lesterol value, iron content, and the transitional hypertrophy of a reticuloendothelial system [9]. It was concluded that Hb-vesicles are expected to function adequately in cases of extensive bleeding.

Development of Platelet Substitutes

The history of platelet substitutes is short compared with that of red blood cells, with few examples of research. A platelet has complicated functions, such as adhesion specific to the bleeding site, expansion, aggregation, secretion, and the activation of a blood coagulation system. Needless to say, we cannot make platelet substitutes that have these all. However, a bleeding tendency is strongly apparent in such bleeding diseases as Bernard-Soulier syndrome and thrombasthenia, in which adhesion and aggregation ability are lacking. In these conditions, a hemostatic effect can be expected by the infusion of particles having functions such as adhesion and the aggregation of platelets due to the assistance of the function of the remaining platelets. Although clinical tests were carried out with human red blood cells [10] or albumin microcapsules [11] conjugating fibrinogen, and with the dried powder of human platelets [12], all clinical tests were suspended due to problems of safety and efficacy. Moreover, since the blood components of human origin were used, the risk of infection cannot be avoided completely. The platelet substitutes created by our group use liposomes and recombinant human albumin as biocompatible particles. They also use recombinant proteins of the part of platelet membrane or synthetic oligopeptides by conjugating to those particles for the purpose of accumulation to the bleeding site involving native platelets, expecting to achieve hemostasis.

The mechanisms of platelet adhesion differ between the blood flow of high shear rate and that of low shear rate. As shown in Fig. 2, the hemostasis of the platelets to bleeding in a high shear rate begins from a platelet recognition of the von Willebrand factor (vWf) bound to the collagen in the subcutaneous tissue of a blood vessel exposed to the bleeding site, followed by platelet adhesion and rolling. This recognition ability comes from the GPIb/V/IX complex containing GPIbα on the surface of a platelet [13]. Next, the platelets will progress and a granule will be secreted if GPIaIIa ($\alpha_2\beta_1$ integrin) or GPVI on the surface of the platelet directly interacts with the collagen and is activated. Fibrinogen recognizes the activated GPIIb/IIIa ($\alpha_{IIb}\beta_3$ integrin), constructs a crosslink between blood platelets, forms an aggregate, and serves as primary hemostasis. Hemostasis is completed by formation (secondary hemostasis) of the fibrin clot by induction of the coagulation system.

The target platelet substitutes bear the water-soluble part of receptor proteins such as GPIbα which recognizes collagen through vWf under the blood

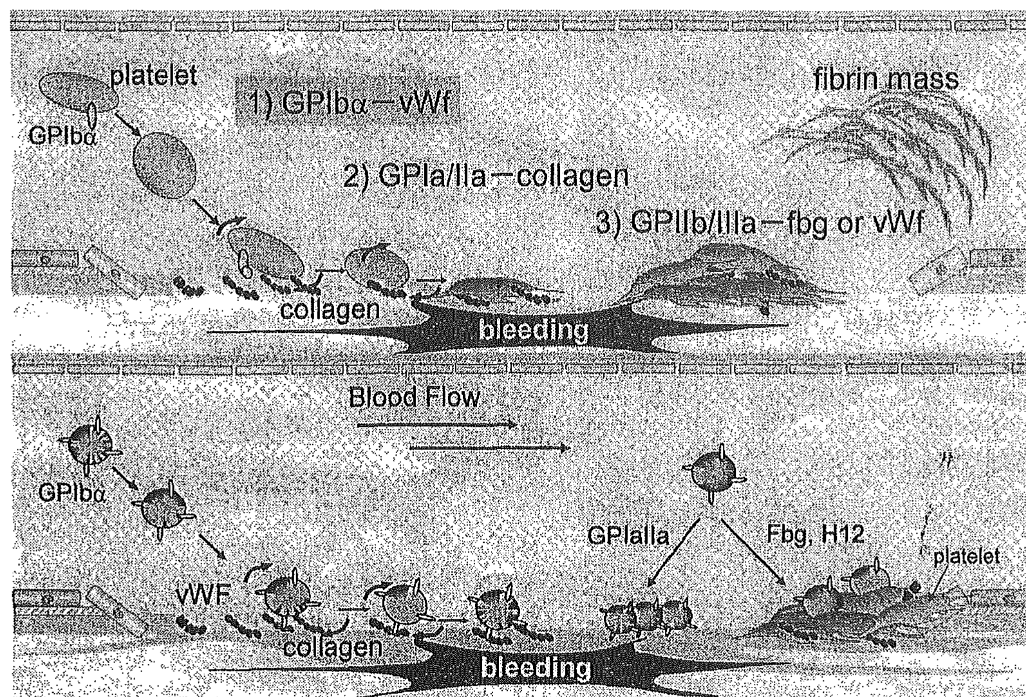

FIG. 2. Design of platelet substitutes studied from functions of natural platelets

flow of high shear rates and GPIaIIa which recognizes collagen directly at low shear rates. Instead of GPIIb/IIIa on the surface of an activated platelet, they bear the fibrinogen or its oligopeptide to assist the platelet aggregation as ligands of the GPIIb/IIIa.

If an anti-GPIIb/IIIa antibody is added to a platelet dispersion to inhibit GPIIb/IIIa activity, the platelets roll on the vWf-immobilized plate along the flow direction by the interaction of rGPIbα on the platelet and the vWf. Interestingly, phospholipid liposomes conjugating rGPIbα roll on the vWf-immobilized plate as well [14]. The number of the rolling liposomes increased with the shear rate, indicating the characteristic of rGPIbα. Moreover, the rolling speed was correlated with the membrane fluidity of the liposomes. That is, the rolling speed of "soft" liposomes was low, whereas that of the "hard" liposomes was high [15]. However, the rGPIbα-liposomes did not continue rolling but departed from the plate after rolling some length. This was remarkable as the "soft" liposomes. When the amount of rGPIbα on the surface of the liposome after the experiment was measured, it was suggested that rGPIbα-lipid should dissociate from the bilayer membrane during the rolling on the vWf-plate. Now rGPIbα-lipid which cannot dissociate serves as a point of a molecular design.

On the other hand, the rGPIaIIa-liposomes directly recognize collagen under the blood flow of low shear rates and adhered to (stopped at) the collagen-immobilized plate [16]. In this case, the number of the adhering liposomes decreased as the shear rate rose. However, liposomes conjugating both rGPIbα and GPIaIIa adhered on the collagen plate under the blood flow from low to high shear rates [17]. If the liposomes having platelet activation factors

or coagulation factors in the internal aqueous phase accumulate at the bleeding site, they will be able to contribute effectively to hemostasis by releasing their contents. We also focused on using polymerized albumin particles as effective platelet substitutes and obtained some unique in vitro and in vivo results [18,19].

Conclusions

For red-blood-cell substitutes the present target is the supportive treatment of transfusion therapy in emergency, and nonclinical and clinical studies will be scheduled within 2 years.

On the other hand, the research of platelet substitutes has just started. There is a conflict between the carrier design for the extension of circulation lifetime and the carrier design to show the hemostatic activity by recognizing the bleeding site; therefore, we need to resolve this conflict and to design platelet substitutes for prophylactic or chronic treatments. And we also need a method to confirm that the candidate does not create a thrombus in blood circulation.

Although profitability is important in the development of blood substitutes, one that is based on a long-term view with consideration for human beings is expected first. At present, as many discoveries about the dynamic function of platelets have accumulated in a short period of time with the progress of biotechnology and opto-electronics, and the manufacturing technology of recombinant proteins or carriers is progressing, a usable product is sure to be invented in the near future.

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Hemostatic effects of fibrinogen γ-chain dodecapeptide-conjugated polymerized albumin particles in vitro and in vivo

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BACKGROUND: Prototypes of platelet (PLT) substitutes have been studied and the focus was on a dodecapeptide, HHLGGAKQAGDV (H12), which is a fibrinogen γ-chain carboxy-terminal sequence (γ 400-411) and exists only in the fibrinogen domain.

STUDY DESIGN AND METHODS: H12 was conjugated to the surface of polymerized albumin particles (polyAlb) as biocompatible and biodegradable particles with a mean diameter of 260 ± 60 nm, and the hemostatic ability of H12-conjugated polyAlb (H12-polyAlb) under flow conditions and thrombocytopenic rats have been studied.

RESULTS: H12-polyAlb enhanced the in vitro thrombus formation of activated PLTs on a collagen-immobilized plate when exposed to the flowing thrombocytopenic imitation blood. Furthermore, the analysis of the tail bleeding time of rats that were made thrombocytopenic by busulfan injection showed that H12-polyAlb had a hemostatic effect. Based on the bleeding time and the amount injected, the hemostatic capacity of 20 H12-polyAlb was estimated to correspond to that of one PLT.

CONCLUSION: These results were important first steps toward the development of PLT substitutes and indicated that H12-polyAlb may be a suitable candidate for an alternative to human PLT concentrates transfused into thrombocytopenic patients in the future.

Platelet (PLT) transfusion plays an important role in supportive therapy of patients with thrombocytopenia caused by hematologic malignancies, cancer, or during surgical procedures and radiotherapy. The shortage of PLTs, however, has always been a serious problem because of the short storage life of PLT concentrates (72 hr in Japan). In addition, the risk of viral and bacterial infections by transfusion is a serious concern. PLT substitutes such as solubilized PLT membrane protein-conjugated liposomes (plateletsome),[1] infusible PLT membranes,[2] fibrinogen-bonded red blood cells (RBCs),[3] fibrinogen-coated albumin microcapsules (synthocyte),[4] and arginine-glycine-asparaginic acid (RGD) peptide-bound RBCs (thromboerythrocyte)[5] have been developed to solve these problems. Despite their usefulness in enhancing PLT aggregation and reducing bleeding

ABBREVIATIONS: cH12 = control H12; cH12 polyAlb = cH12-conjugated polyAlb; GP = glycoprotein; H12 = HHLGGAKQAGDV dodecapeptide; H12-polyAlb = H12-conjugated polyAlb; polyAlb = polymerized albumin particles; PRP = platelet-rich plasma; SPDP = *N*-succinimidyl 3-(2-pyridyldithio)propionate.

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time in vivo, these PLT substitutes consist of materials derived from blood components.

PLT membrane glycoprotein (GP) Ibα and GPIa/IIa are the receptors for von Willebrand factor (VWF) and collagen, respectively.[6-8] We have focused our studies on recombinant forms of these proteins (rGPIbα[9,10] or rGPIa/IIa complex[11-13]) and have conjugated them to biocompatible carriers such as polymerized albumin particles (polyAlb)[11,14,15] and phospholipid vesicles (liposomes).[12,13,16] In particular, we found that rGPIa/IIa-conjugated polyAlb reduced the bleeding time of thrombocytopenic mice in vivo.[11]

PLTs that adhere to the collagen-immobilized surface are activated, and the conformation of GPIIb/IIIa that exists on PLT membrane changes from a silent state to an activated state.[17] This leads to the binding of fibrinogen and VWF[18,19] followed by PLT aggregation.[20,21] Fibrinogen contains three putative PLT interaction sites, namely, a tetrapeptide containing RGD sequences such as RGDF and RGDS at α95-98 and α572-575, respectively,[22] and a dodecapeptide (HHLGGAKQAGDV, H12) corresponding to a γ-chain carboxy-terminal segment (γ400-411).

We have also developed fibrinogen-conjugated polyAlb, which was shown to facilitate the accumulation of flowing PLTs into polyAlb aggregates after their attachment to an activated PLT-immobilized surface in vitro.[14] These findings confirmed that such conjugates could emulate the function of PLTs in primary hemostasis. Fibrinogen from human blood, however, is not stable, and its activity in solution is extremely low.[14]

Recently, we focused on H12, which exists only in fibrinogen domain.[23-30] Based on our result obtained from flow cytometric analyses of agglutination, the H12-conjugated latex beads showed minimal interaction with nonactivated PLTs in comparison with RGD-conjugated latex beads.[31] Furthermore, H12-conjugated latex beads enhanced the in vitro PLT thrombus formation on collagen-immobilized plates when exposed to flowing thrombocytopenic imitation blood.[31]

In this study, we conjugated H12 to the surface of polyAlb to produce biocompatible and biodegradable particles and evaluated their effect on enhancement of thrombus formation on a collagen-coated surface in the presence of the H12-polyAlb when exposed to flowing thrombocytopenic imitation blood in vitro. We prepared thrombocytopenic rats by busulfan administration, intravenously administered the H12-polyAlb into the rats, and measured the tail bleeding time for evaluation of the hemostatic properties of the particles.

MATERIALS AND METHODS

Reagents

A fibrinogen γ-chain dodecapeptide (C-HHLGGAKQAGDV, H12) or a reverse sequence of H12 (C-VDGAQKAGGLHH, control H12 [cH12]) was synthesized with a solid-phase synthesizer by BEX (Tokyo, Japan). *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and an anticoagulant D-Phe-Pro-Arg-chloromethylketone were purchased from Pierce Chemical Co. (Rockford, IL) or Calbiochem (San Diego, CA). 3,3′-Dihexyloxacarbocyanine iodide, which is a PLT fluorescent dye, was purchased from Molecular Probes (Eugene, OR). Both busulfan and polyethylene glycol (PEG; mean molecular weight, 400) were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant human serum albumin (rHSA) was donated by Mitsubishi Pharma (Osaka, Japan).

Preparation of H12-polyAlb or cH12-conjugated polyAlb

A solution of rHSA (250 mg/mL) was dialyzed against distilled water for 6 hours at 4°C to remove stabilizers such as *N*-acetyl-D,L-tryptophan and sodium caproate. The rHSA solution (25 mL) was diluted with saline to 10 mg per mL, and the pH was adjusted to 10.7 (at room temperature) by titration with 0.1 N NaOH (800 μL). The solution was heated to 80°C for 10 minutes, rapidly cooled in an ice bath, and then brought to room temperature. The pH was adjusted to 6.1 at room temperature by dropwise addition of 0.1 N HCl (900 μL) and then the solution was stirred at 40°C for 120 minutes until the turbidity reached 0.4 ± 0.1. Excess iodoacetamide (25 mg) was added to terminate polymerization, and the solution was dialyzed against PBS at 4°C for 24 hours. A 25-mL dispersion of polyAlb ([rHSA] = 9.0 mg/mL, pH 7.4) was thus prepared. Mean diameter was determined by a dynamic scattering method (Coulter N4 Plus submicron particle sizer, Beckman-Coulter, Miami, FL). H12 was conjugated to the surface of polyAlb as previously described.[15] A solution of SPDP in ethanol (20 mmol/L, 15 μL) was added to the polyAlb suspension (18 mg/mL, 10 mL), and the suspension was stirred for 30 minutes at room temperature. The unreacted SPDP and the by-products were separated by repeated centrifugation and washing with saline ($30\,000 \times g$, 10 min, 4°C, three times), and the pyridyl disulfide-bonded polyAlb was collected. A suspension of pyridyl disulfide–bonded polyAlb (15 mg/mL, 10 mL) was mixed with a solution of H12 (100 mmol/L, 20 μL) and allowed to react at 20°C for 12 hours. The unreacted reagents were removed by repeated centrifugation and washing with saline ($30\,000 \times g$, 10 min, 4°C) to obtain the purified H12-conjugated polyAlb (H12-polyAlb, 10 mg/mL, 10 mL). The concentration of the H12 conjugated on the polyAlb was determined by the quantification of the 2-thiopyridone that was liberated by the thiol-disulfide exchange reaction, with high-pressure liquid chromatography on a TSK-GEL G3000SW$_{XL}$ column (7.8 mm o.d. × 300 mmh in PBS at 1 mL/min), by measuring the absorbance of the column effluent at 343 nm. The cH12-conjugated polyAlb (cH12-

polyAlb) were prepared by the same method as mentioned above.

PLT aggregation study

Blood withdrawn from healthy volunteers was mixed with 10 percent volume of 3.8 percent (wt/vol) sodium citrate. PLT-rich plasma (PRP) was prepared by centrifugation ($100 \times g$, 15 min, 22°C), and the PLT concentration of PRP was adjusted to 200×10^3 per μL by PLT-poor plasma prepared by centrifugation ($2200 \times g$, 10 min, 22°C). The PLT concentration was determined with an automated hematology analyzer (K-4500, Sysmex, Kobe, Japan). A 20 μmol per L ADP solution was added to the PRP containing H12 or cH12 solutions adjusted to final concentration of 1 mmol per L, and the light transmittance was measured with an aggregometer (Hema Tracer T-638, Nico Bioscience, Tokyo).

Preparation of a collagen-immobilized surface

Collagen I-A (3.0 mg/mL, Cellmatrix, Nitta Gelatin, Osaka, Japan) was suspended in PBS at 4°C to give a final concentration of 30 μg per mL. A glass plate (diameter, 24 mm; thickness, 0.5 mm) was immersed into the collagen suspension at 4°C for 8 hr, carefully rinsed with PBS, and then immersed in a bovine serum albumin solution (20 mg/mL) at room temperature for 2 hr.

Measurement of the interaction of PLTs with the collagen surface in the presence of H12-polyAlb with thrombocytopenic imitation blood

Blood withdrawn from healthy volunteers was treated with the thrombin inhibitor D-Phe-Pro-Arg-chloromethylketone (final concentration, 40 μmol/L) and was filtered through a white blood cell (WBC) removal filter (NEO1J, Nihon Poll, Tokyo, Japan), which could remove PLTs as well as WBCs. The residual PLT concentration of the filtered blood was determined to be $5.0 \times 10^3 \pm 3.0 \times 10^3$ per μL, and the final PLT concentration was adjusted to 20×10^3 per μL by addition of PRP, which was prepared by centrifugation ($100 \times g$, 15 min, 22°C) of sodium citrate-treated blood. The PLT concentration was determined with an automated hematology analyzer (K-4500). This blood preparation was termed as thrombocytopenic imitation blood.

The thrombocytopenic imitation blood and H12-polyAlb (10 mg/mL, 70 μL) mixtures were placed in a recirculating chamber mounted on an epifluorescent microscope (ECLIPS TE300, Nikon, Tokyo, Japan) equipped with a CCD camera, and the interaction of PLTs with the collagen immobilized on the surface was observed. Single-frame images of adhesion and aggregation of PLTs in the presence of H12- or cH12-polyAlb were obtained with an image processor (Argus-50, Hamamatsu Photonics, Hamamatsu, Japan), and the surface coverage of the adhered PLTs on the plate was calculated with an image processor (Argus-20, Hamamatsu Photonics). All perfusion studies were performed at 37°C.

Measurement of the tail bleeding time of the thrombocytopenic rats

All animal studies were approved by the Animal Subject Committee of Keio University, School of Medicine, and performed according to NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication 85-23, Rev. 1985). Experiments were carried out with male Wistar rats (230-250 g, CLEA Japan, Tokyo, Japan). A busulfan solution was prepared at a final concentration of 5 mg per mL in PEG (mean molecular weight, 400).[32,33] Rats were anesthetized with diethyl ether and injected on Day 0 and Day 3 with 10, 15, or 20 mg per kg on each dosing day, to produce a total dosage of 20, 30, or 40 mg per kg busulfan, respectively. Blood samples for cell counting were obtained from ether-anesthetized rats by inserting a 25-gauge needle into a tail vein, and the cell concentration was determined with an automated hematology analyzer (K-4500).

On Day 10, thrombocytopenic rats were anesthetized with a general anesthetic sedative (Sevofrane), and the sample suspension was infused into the tail vein. The samples were H12-polyAlb, cH12-polyAlb, or polyAlb at a dose of 4 mL per kg; saline was used to obtain the control value. Five minutes after administration, a 2.5 mm length × 1.0 mm depth template-guided incision (Quikheel, Becton-Dickinson, San Jose, CA) was made 1 cm from the tip of the tail. A tail was immersed in a 50 mL cylinder of saline, and the time taken for bleeding to stop was measured. In addition, cell concentrations were determined with an automated hematology analyzer (K-4500) before (–5 min) and after (30 min) samples injection.

Statistical analysis

Significance of the Day 10 group versus the normal group as shown in Fig. 4, and the H12-polyAlb group versus the saline group, the polyAlb group, and the cH12-polyAlb group as shown in Fig. 5, was tested with Tukey-Kramer tests. A p value of less than 0.05 was considered to be significant. Analytic software was used (StatView, SAS Institute Inc., Cary, NC).

RESULTS

PLT aggregation study

By use of an aggregometer, we confirmed that H12 showed a concentration-dependent suppression of PLT aggrega-

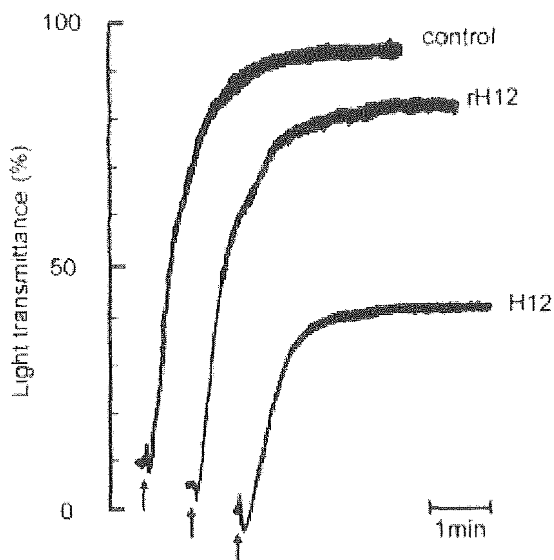

Fig. 1. ADP-induced (20 μmol/L) PLT aggregation (200 × 10^3/μL) was monitored with an aggregometer in the presence of 1 mmol per L H12, cH12, or PBS (control).

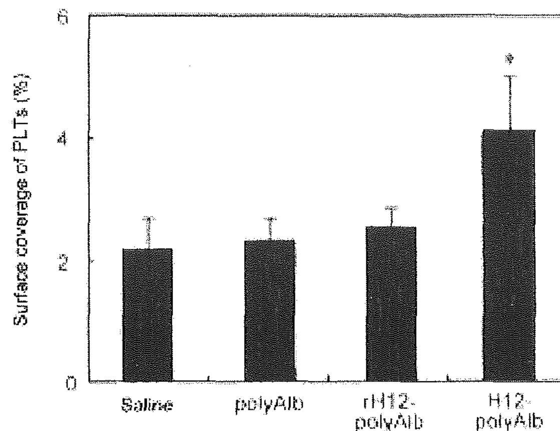

Fig. 2. Comparison of surface coverage of PLTs in the thrombocytopenic imitation blood on the collagen surface after addition of H12-polyAlb, cH12-polyAlb, or polyAlb at a shear rate of 150 per second (n = 4). The amount of surface coverage was recorded after blood was circulated for 210 seconds. The concentration of PLTs was 20 × 10^3 per μL. *$p < 0.05$ for the H12-polyAlb group versus the saline group, the polyAlb group, and the cH12-polyAlb group.

tion.[31] In particular, when H12 was added at a concentration of 1 mmol per L, PLT aggregation was significantly suppressed in comparison with the control experiment (Fig. 1). In contrast, cH12 at the same concentration did not inhibit PLT aggregation.

Characterization of H12-polyAlb and cH12-polyAlb

In the conjugation reaction, the mercapto group of the terminal cysteine of H12 or cH12 reacted with the pyridyl disulfide–bonded group of the polyAlb, which have diameters of 260 ± 60 nm, and 2-thiopyridone was liberated after the formation of disulfide linkage owing to the thiol-disulfide exchange reaction. With the absorption of the liberated 2-thiopyridone at 343 nm, the number of the H12 and cH12 molecules conjugated to one polyAlb was estimated to be approximately 9.6 × 10^3 and 9.1 × 10^3, respectively. The endotoxin concentration in the H12- or cH12-polyAlb suspension was below 0.2 EU per mL.

Measurement of the interaction of PLTs with the collagen surface in the presence of H12-polyAlb with thrombocytopenic imitation blood under flow conditions

We prepared the thrombocytopenic imitation blood and adjusted the number of PLTs to 20 × 10^3 per μL. The PLTs were labeled with a fluorescent marker, 3,3′-dihexyloxacarbocyanine iodide, to observe the adherence of PLTs to the collagen surface at a shear rate of 150 per second. In the presence of the control polyAlb, the total surface coverage of PLTs was 2.1 ± 0.4 percent after 210 seconds of flow (Fig. 2). When PLTs were allowed to flow over the collagen-immobilized surface in the absence of polyAlb, the same value was obtained. When the H12-polyAlb were added to the blood instead of the polyAlb, there was a significant increase in the surface coverage to 3.9 ± 1.1 percent. By contrast, when cH12-polyAlb was used, the surface coverage did not change from that of the control polyAlb (2.4 ± 0.4%).

Preparation of the thrombocytopenic rats

Rats that received busulfan at total doses of 40 or 30 mg per kg developed profound thrombocytopenia with a decline in the level of PLTs to half-maximal value on Day 7 or 8, respectively, which reached the lowest value between Day 10 and Day 14 (Fig. 3). Unfortunately, the rats became anorexic, lost weight, became anemic, and were all dead by Days 12 and 16, respectively (data not shown). At a total dose of 20 mg per kg, however, thrombocytopenia was just as profound as at the higher doses of busulfan, but all rats survived with no major problems. As shown in Fig. 3, the rats treated with the 20 mg per kg dose had a decline in PLT count to half-maximal value (410 × 10^3 ± 30 × 10^3/μL) on Day 9. On Day 10, their PLT concentration was 200 × 10^3 ± 30 × 10^3 per μL, which was equivalent to one-fourth to one-fifth of the PLT count of normal rats. There was a slight decrease in the WBCs concentration of the busulfan-treated rats compared with that of uninjected

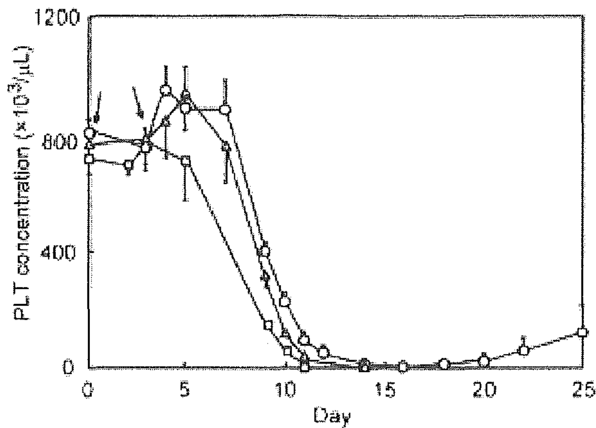

Fig. 3. Dose-response effect of busulfan on rats. Rats were injected with busulfan at a total dose of 20 (○), 30 (△), or 40 mg per kg (□) as described under Materials and Methods. Tail vein blood was collected at intervals and PLT concentration measured. Arrows show injection day of busulfan (n = 5).

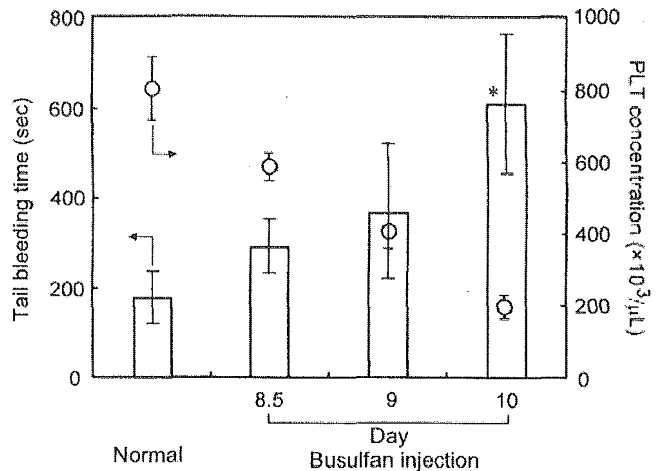

Fig. 4. Correlation of tail bleeding time (white bars) with PLT concentration (○) in the rat (n = 5). *p < 0.05 for the Day 10 group vs. the normal group.

TABLE 1. Weights and hematologic measures before and after (on Day 10) busulfan injection

Rat	Initial weight (g)	Final weight (g)	RBCs (×10³/μL)	WBCs (×10³/μL)	PLTs (×10³/μL)
Normal (n = 6)	251 ± 11	304 ± 15	7160 ± 300	12.7 ± 2.8	809 ± 86
Busulfan 20 mg/kg (n = 6)	258 ± 10	288 ± 12	7000 ± 230	8.1 ± 1.2	198 ± 28

control rats; however, the weight of busulfan-injected rats increased and their RBC counts were unchanged (Table 1). Predictably, there was a gradual decrease in the PLT count of the busulfan-treated rats to Day 11 ($97 \times 10^3 \pm 25 \times 10^3$/μL); however, RBC count also began to decline compared with that of uninjected control rats (data not shown). After the PLT count reached the lowest point on Day 14, the low count of $3 \times 10^3 \pm 2 \times 10^3$ per μL persisted for at least 6 days before starting to increase gradually.

Measurement of the tail bleeding time of the thrombocytopenic rats in the presence of H12-polyAlb

The tail bleeding times of the normal rats ([PLT] = $810 \times 10^3 \pm 90 \times 10^3$/μL) and thrombocytopenic rats on Day 10 after the busulfan injection ([PLT] = $200 \times 10^3 \pm 30 \times 10^3$/μL) were 187 ± 51 and 609 ± 153 seconds, respectively (Fig. 4). In contrast, PLT concentrations in the course of preparation of thrombocytopenic rats on Days 8.5 and 9 were $570 \times 10^3 \pm 40 \times 10^3$ and $410 \times 10^3 \pm 30 \times 10^3$ per μL, respectively, and their bleeding time was 288 ± 56 and 366 ± 153 seconds, respectively. In addition, after Day 11, thrombocytopenia became progressive rapidly but the measurement of bleeding time was hard to be standardized (data not shown). In fact, the bleeding time of the busulfan (20 mg/kg)-injected rats on Day 11 (PLTs; $97 \times 10^3 \pm 25 \times 10^3$/μL) was found to be immeasurable, because bleeding did not stop for more than 30 minutes. Therefore, for in vivo experiment we used animals on Day 10 after the busulfan treatment.

The intravenous administration of H12-polyAlb at a dose of 1 mg per kg slightly reduced the bleeding time to 581 ± 110 seconds in comparison with those of saline group (609 ± 153 sec) or control polyAlb at a dose of 1 mg per kg (679 ± 102 sec) (Fig. 5). At doses of 4 and 10 mg per kg, we confirmed a dose-dependent reduction of the bleeding time, and the time decreased significantly to 342 ± 73 and 288 ± 120 seconds, respectively. By comparison, control polyAlb groups at doses of 4 and 10 mg per kg were 553 ± 104 and 436 ± 102 seconds, respectively.

DISCUSSION

In the previous studies on PLT substitutes[2-5] despite their usefulness in reinforcing PLT aggregation and reducing bleeding time in vivo, their raw materials are dependent on human blood. In our previous studies, we have focused on H12, which is a synthetic peptide of human fibrinogen, and we conjugated H12 to the surface of fluorescein isothiocyanate (FITC)-labeled latex beads.[31] H12-latex

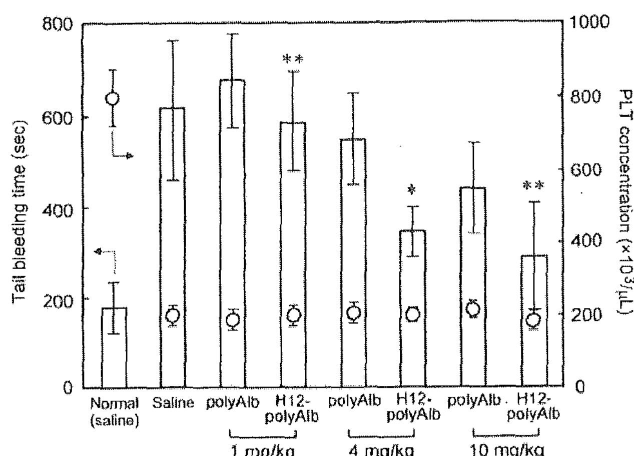

Fig. 5. Effects of the administration of H12-polyAlb on tail bleeding time (white bars). The administered amount of H12-polyAlb was 1, 4, and 10 mg/kg equivalent of albumin. (○) PLT concentration in the rats (n = 6-10). *$p < 0.05$ or **not significant for the H12-polyAlb group vs. the polyAlb group at the same dose.

beads in an RBC suspension were allowed to flow over the activated PLTs that were immobilized on a collagen surface at a shear rate of 150 per second. They adhered and accumulated to the surface in a time-dependent manner, whereas control latex beads did not adhere. The adhesion of H12-latex beads was suppressed in the presence of free H12 as an inhibitor of GPIIb/IIIa binding, showing that the adhesion was specific. Furthermore, the H12-latex beads showed minimal interaction with nonactivated PLTs based on the results of flow cytometric analyses of agglutination with the FITC-labeled latex beads. Therefore, H12 is a noninfective promising part as a recognition site of PLT substitutes. We prepared H12 particles with rHSA, which was not derived from human blood, to obtain information about the possibility to construct biocompatible and biodegradable particles with hemostatic ability in thrombocytopenic conditions.

First, we confirmed a specific binding of H12 in comparison with cH12 from a study of the inhibition of fibrinogen-mediated PLT aggregation. Because H12 is a fibrinogen sequence, which is responsible for the recognition of GPIIb/IIIa on activated PLTs,[22-28] it significantly suppressed the PLT aggregation by competitive binding to the fibrinogen-binding site of the PLT, and the binding of H12 for GPIIb/IIIa was maintained even after the introduction of the N-terminal cysteine. In contrast, cH12 showed a minimal inhibitory effect. These results were also supported by the other publications that the IC_{50} of inhibition of ^{125}I-fibrinogen binding to activated PLTs by H12 and cH12 was 28 and 85 µmol per L, respectively, indicating that binding avidity for GPIIb/IIIa of H12 was stronger than that of cH12.[23,24]

We sufficiently conjugated either H12 or cH12 to the surface of polyAlb and estimated the conjugation density on the polyAlb surface to approximately 46×10^3 or 43×10^3 molecules per μm^2, respectively. The conjugation densities were similar to that of H12-latex beads (diameter, 1 µm), which enhanced PLT thrombus formation.[31] In thrombocytopenic imitation blood flowing on the collagen surface, the surface coverage of PLTs in the presence of control polyAlb was very low and the same in the absence of polyAlb. Furthermore, we confirmed that H12-polyAlb enhanced the thrombus formation of the remaining PLTs, whereas cH12-polyAlb did not. They indicate that the particles such as the polyAlb and the cH12-polyAlb did not interfere the PLT adhesion and aggregation, and the enhancement effect was specific for H12 conjugated to the surface of polyAlb.

Next, we prepared the thrombocytopenic rats with busulfan to evaluate a hemostatic ability of H12-polyAlb in vivo. We succeeded in obtaining the extinction curve of PLTs similar to that of the previous studies.[32] It was reported that a low hematocrit resulted in a significant prolongation in the bleeding time.[34] In fact, the bleeding time of the busulfan (20 mg/kg)-injected rats on Day 11 (PLTs; $97 \times 10^3 \pm 25 \times 10^3/\mu L$) was found to be immeasurable, because bleeding did not stop for more than 30 minutes. From the data of hematologic indices, in which the PLT counts were sufficiently decreased and the RBC counts were maintained, as shown in Table 1, we determined that the appropriate dose of busulfan for the rats was 20 mg per kg and the incision of the tail was to be made on Day 10. The bleeding time of the thrombocytopenic rats on Day 10 was extended significantly in comparison with that of normal rats as shown in Fig. 4. The bleeding time approached that of normal rats as their PLT concentration increased. We confirmed that the bleeding time was correlated with their PLT concentration and inferred that the tail bleeding time was an effective evaluation measure for measuring the hemostatic capacity of the PLT substitutes.

The endotoxin concentration in the H12- or cH12-polyAlb suspension was below 0.2 EU per mL, acceptable for the in vivo study. In preliminary experiments we studied the systemic clearance of H12-polyAlb. At a dose of 4 mg per kg (equivalent albumin concentration), the amount of FITC-labeled H12-polyAlb rapidly decreased to 88.8, 51.1, 27.2, and 9.0 percent after 5, 10, 15, and 30 minutes, respectively (taken as 100% just after injection) with a spectrofluorometer (FP-750, Jasco, Tokyo, Japan). The half-life of the H12-polyAlb was estimated to be approximately 10 minutes from the above data. Therefore, we measured the tail bleeding time 5 minutes after the injection. We are now studying the prolongation of the half-life of the H12-polyAlb with PEG modification.

Although not tested in animals with severe thrombocytopenia, we confirmed the hemostatic effect of H12-

polyAlb in vivo with moderately thrombocytopenic rats, suggesting that the polyAlb may be a promising candidate for PLT substitutes (Fig. 5). The circulating H12-polyAlb at the dose of 4 mg per kg significantly reduced the bleeding time of the thrombocytopenic rats, whereas at a lower concentration of 1 mg per kg, the hemostatic effect did not reach a significant level. At a higher concentration of 10 mg per kg, however, the bleeding time of H12-polyAlb was significantly reduced in comparison with that of saline group, but not the polyAlb group, because the bleeding time of the control polyAlb was also slightly reduced, indicating that the polyAlb itself possessed a hemostatic activity at its higher concentration. When we used PEG-modified polyAlb, the bleeding time became comparable to that obtained with saline group (manuscript in preparation), suggesting that negative charge of the control polyAlb at pH 7.4 may nonspecifically cause the shortening of the tail bleeding time.

We estimated that the hemostatic ability of the H12-polyAlb at a dose of 4 mg per kg (approx. 1.0×10^{11} particles) would correspond to 1/20th of the hemostatic ability of one PLT, which was based on the bleeding time of the thrombocytopenic rats and PLT concentration. Furthermore, we confirmed that hematologic indices were stable before and after the administration of H12-polyAlb.

In conclusion, H12-polyAlb was shown to preferentially interact with an activated PLT surface via GPIIb/IIIa receptors and to facilitate PLT accumulation at sites of hemostasis. Furthermore, H12-polyAlb had the hemostatic ability in the tail bleeding model of thrombocytopenic rats. Thus, H12-polyAlb may be a suitable candidate for an alternative to human PLT concentrates infused into thrombocytopenic patients.

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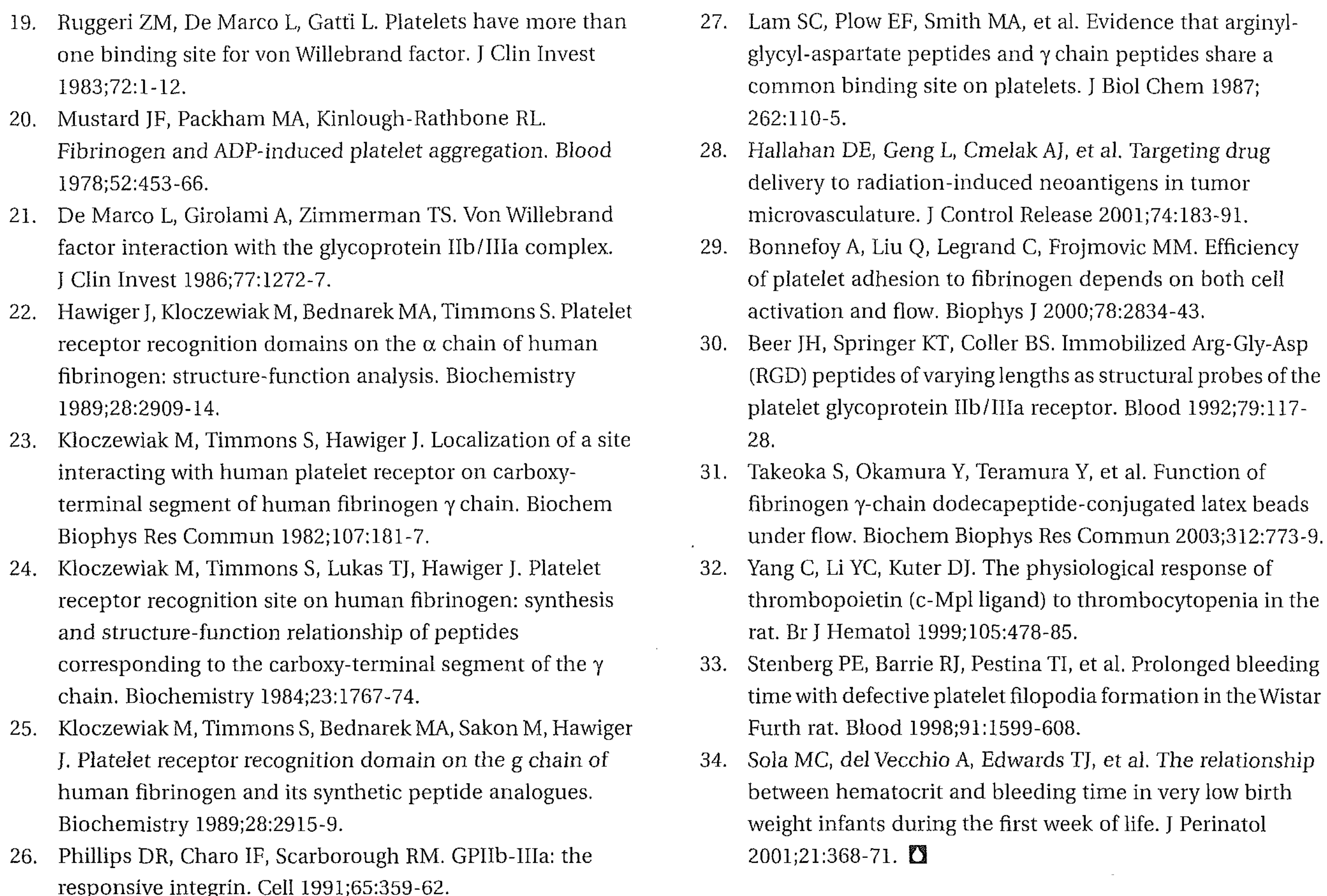

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Hemostatic Effects of Phospholipid Vesicles Carrying Fibrinogen γ Chain Dodecapeptide in Vitro and in Vivo

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We studied prototypes of platelet substitutes that bear on their surface a dodecapeptide, HHLGGAKQAGDV (H12). The peptide is a fibrinogen γ chain carboxy-terminal sequence (γ400–411) and recognizes specifically the active form of glycoprotein (GP) IIb/IIIa on the surface of activated platelets. We conjugated H12 to the end of poly(ethylene glycol) chains on the surface of a phospholipid vesicle with an average diameter of 220 nm to prepare H12–PEG–vesicles. The half-life of the H12–PEG–vesicles was significantly prolonged by PEG modification, and the ability of H12 on the surface of the vesicle to recognize GPIIb/IIIa was maintained even though the surface was modified with PEG chains. The H12–PEG–veiscles enhanced the in vitro thrombus formation of platelets that were adhering to a collagen-immobilized plate, when thrombocytopenia-imitation blood was passed over the plate. Based on the flow cytometric analyses of PAC-1 binding and P-selectin expression, the H12–PEG–vesicles were shown not to cause platelet activation. Furthermore, the H12–PEG–vesicles dose-dependently shortened the tail bleeding time of thrombocytopenic rats. It was confirmed that the H12–PEG–vesicles had a hemostatic effect and may be a suitable candidate for an alternative to human platelet concentrates transfused into thrombocytopenic patients.

INTRODUCTION

Platelet transfusion plays an important role in prophylactic therapy for patients with thrombocytopenia caused by hematologic malignancies or intensive chemotherapy for solid tumors or as a result of surgical procedures and radiotherapy. However, due to the short storage life of platelets (3 days in Japan), the shortage has become a serious concern in an aging society. Furthermore, the risk of viral and bacterial infections accompanied by transfusion is also a serious issue. To solve these problems, various platelet substitutes (*1*) have been developed, such as, solubilized platelet membrane protein-conjugated liposomes (Plateletsome) (*2*), infusible platelet membranes (IPM) (*3*), fibrinogen-coated albumin microcapsules (Synthocyte) (*4*), fibrinogen-bonded red blood cells (*5*), liposomes bearing fibrinogen (*6*), and arginine-glycine-aspartic acid (RGD) peptide-bound red blood cells (Thromboerythrocyte) (*7*). These platelet substitutes consist of materials derived from blood components.

We have developed platelet substitutes using polymerized albumin particles (polyAlb) (*8–11*) and phospholipid vesicles (*12–15*) as biocompatible and biodegradable carriers and shown that those carriers carrying recombinant fragments of platelet membrane proteins (rGPIbα (*16*, *17*) and rGPIa/IIa complex (*18*, *19*)) specifically interact with a surface that mimics the site of bleeding injury, such as von Willebrand factor (VWF) and collagen immobilized on a surface under flow conditions in vitro. Moreover, we demonstrated that rGPIa/IIa-conjugated polyAlb reduced the tail bleeding time of thrombocytopenic mice (*8*). These carriers have the ability to induce platelet hemostasis; however, they cannot recruit flowing platelets to induce platelet aggregation.

When platelets are made to adhere to collagen immobilized on a surface and activated, the conformation of GPIIb/IIIa which exists on the platelet membrane changes from a silent form to an activated form (*20–22*). The activated form acts as a receptor for fibrinogen and VWF (*23–25*), which is followed by platelet aggregation (*26*, *27*). This is because fibrinogen contains three putative platelet interaction sites, namely, a tetrapeptide containing RGD sequences such as RGDF and RGDS at α95–98 and α572–575, respectively, and a dodecapeptide (HHLGGAKQAGDV, H12) at a γ chain carboxy-terminal segment (γ400–411) (*28*).

To study the usefulness of fibrinogen as a recognition site of platelet substitutes, we prepared fibrinogen-conjugated polyAlb, and we confirmed that the polyAlb enhanced adhesion of the flowing platelets to an activated platelet-immobilized surface in vitro (*9*). However, fibrinogen is derived from human blood and when isolated in solution tends to precipitate at 4 °C within a few hours (*29*).

Recently, we have focused on the stable dodecapeptide, H12, instead of fibrinogen (*28*, *30–33*). Based on our results obtained from flow cytometric analyses of agglutination, H12-conjugated latex beads showed minimal interaction with nonactivated platelets in comparison

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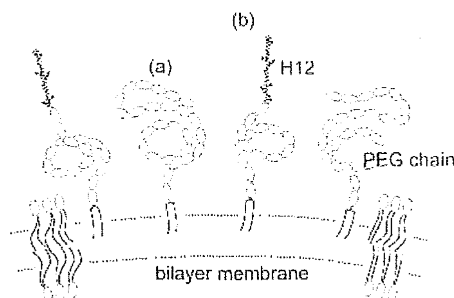


Figure 1. The structure of H12–PEG–vesicles: (a) PEG–DSPE (PEG 5.0 kDa) and (b) H12–PEG–Glu2C18 (PEG: 3.1 kDa, H12: 1.3 kDa) are incorporated into the bilayer membrane.

with RGD-conjugated latex beads (*34*). Furthermore, the H12-conjugated latex beads enhanced in vitro thrombus formation on the collagen-immobilized plate when thrombocytopenia-imitation blood was passed over the plate (*34*).

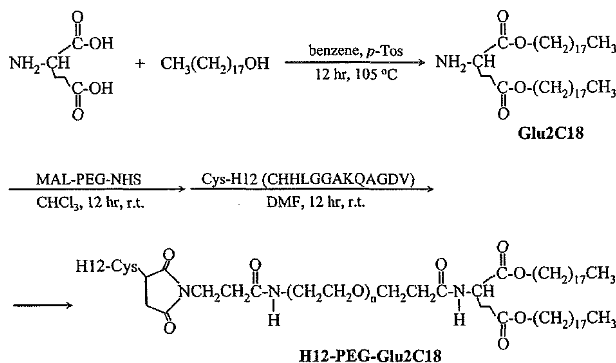
Phospholipid vesicles have been extensively studied as carriers for biologically important substances such as drugs, hemoglobin, enzymes, hormones, and DNA. In particular, recognition proteins such as antibodies and various ligands have been conjugated to the surface of vesicles to bind and interact with tissue epitopes or specific cells. Recently, it was reported that H12-liposomes enhanced platelet aggregation and specifically adhered to the platelets activated on the collagen surface in a state of rest (*35*). On the other hand, poly(ethylene glycol) (PEG) modification of the surface of the vesicles has been widely used to prolong the half-life or to stabilize their dispersion states (*36–40*). We previously reported that PEG modification of the vesicles was effective in preventing intervesicular access and aggregation as determined using a capillary viscosimeter and optical microscopy. Also, subcutaneous microvascular studies showed that PEG conjugates significantly improved microcirculation (flow rate, functional capillary density, and vessel diameter) (*38, 39*).

In this study, we prepared phospholipid vesicles carrying both H12 and PEG chains to enhance the biocompatibility as a platelet substitute and to enhance the stabilization of the vesicles in vitro and in vivo, as noted above. Our purpose was to produce a platelet substitute for the indication of prophylactic transfusion. However, the excluded volume effect of the neighboring PEG chains might shield the recognition ability of H12. Therefore, we conjugated H12 to the end of the PEG chain by using PEG–lipids and prepared the PEG-modified vesicles carrying H12 (H12–PEG–vesicles, Figure 1). Then we studied the recognition ability toward platelets activated on collagen immobilized on a surface when thrombocytopenia-imitation blood was passed over the surface. Furthermore, we intravenously administered the H12–PEG–vesicles into thrombocytopenic rats and measured the tail bleeding time for evaluation of the hemostatic ability of the vesicles.

MATERIALS AND METHODS

Materials. All reagents were used without further purification. L-Glutamic acid, *p*-toluenesulfonic acid monohydrate (*p*-Tos), stearyl alcohol, and *N,N'*-dicyclohexylcarbodiimide were purchased from Kanto Chemical (Tokyo). 1,2-Distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[monomethoxy poly(ethylene glycol)] (5000) (PEG–DSPE, M_w = 5.1 kDa), and α-maleimidyl-ω-hydroxy succinimidyl poly(ethylene glycol) (MAL–PEG–

Scheme 1. Syntheses of H12–PEG-Glu2C18 (PEG 3.1 kDa, H12 1.3 kDa)



NHS, M_w = 3.5 kDa) were purchased from NOF (Tokyo). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) and cholesterol were purchased from Nippon Fine Chemical (Osaka). 1,5-Dihexadecyl-*N*-succinyl-L-glutamate (DHSG) was synthesized in our laboratory (*41, 42*). A fibrinogen γ chain dodecapeptide (C-HHLGGAKQAGDV, Cys-H12) was synthesized with a solid-phase synthesizer by BEX (Tokyo). 3,3'-Dihexyloxacarbocyanine iodide ($DiOC_6$), which is a platelet fluorescent dye, and dioctadecyloxacarbocyanine perchlorate ($DiOC_{18}$), which is a vesicle fluorescent dye, were purchased from Molecular Probes (Eugene, OR). Collagen I-A (Cellmatrix) was purchased from Nitta Gelatine (Osaka). D-Phe-Pro-Arg-chloromethyl ketone (PPACK) was purchased from Calbiochem (San Diego, CA). FITC-labeled PAC-1, which is anti-GPIIb/IIIa antibody, and FITC-labeled anti-P-selectin (CD62P) antibody were obtained from Becton Dickinson (San Jose, CA) and BD-Pharmingen (San Diego, CA), respectively. Both busulfan and poly(ethylene glycol) (average molecular weight 400) were purchased from Sigma-Aldrich (St Louis, MO).

Synthesis of H12–PEG–Glu2C18. The syntheses were performed according to Scheme 1. L-Glutamic acid (2.96 g, 20 mmol) and *p*-Tos (4.56 g, 24 mmol) were dissolved in benzene and refluxed for 1 h at 105 °C. Stearyl alcohol was added to the solution, and the solution was refluxed for 12 h at 105 °C. The crude product was washed with an aqueous solution of saturated Na_2CO_3 and then distilled water. After recrystallization from methanol at 4 °C, 1,5-distearoyl-L-glutamate (Glu2C18) was obtained (9.5 g, yield 80%). Glu2C18 (115 mg, 176 μmol) and MAL–PEG–NHS (300 mg, 88 μmol) were dissolved in chloroform and stirred for 12 h at room temperature (rt). The crude product was purified by reprecipitation from diethyl ether. After recrystallization from ethanol, the MAL–PEG–Glu2C18 was obtained as a white solid (265 mg, yield 64%). MAL–PEG–Glu2C18 (100 mg, 25 μmol) and Cys-H12 (33 mg, 25 μmol) were reacted in *N,N*-dimethylformamide (DMF) for 12 h at rt. The crude product was purified by reprecipitation from diethyl ether and extracted with distilled water. The H12–PEG–Glu2C18 was obtained as a white solid (63 mg, 12 μmol, 47%).

Physicochemical Properties of H12–PEG–Glu2C18. TLC (silica gel, chloroform/methanol = 4/1, v/v): R_f 0.17. IR (film; cm^{-1}): 1736($\nu_{C=O}$, ester). ^1H NMR ($CDCl_3$, 500 MHz, δ ppm): 0.89 (t, 6H, $-CH_3$), 1.26 (br, 60H, $-CH_2-$), 1.63 (m, 4H, $-COOCH_2CH_2-$), 2.00 (m, 2H, $-CH_2CHCON-$), 2.46 (m, 2H, $-CH_2COO-$), 4.08 (m, 4H, $-COOCH_2-$), 3.63 (br, 360H, PEG).

Preparation of H12–PEG–vesicles. DPPC (100 mg, 136 μmol), cholesterol (52.7 mg, 136 μmol), DHSG (18.9

mg, 27.2 μmol), PEG–DSPE (5.2 mg, 0.90 μmol), and H12–PEG–Glu2C18 (0, 0.47, 1.57, 4.7, or 9.4 mg; 0, 0.090, 0.30, 0.90, or 1.8 μmol, respectively) were dissolved in benzene and then freeze-dried. The resulting white powder was hydrated with distilled water and extruded with membrane filters (pore sizes 3.00, 0.80, 0.65, 0.30, or 0.22 μm, FM filter, Fuji Film, Tokyo). The vesicles were washed with phosphate-buffered saline (PBS, pH 7.4) by suspension and centrifugation (100 000*g*, 30 min, 4 °C, twice). The particle diameter was analyzed by a dynamic light scattering method (N4 PLUS, Beckman-Coulter, Fullerton, FL). We prepared PEG–vesicles or vesicles using lipids in the absence of H12–PEG–Glu2C18 or H12–PEG–Glu2C18 and PEG–DSPE, respectively.

Measurement of the Stability of H12–PEG–vesicles. For the measurement of the incorporation ratio of H12–PEG–Glu2C18 to the vesicle, the H12–PEG–vesicles dispersion was freeze-dried to dissolve in $CDCl_3$. The peak area ratio (R_1) of the choline methyl proton of DPPC (3.37 ppm) to the methylene proton of the PEG chain in H12–PEG–Glu2C18 (3.64 ppm) was measured by ^1H NMR spectroscopy (JNM-Lambda 500, JEOL, Tokyo) to determine the incorporation ratio of the H12–PEG–Glu2C18. To study the change of the incorporated PEG lipids in the vesicles, the 30-fold diluted sample dispersion ([lipid] = 0.58 M in PBS, pH 7.4) was incubated at 37 °C, and R_1 was calculated from the ^1H NMR spectrum as described above. The peak area ratio before the incubation was defined as R_0. The incorporation ratio was calculated from a percentage of R_1 to R_0.

Preparation of Collagen Immobilized on a Surface. Collagen I-A (3.0 mg/mL) was suspended in PBS at 4 °C (30 μg/mL), and glass plates (diameter 24 mm, thickness 0.5 mm) were immersed into the collagen suspension at 4 °C for 8 h. The glass plates were carefully rinsed with PBS and immersed in a bovine serum albumin solution (20 mg/mL) at rt for 2 h before perfusion studies were performed, as described below.

Preparation of Platelets Immobilized on a Surface. Blood withdrawn from three healthy volunteers was mixed with a 10% volume of 3.8% (w/v) sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation (100*g*, 15 min, 22 °C). The PRP was mixed with a 15% volume of acid–citrate–dextrose composed of 2.2% (w/v) sodium citrate, 0.8% (w/v) citric acid, and 2.2% (w/v) glucose (ACD) containing 1 μM prostaglandin E_1 (PGE_1, Sigma, St. Louis, MO). The suspension was centrifuged (2200*g*, 10 min, 22 °C), and the plasma was replaced with a Ringer's–citrate–dextrose solution (RCD solution, composition 0.76% (w/v) citric acid, 0.090% (w/v) glucose, 0.043% (w/v) $MgCl_2$, 0.038% (w/v) KCl, 0.60% (w/v) NaCl, pH 6.5) containing 1 μM PGE_1. After the pellets were resuspended in the RCD solution, the suspension was centrifuged (2200*g*, 10 min, 22 °C), and the concentrated platelets were resuspended at 2.0 × $10^5/\mu$L in a Hepes–Tyrode buffer (H–T buffer, pH 7.4). The platelet counts were determined using an automated hematology analyzer (K-4500, Sysmex, Kobe, Japan). A collagen-coated glass plate was immersed into the platelet suspension for 1 h at 37 °C and carefully rinsed with PBS.

Measurement of Interaction of the H12–PEG–vesicles with Platelets Immobilized on a Glass Plate using Reconstituted Blood. Blood containing a 10% volume of 3.8% (w/v) sodium citrate was centrifuged (100*g*, 15 min, 22 °C) to replace PRP with an equal volume of a 0.9% (w/v) NaCl solution containing 10% (v/v) ACD (10% ACD–saline). The red blood cell suspension was centrifuged (2200*g*, 10 min, 22 °C) and washed with 10% ACD–saline three times to remove the buffy coat and plasma completely. After the final centrifugation, the red blood cells were resuspended in a H–T buffer to adjust the hematocrit (Hct) to 50%. The residual platelet counts were determined to be (5.0 ± 2.0) × $10^3/\mu$L.

A solution of $DiOC_{18}$ in DMSO (10 mM, 50 μL) was added to the H12–PEG–vesicle suspension (20 mg/mL, 5 mL), and the suspension was incubated at rt for 30 min. The free $DiOC_{18}$ was separated by washing with PBS and centrifugation at 100 000*g* for 30 min at 4 °C twice, and the $DiOC_{18}$-labeled H12–PEG–vesicles were collected.

The reconstituted blood and the $DiOC_{18}$-labeled H12–PEG–vesicle mixtures were placed in a recirculating chamber mounted on an epifluorescent microscope (ECLIPS TE300, Nikon, Tokyo) equipped with a CCD camera, and the interaction of the H12–PEG–vesicles with the platelets immobilized on the surface was observed. All perfusion studies were performed at 37 °C. Single-frame images of the H12–PEG–vesicles on the plates were obtained with an image processor, Argus-50 (Hamamatsu Photonics, Hamamatsu).

Measurement of the Dynamic Interaction of Platelets in Thrombocytopenia-Imitation Blood with a Collagen-Coated Surface in the Presence of the H12–PEG–vesicles. Blood containing PPACK (40 μM), a thrombin inhibitor, was filtered with a leukocyte removal filter (NEO1J, Nihon Poll, Tokyo), which can remove both leukocytes and platelets from whole blood. The residual platelet count in the blood was (6.0 ± 2.0) × $10^3/\mu$L. The platelet count was adjusted to 5.0 × $10^4/\mu$L with PRP. This blood preparation was termed thrombocytopenia-imitation blood.

Platelets in the blood were labeled with $DiOC_6$ (final concentration, 2 μM), and the H12–PEG–vesicles were added to the blood for 5 min at 37 °C ([lipid] = 0.89 mM). The blood was placed in a recirculating chamber mounted on an epifluorescent microscope as described above, and the interaction of platelets with the collagen immobilized on the surface was observed. The surface coverage of the platelets that adhered on the plate was calculated with an image processor, Argus-20 (Hamamatsu Photonics).

Measurement of PAC-1 Binding and P-Selectin Expression of Resting Platelets in the Presence of the H12–PEG–vesicles Using Flow Cytometry. H12–PEG–vesicles or PEG–vesicles (final lipid concentration 0.82 μM) were added to PRP ([platelet] = 5.0 × $10^4/\mu$L), and the mixtures were stirred at 37 °C for 30 min. Before and after stirring, FITC-labeled anti-P-selectin antibody (approximately 0.5 μg) was added to the mixture, incubated at 37 °C for 10 min, and then fixed with formaldehyde (final concentration 1.5% (v/v)). Alternatively, FITC-labeled PAC-1 (approximately 0.5 μg) was added to the mixture of platelets and samples and stirred at 37 °C for 10 min, followed by fixing with formaldehyde. As a positive control, ADP-stimulated platelets (final concentration of ADP; 20 or 100 μM) were prepared from PRP. The platelets were gated according to their characteristic forward versus side scatter, and 20 000 platelets were analyzed using a FACSCalibur flow cytometer (Nihon Becton Dickinson, Tokyo). PAC-1 binding or P-selectin expression was quantified as a fraction of the fluorescent-positive platelets. Each experiment was performed at least three times.

Measurement of Tail Bleeding Time and Half-Life of H12–PEG–vesicles Using Thrombocytopenic Rats. All animal studies were approved by the Animal Subject Committee of Keio University School of Medicine and performed according to NIH guidelines for the care

and use of laboratory animals. Experiments were carried out using male Wistar rats (230–250 g, CLEA Japan, Tokyo). A busulfan solution was prepared at a final concentration of 5 mg/mL in poly(ethylene glycol) (average molecular weight 400) (*11, 43, 44*). Rats were anesthetized with diethyl ether and injected on day 0 and day 3 with 10 mg/kg on each dosing day to produce a total dosage of 20 mg/kg of busulfan. On day 10, thrombocytopenic rats were anesthetized with sevofrane, and the sample suspension was infused into the tail vein. The samples were the H12–PEG–vesicles and the PEG–vesicles at a dose of 4 mL/kg; saline was used for control. Five minutes after administration, a template-guided incision (Quikheel, Becton-Dickinson, San Jose, CA) 2.5 mm in length and 1.0 mm in depth was made 1 cm from the tip of the tail. The tail was immersed in a 50 mL cylinder of saline, and the time taken for bleeding to stop was measured. In addition, blood was sampled from ether-anesthetized rats 5 min before and 30 min after sample injection by inserting a 25-gauge needle into the tail vein, and the cell counts were determined using an automated hematology analyzer (K-4500).

Half-life was measured from the β-phase of the clearance curve as follows: the $DiOC_{18}$-labeled H12–PEG–vesicles, PEG–vesicles, or vesicles were infused into the tail vein of thrombocytopenic rats at a dose of 40 mg/kg (equivalent lipid concentration). Following this, 400 μL of blood was collected from the tail vein of the rats at time intervals and centrifuged (2200*g*, 10 min, 22 °C), and 200 μL of plasma containing vesicles was collected. The plasma was mixed with deca(oxyethylene) dodecyl ether ($C_{12}E_{10}$) and was heated at 42 °C for 2 min to enhance the solubilization of the vesicles. The fluorescent intensity was then measured (E_x = 485 nm, E_m = 500 nm), and the half-life was calculated using a spectrofluorometer (FP-750, JASCO, Tokyo).

Statistical Analysis. Statistical significance of the results for H12–PEG–vesicles group vs the PEG–vesicles group was tested with Tukey–Kramer tests (Figure 4). A P value of less than 0.05 was considered to be statistically significant. Statistical analyses were performed using Stat View software (SAS Institute).

RESULTS

Characterization of H12–PEG–vesicles. L-Glutamic acid was selected as a connector to synthesize PEG–lipid derivatives as shown in Scheme 1. Each of the two carboxyl groups of the glutamic acid were bonded to a stearyl alcohol molecule by an ester bond to produce a hydrophobic moiety. The product, Glu2C18, was obtained after reprecipitation and recrystallization with a yield of 80%. MAL–PEG–Glu2C18 was obtained from the reaction of MAL–PEG–NHS and Glu2C18 with a yield of 64%. Cys-H12, where cysteine was introduced at the N terminal end of H12, was reacted with MAL–PEG–Glu2C18 in DMF to obtain H12–PEG–Glu2C18. In the ^{1}H NMR spectrum of H12–PEG–Glu2C18, the peak (δ = 6.70 ppm) that was attributed to the maleimide group of MAL–PEG–Glu2C18 disappeared, indicating the bonding of the thiol group of the cysteine with the maleimide group of MAL–PEG–Glu2C18 (yield 47%).

Next, we prepared H12–PEG–vesicles (220 ± 70 nm), DPPC/cholesterol/DHSG = 5/5/1, by molar ratio (Figure 1), which were modified with PEG–DSPE (0.3 mol %) and H12–PEG–Glu2C18 (from 0.03 to 0.6 mol %). The molecular weights of the PEG chain of the PEG–DSPE and H12–PEG–Glu2C18 were 5.0 and 3.1 kDa, respectively. The incorporated ratio (R_1/R_0) of the H12–PEG–Glu2C18 (0.3 and 0.6 mol %) was measured after incubation at 37 °C in a 30-fold dilution to analyze the stability of the lipids in the vesicle. From the ^{1}H NMR analyses, the R_1/R_0 of the H12–PEG–Glu2C18 did not change after the incubation for 12 h at 37 °C. The endotoxin concentration in the H12–PEG–vesicle suspension ([lipid] = 53.5 mM) was below 0.2 EU/mL. Furthermore, the half-life of H12–PEG–vesicles was estimated to be 200 ± 23 min from the β-phase of the clearance curve. In the cases of the control PEG–vesicles and the bare vesicles, the half-life was calculated to be 268 ± 2 min and 29 ± 1 min, respectively.

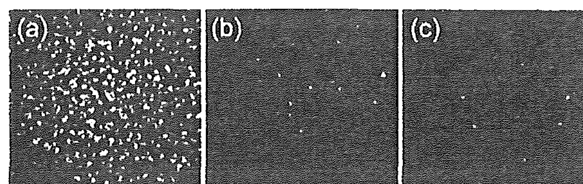


Figure 2. *Specific adhesion of H12–PEG–vesicles in the* reconstituted blood (Hct. 50%, platelet counts = (5.0 ± 2.0) × $10^3/\mu$L) to platelets activated on the collagen surface at a shear rate of 150 s^{-1}: (a) H12–PEG–vesicles; (b) H12–PEG–vesicles in the presence of PAC-1; (c) PEG–vesicles. Images a–c were obtained after blood was circulated for 500 s ([lipid] = 0.89 M).

Interaction of the H12–PEG–vesicles with Platelets Immobilized on a Surface under Flow Conditions. $DiOC_{18}$-prelabeled H12–PEG–vesicles (modification ratio of H12 0.3 mol %) were mixed with the reconstituted blood, and the mixture was allowed to flow over the activated platelets that were adhering to the collagen surface at a shear rate of 150 s^{-1}. The vesicles adhered and accumulated on the surface in a time-dependent manner, whereas the control PEG–vesicles did not adhere (Figure 2). The adhesion of the H12–PEG–vesicles was suppressed in the presence of PAC-1, an inhibitor of GPIIb/IIIa of the activated platelets.

Platelet Adhesion to a Collagen-Coated Surface in Thrombocytopenia-Imitation Blood Containing the H12–PEG–vesicles. We prepared a thrombocytopenia-imitation blood and adjusted the platelet counts to 5.0 × $10^4/\mu$L. The platelets were labeled with a fluorescent reagent, $DiOC_6$, to observe the dynamic interactions of the platelets with the collagen-coated surface at a shear rate of 150 s^{-1}. In this case, vesicles were not labeled. In the presence of the control PEG–vesicles, the surface coverage of platelets gradually increased to 10.6% ± 1.3% after 300 s of flow (Figure 3A, filled triangle). The value was not appreciably different (9.1% ± 0.4%) in the absence of the PEG–vesicles (Figure 3A, filled circle). When the H12–PEG–vesicles (modification ratio of H12 0.3 mol %) were added to the blood in the same way, the initial adhesion rate was comparable to that obtained with the PEG–vesicles; however, the adhesion rate increased dramatically with time, and the surface coverage reached 17.2% ± 1.9% (Figure 3A, opened circle).

We carried out the same experiments using the H12–PEG–vesicles with various modification ratios of the H12–PEG–Glu2C18 from 0 to 0.6 mol % (Figure 3B). The surface coverage of the PEG–vesicles and the H12–PEG–vesicles having the modification ratio of 0.1 mol % was 8.8% ± 1.8% and 9.5% ± 4.2% after 300 s of flow, respectively. When the H12-modification ratio was higher than 0.3 mol %, the surface coverage increased significantly to 17.2% ± 1.9% and then saturated.

PAC-1 Binding to and P-Selectin Expression of Platelets in the Presence of the H12–PEG–vesicles.

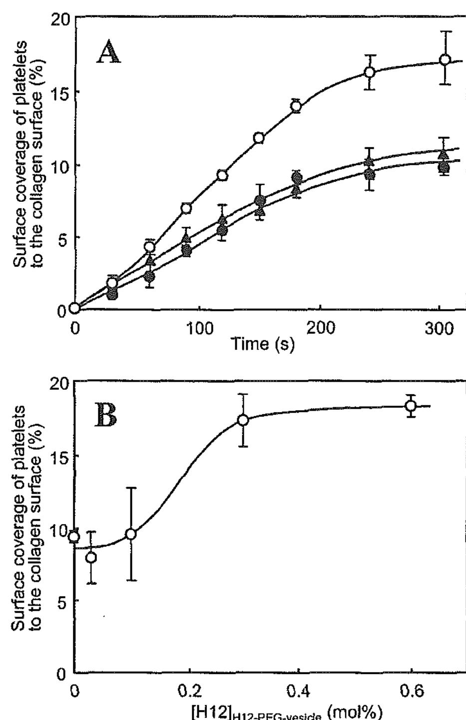


Figure 3. (A) Time course studies of surface coverage of platelets *in the thrombocytopenic-imitation blood on the collagen* surface after addition of (○) H12–PEG–vesicles, (●) PEG–vesicles, and (▲) PBS and (B) comparison between surface coverage of platelets and modification ratio of H12 on the surface of vesicles (shear rate 150 s^{-1}, [platelets] = 5.0 × $10^4/\mu L$, [lipid] = 0.89 M, N = 3). The surface coverage was measured after 300 s.

Table 1. Binding Ratios of PAC-1 and Anti-P-selectin Antibody to the Resting Platelets in the Presence of H12–PEG–vesicles (N = 3)

stirring time (min)	PAC-1 binding (%) 0	PAC-1 binding (%) 10	P-selectin expression (%) 0	P-selectin expression (%) 10
PBS	0.37 ± 0.12	3.77 ± 0.89	0.35 ± 0.11	2.52 ± 0.52
PEG–vesicles	0.52 ± 0.11	3.35 ± 0.32	0.50 ± 0.09	2.27 ± 0.54
H12–PEG–vesicles ([H12] = 0.3 mol %)	0.34 ± 0.16	3.72 ± 0.53	0.40 ± 0.10	2.43 ± 0.21
	89.80 ± 10.90[a]		33.42 ± 6.87[b]	

[a] ADP = 100 μM. [b] ADP = 20 μM.

We measured the ratios of PAC-1 binding to platelets and the P-selectin expression of platelets in the presence of the H12–PEG–vesicles (modification ratio of H12 0.3 mol %) (Table 1). In the ADP-stimulated and the resting platelets, the PAC-1 binding ratio was 89.8% ± 10.9%, and 0.37% ± 0.12%, respectively. When the resting platelets were stirred for 10 min at 37 °C, the ratio increased slightly to 3.77% ± 0.89%. Before and after mixing with the H12–PEG–vesicles, the ratio was 0.34% ± 0.16% and 3.72% ± 0.53%, respectively. In the case of the PEG–vesicles, the values (before 0.52% ± 0.11%, after 3.35% ± 0.32%) were approximately the same as those of the H12–PEG–vesicles.

On the other hand, the P-selectin expression ratios before and after mixing with the H12–PEG–vesicles were 0.40% ± 0.10% and 2.43% ± 0.21%, respectively, whereas in the PEG–vesicles, the values did not change (before 0.50% ± 0.09%, after 2.27% ± 0.54%). The positive and negative controls were 33.42% ± 6.87% and 0.35% ± 0.11%, respectively.

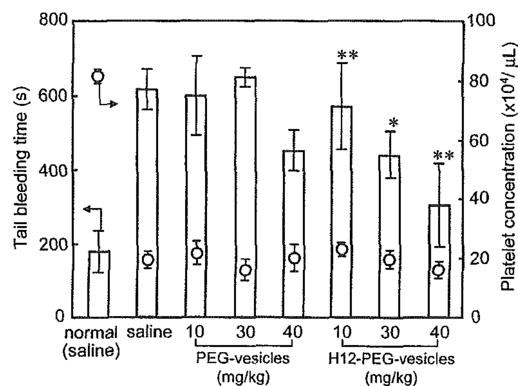


Figure 4. Effects of the administration of H12–PEG–vesicles on tail bleeding time. The administered amount of H12–PEG–vesicles was 10, 30, or 40 mg/kg equivalent of lipid. ○ indicates *the platelet concentration in the rats (N = 6–10); ∗ indicates P* < 0.05 or ∗∗ indicates not significant for H12–PEG–vesicles vs PEG–vesicles group at the same dose.

Measurement of the Tail Bleeding Time of Thrombocytopenic Rats in the Presence of the H12–PEG–vesicles. Thrombocytopenic rats were prepared by administering busulfan at a total dose of 20 mg/kg as described in our previous study (*11*). The tail bleeding times of normal rats ([platelet] = (8.1 ± 0.9) × $10^5/\mu L$) and thrombocytopenic rats ([platelet] = (2.0 ± 0.3) × $10^5/\mu L$) were 178 ± 56 and 618 ± 51 s, respectively (Figure 4). The bleeding time of the thrombocytopenic rats was about 3.5 times longer than that of the normal rats. Intravenous administration of the H12–PEG–vesicles at a dose of 10 mg/kg slightly reduced the bleeding time to 573 ± 127 s in comparison with those of a saline group (618 ± 51 s) or a control PEG–vesicle group at the same dose of the H12–PEG–vesicles (598 ± 115 s). At doses of 30 and 40 mg/kg, we confirmed a dose-dependent reduction of the bleeding time as shown in Figure 4; the time decreased significantly to 441 ± 66 and 335 ± 96 s, respectively. By comparison, the bleeding times of the control PEG–vesicles groups at doses of 30 and 40 mg/kg were 662 ± 21 and 432 ± 22 s, respectively. At the dose of 30 mg/kg, H12–PEG–vesicles significantly reduced the bleeding time in comparison with controls, whereas the H12–PEG–vesicles tended to reduce the bleeding time at the dose of 40 mg/kg.

DISCUSSION

We previously showed that fibrinogen-related dodecapeptide (H12) did not interact with the nonactivated platelets and did not cause platelet activation by using H12-latex beads (*34*). This indicated that H12 is a promising candidate as a key material of platelet substitutes. In this paper, we prepared H12–vesicles modified with PEG to obtain information about the possibility of constructing biocompatible and biodegradable particles with hemostatic ability in thrombocytopenic conditions. On the other hand, PEG-conjugated phosphatidylethanolamines (PEG–phospholipids) have been widely used for modification of the surface of vesicles to prolong the blood circulation time or to stabilize their dispersion states (*40–44*). Furthermore, PEG–phospholipids in which the terminus has an active group such as maleimido and pyridyl disulfide groups were also utilized to conjugate receptor proteins or ligands. We are proposing the use of amino acid-based lipids instead of phospholipid because various modifications are possible by simple syntheses. In this study, we used the PEG-conjugated amino acid-based lipid, as in our previous studies (*41*,

42). L-Glutamic acid was used as a connector between the PEG chain and two hydrophobic chains, in this case, stearyl alcohol (Glu2C18). The length of the alkyl chain is important because shorter alkyl chains should result in the dissociation of the PEG−lipid from the surface of the vesicle. The amino group of Glu2C18 reacted with the NHS group of MAL−PEG−NHS to produce MAL−PEG−Glu2C18 with a yield of 64%. The low yield is understandable because the NHS group was degraded before or during the reaction by hydrolysis. H12−PEG−Glu2C18 was thus obtained in a relatively high yield via an addition reaction between the maleimido and the mercapto groups of H12.

Next, we prepared vesicles consisting of DPPC/cholesterol/DHSG/PEG−DSPE at a molar ratio of 5/5/1/0.033. The vesicles with this composition were confirmed to be very stable in the state of aqueous dispersion and had excellent blood compatibility with an adequate blood circulation time (*41*). In the case of the PEG modification ratio of 0.3 mol %, the PEG chain is considered to take a so-called mushroom structure on the surface of the vesicle (*45*). For example, if 0.3 mol % of PEG−DSPE (M_w 5.0 kDa) and 0.3 mol % of H12−PEG−Glu2C18 (M_w of PEG chain 3.4 kDa) were incorporated in the surface of the vesicle, the excluded volume of the PEG chains would be estimated from the globular polymer chain as a radius of 6.2 and 4.9 nm, respectively, and the linear dodecapeptide (ca. 4.5 nm) was conjugated to the end of the PEG chain. This means that the H12 should locate on the more outer side more than the neighboring PEG chains, and therefore, the H12 has a possibility to be recognized by the platelets activated on the collagen plate.

The H12−PEG−Glu2C18 was incorporated into the bilayer membrane of the H12−PEG−vesicles in an equilibrium state. We have to determine whether the PEG−lipid tends to dissociate from the vesicular surface after dilution of the H12−PEG−vesicle suspension. We measured the incorporated ratio of H12−PEG−Glu2C18 (0.3 and 0.6 mol %) after incubation at 37 °C in a 30-fold dilution to analyze the stability of the lipids incorporated in the vesicles. From the ^{1}H NMR analyses, the incorporated ratio of the H12−PEG−Glu2C18 did not change. This indicated that the H12−PEG−Glu2C18 with the molecular weight of 4.4 kDa could be tethered to the vesicular surface with two stearoyl chains of Glu2C18. Therefore, the H12−PEG−vesicles would circulate stably in vivo without dissociation of the PEG−lipids. Furthermore, we confirmed that the half-life of the H12−PEG−vesicles was approximately 7-fold longer than that of the bare vesicles due to the effect of PEG modification, indicating that the H12−PEG−vesicles were useful for prophylactic transfusion. We are now studying the prolongation of the hemostatic effects at 180 min and longer after the intravenous administration of the H12−PEG−vesicles.

We prepared the immobilized-platelet surface and reconstituted blood to determine whether the H12−PEG−vesicles specifically bind to the immobilized platelets. In our previous studies, the flowing platelets specifically tethered and adhered to the platelets immobilized on the collagen plate, and the adhesion was suppressed in the presence of PAC-1 (*46*), soluble RGD, or soluble H12, which are GPIIb/IIIa antibodies or inhibitors, indicating that the immobilized-platelet plate should have GPIIb/IIIa exposed on the surface (*9, 47*) (data not shown). Especially, PAC-1 recognizes an epitope on the GPIIb/IIIa complex of activated platelets at or near the platelet fibrinogen receptor (*46*). In reconstituted blood flowing over the immobilized-platelet plate, the H12−PEG−vesicles adhered and accumulated on the surface, whereas the control PEG−vesicles did not adhere as shown in Figure 2. When PAC-1 was added to the blood containing the H12−PEG−vesicles, the adhesion of the H12−PEG−vesicles was completely inhibited, indicating that the H12−PEG−vesicles were specifically bound to the activated GPIIb/IIIa on the surface of the platelets even with PEG modification. This was supported by the results showing that the number of the adherent H12−PEG−vesicles increased with increasing H12-modification ratio to the vesicle from 0.03 to 0.6 mol % (data not shown).

To determine whether the H12−PEG−vesicles have an ability to reinforce thrombus formation under thrombocytopenic conditions, we prepared a thrombocytopenic blood and adjusted the number of platelets to 5.0 × $10^4/\mu$L (one-fifth of normal). When the thrombocytopenic blood flowed over the collagen surface, the control PEG−vesicles did not influence the surface coverage of platelets as shown in Figure 3A (filled circle). In the case of the H12−PEG−vesicles having 0.3 mol % H12, the enhancement of the platelet thrombus formation was confirmed as shown in Figure 3A (opened circle). These results suggest that the H12−PEG−vesicles cross-link the activated platelets to enhance the platelet aggregation. In the initial 30 s, the adhesion rate of platelets in the presence of the H12−PEG−vesicles was similar to that of the PEG−vesicles, and then the surface coverage increased dramatically with time in the case of the H12−PEG−vesicles. Furthermore, we confirmed that the H12−PEG−vesicles labeled with rhodamine were involved in the platelet thrombus labeled with $DiOC_6$ by a double-stain method (data not shown). The above results suggest that (1) the adhesion of the H12−PEG−vesicles could be initiated by the activated platelets, which had already adhered on the surface of the immobilized-collagen plate, (2) the H12−PEG−vesicles adhering to the surface of the platelet could act as binding sites for the activated platelets, and (3) the H12−PEG−vesicles could accelerate the formation of the thrombus of the remaining flowing platelets in the thrombocytopenic blood.

Figure 3B summarized the results of the surface coverage of platelets after 300 s in the presence of the H12−PEG−vesicles in which the modification ratio of H12 is from 0 to 0.6 mol % in thrombocytopenic blood. From 0 to 0.1 mol %, the constant surface coverage in the region of the modification ratios suggests that the recognition ability of the H12−PEG−vesicles to the GPIIb/IIIa on the activated platelets was very low because of the low modification density of H12. Surprisingly, the surface coverage increased significantly when the modification ratio was raised above 0.3 mol %. This indicated that the H12−PEG−vesicles had the ability to cross-link the activated platelets. Therefore, we adopted the H12 modification ratio of 0.3 mol %.

Before in vivo studies, we used platelet-activation markers, PAC-1 or anti-P-selectin antibody (*48*), to determine whether the H12−PEG−vesicles caused the activation of platelets. P-selectin is a membrane glycoprotein existing in the α granules of platelets and Weibel−Palade bodies of vascular endothelial cells, and following cellular activation, it becomes surface-expressed by exocytosis (*48*). When a mixture of platelets and the control PEG−vesicles was stirred with PAC-1 or anti-P-selectin antibody, the binding ratios of both antibodies to the platelets were slightly increased after stirring as shown in Table 1. These phenomena were also observed in the absence of the control PEG−vesicles or in the

presence of H12–PEG–vesicles as shown in Table 1. This suggested that the increase was caused by stirring and was within the error limit in comparison with that of PAC-1 binding and P-selectin expression to ADP-stimulated platelets. We concluded that the H12–PEG–vesicles did not activate platelets and did not interact with the nonactivated platelets under flow and that the adhesion of the H12–PEG–vesicles was induced by the platelets activated by the collagen surface. Therefore, H12–PEG–vesicles have the potential to be a candidate for platelet substitutes.

Next, we evaluated the hemostatic ability of the H12–PEG–vesicles using thrombocytopenic rats. In our previous studies, we succeeded in preparing thrombocytopenic rats by busulfan administration and getting the extinction curve of platelets reproducibly (*11*). From the data of hematologic indices, where the platelet counts were sufficiently decreased and the erythrocyte and leukocyte counts were maintained, we determined that the appropriate total dosage of busulfan for the rats (8 weeks) was 20 mg/kg. The incision of the tail was to be made on Day 10 after busulfan infusion. We also confirmed that the bleeding time correlated with their platelet concentration, indicating that the tail bleeding time was an effective evaluation parameter for measuring the hemostatic capability of the platelet substitutes.

As shown in Figure 4, the bleeding time of the thrombocytopenic rats was significantly extended in comparison with that of normal rats 5 min after the intravenous administration of saline, and we determined that it was possible to evaluate the hemostatic effect of the H12–PEG–vesicles in vivo using thrombocytopenic rats. The circulating H12–PEG–vesicles at the dose of 30 mg/kg significantly reduced the bleeding time of the thrombocytopenic rats, whereas at a lower concentration of 10 mg/kg, the hemostatic effect did not reach a significant level. At a higher concentration of 40 mg/kg, however, the bleeding time was significantly reduced in comparison with that of the saline group but not with the control PEG–vesicles group (40 mg/kg). This suggested that the PEG–vesicles themselves possessed a hemostatic activity at the higher concentration, because the PEG–vesicles having negative charge at pH 7.4 might nonspecifically bind to site of bleeding injury.

We estimated that the hemostatic ability of the H12–PEG–vesicles at a dose of 30 mg/kg (approximately 2.0×10^{12} particles) would correspond to 1/500th of the hemostatic ability in particle base, which was based on the bleeding time of the thrombocytopenic rats and platelet concentration. Furthermore, we confirmed that hematologic indices such as the platelet and leukocyte counts did not change before and after the administration of the H12–PEG–vesicles.

In conclusion, we demonstrated that the H12–PEG–vesicles did not interact with nonactivated platelets, and the vesicles were shown to preferentially interact with an activated platelet surface via GPIIb/IIIa receptors and to facilitate platelet accumulation at sites of hemostasis. Furthermore, the H12–PEG–vesicles could reduce the tail bleeding time of thrombocytopenic rats. Thus, the H12–PEG–vesicles may be a suitable candidate for an alternative to human platelet concentrates infused into thrombocytopenic patients.

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