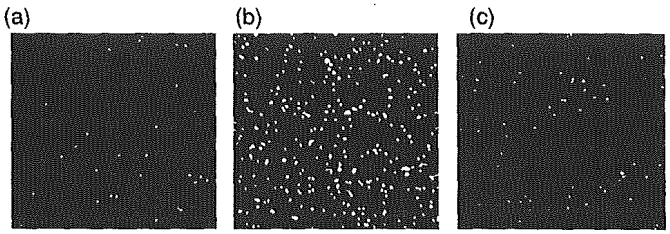

Figure 4. Observation of the interaction of (a) latex beads, (b) H12-latex beads, and (c) H12-latex beads and free H12 (4 mM) with the platelet-immobilized surface in the reconstituted blood at the shear rate of 150 s^{-1}. (Hct. 50 %, [platelet]= 5.0 x 10^5/μL). These images were taken at 180 s after blood circulation.

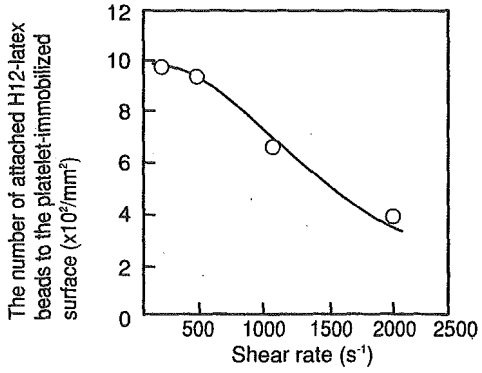

Figure 5. Changes of attached H12-latex beads to the platelet-immobilized surface at the various shear rates (Hct. 50 %, [platelet]= 5.0 x 10^5/μL). These values were calculated from the images taken at 180 s after blood circulation.

り速度の比較的低い領域で活性化血小板と凝集することが期待される．

次に，血小板減少モデル血液中（2.0x10⁴/μL，正常の10分の1）における，コラーゲン基板へのH12-ラテックスビーズと血小板の粘着挙動を観察した．まず血小板にのみ蛍光標識後，コントロール群としてラテックスビーズを混合したところ，流動直後から血小板のコラーゲン基板への粘着が認められ，その粘着占有率は時間の経過にともない増大し，180秒後では3.1±0.4%となった（Fig. 6 ●）．しかし，この挙動はラテックスビーズを添加しない血液の粘着占有率（3.0±0.6%，data not shown）とほとんど変わらないことから，血中のラテックスビーズは血小板粘着に影響を及ぼさないことを確認した．H12-ラテックスビーズを添加した場合，血小板の粘着占有率は180秒後で5.1±0.3%となり，コントロール群よりも約1.7倍増幅された（Fig. 6 ○）．他方、同条件におけるH12-ラテックスビーズの粘着挙動を観察したところ，コラーゲン基板に対する粘着は，血小板のそれと比較して非常に緩やかであったが，およそ100秒後からH12結合ラテックスビーズの粘着占有率が徐々に増大する挙動が認められた（Fig. 6 □）．また，ラテックスビーズ

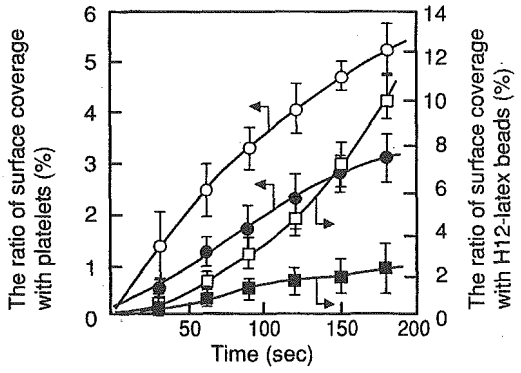

Figure 6. Changes of the ratios of surface coverage with flowing platelets (○) and H12-latex beads (□) to the collagen-immobilized surface under flow conditions of thrombocytopenic-imitation blood ([platelet]= 2.0 x 10⁴/μL, [H12-latex beads]=1.0 x 10⁵/μL). As a control using thrombocytopenic-imitation blood containing bare latex beads, the ratios of surface coverage with flowing platelets (●) and the latex beads (■) were also monitored.

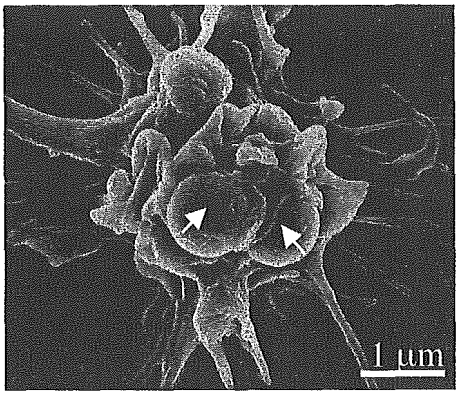

Figure 7. SEM image of platelet aggregation involving H12-latex beads (arrow).

ではコラーゲン基板への粘着はほとんどみられないことから（Fig. 6 ■），コラーゲン基板に対して流動している血小板が粘着し活性化してGPIIb/IIIaを発現後，流動しているH12-ラテックスビーズがその血小板を認識して粘着，さらに流動している血小板が粘着したH12-ラテックスビーズを足場として血小板凝集を促進したために，粘着占有率が増大する機構が考えられた．

同実験の血液流動後の基板をSEM観察したところ，コラーゲン基板上にまず血小板が粘着した後にH12-ラテックスビーズが結合し，さらにこれを巻き込むようにして血小板が凝集している様子が確認できた（Fig. 7）．これは，H12-ラテックスビーズが，血小板凝集を促進することを支持する結果である．現在，H12を担持したアルブミン重合体やリン脂質小胞体の止血能評価を行っている．

4．結論

H12-ラテックスビーズは正常血小板に対する非特異的な相互作用は認めなかった．血小板数減少症を再現した流動下において，本ラテックスビーズは出血部位モデル基板であるコラーゲン基板へ粘着して活性化した血小板に対して凝集し凝集塊形成を促進した．以上より，H12は血小板代替物の認識部位として有効であると考えられ，アルブミン重合体や小胞体に担持させた系による*in vitro, in vivo*評価を進めている．

5．謝辞

H12あるいはRGDの機能評価法に関して御指導，御討論頂きました，慶応義塾大学医学部内科　村田　満博士ならびに横山健次博士に心から御礼申し上げます．本研究の一部は，厚生労働科学研究費補助金（医薬安全総合研究事業、11877172），21COE実践的ナノ化学（文部科学省）および日本学術振興会科学研究費の支援より行われた．

参考文献

1) Fujimoto TT, Kohata S, Suzuki H, Miyazaki H, Fujimura K. Production of functional platelets by differentiated embryonic stem (ES) cells in vitro. Blood 2003;102:4044-4051.
2) Graham SS, Gonchoroff NJ, Miller JL. Infusible platelet membranes retain partial functionality of the platelet GPIb/IX/V receptor complex. Am J Clin Pathol 2001;115:144-147.
3) Rybak M, Renzulli LA. A liposome based platelet substitutes, the plateletsome, with hemostatic efficacy. Biomater Artif Cells Immobilization Biotechnol 1993;21:108-118.
4) Agam G, Livine AA. Erythrocytes with covalently bound fibrinogen as a cellular replacement for the treatment of thrombocytopenia. Eur J Clin Invest 1992;22;105-112.
5) Levi M, Friedrich PW, Middleton S, De Groot PG, Wu