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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*

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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).

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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.

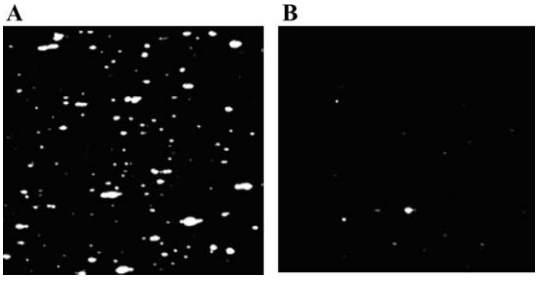

Fig. 1. Observation of the attachment of FITC-labelled rGPIa/IIa–polyAlb to the collagen surface at the shear rate of $350\,s^{-1}$ with a fluorescence microscopy, (A) in the absence of antiGPIa/IIa monoclonal antibody (7E10B) and (B) in the presence of 7E10B.

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

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)

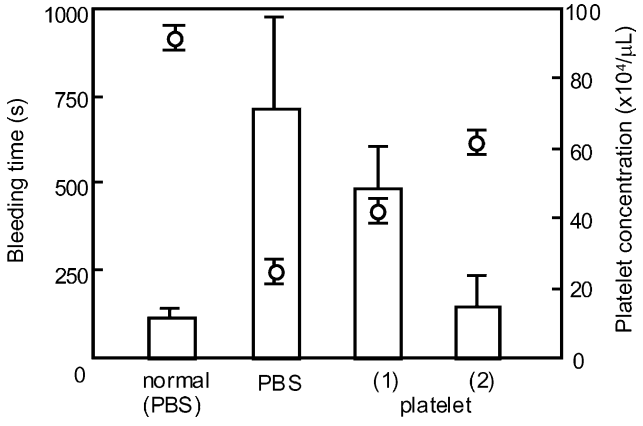

Fig. 2. Effect of the administration of the PBS and the mouse platelets on the tail bleeding time (white bar) in thrombocytopenic mice. The administered amounts of platelets are: (1) 1.0×10^{10} and (2) 3.0×10^{10} particles/kg. ○, platelet concentration in the mouse.

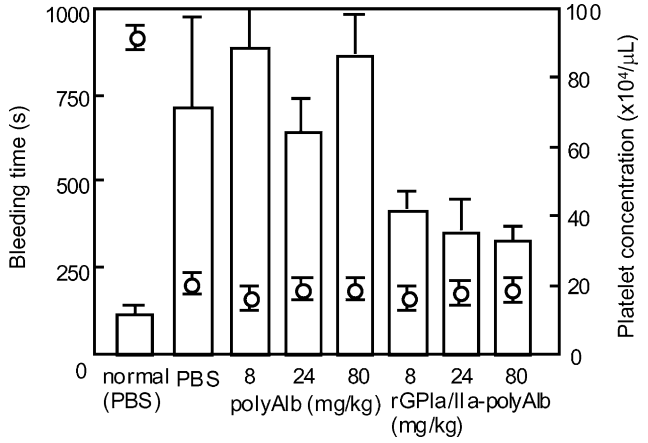

Fig. 3. Effect of the administration of rGPIa/IIa–polyAlb on the tail bleeding time (white bar). The administered amounts of rGPIa/IIa–polyAlb are 8, 24, and 80 mg/kg. ◯, platelet concentration in the mouse.

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.

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