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Decoration of fibrinogen γ-chain peptide on adenosine diphosphate-encapsulated liposomes enhances binding of the liposomes to activated platelets

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ABSTRACT

For the purpose of efficient hemostasis, we previously developed ADP-encapsulated liposomes modified with a dodecapeptide (HHLGGAKQAGDV, H12), H12-(ADP)Lipo. This liposome actually enhanced platelet aggregation *in vitro*, and showed significant hemostatic effect *in vivo*. Since fibrinogen (Fbg) is abundant in the bloodstream, it is unclear why this liposome binds platelets so efficiently, overcoming the competition with Fbg. Therefore, we investigated the relationship between H12 density on the liposome and the binding ability to platelets, and evaluated the inhibitory effect of Fbg on the binding of H12-(ADP)Lipo to platelets. As a result, the binding ability to platelets steeply increased depending on H12 density until it reached about 3×10^{15} H12 molecules/m^2. The 50% inhibition concentration of Fbg on the binding of H12-(ADP)Lipo to platelets was about 25-fold over the concentration of H12 molecules on the liposome. Moreover, almost no inhibition by Fbg was observed at the physiological concentration of it. This result suggests that the ability of H12 to bind to GPIIb/IIIa increased overwhelmingly by the anchoring to the liposome that enabled the cooperative binding of H12 peptides to the platelets.

1. Introduction

Recently, strong chemotherapy and bone-marrow transplantation techniques have appeared as a treatment method for malignant tumors and disorders of the hematopoietic system. Since these treatments often are accompanied by a decrease in the platelet count, the importance of platelet transfusion has come to be recognized as a supportive therapy. However, there are two serious problems with platelet transfusion. One of them is the short supply caused by the short storage life of platelets (4 days in Japan, 5–7 days in USA and Europe). The other is the side effects such as transfusion-transmitted diseases and immune reactions. To solve these problems, various platelet substitutes (Blajchman, 2003), which consist of materials derived from blood components, have been developed, such as solubilized platelet membrane protein-conjugated liposomes, plateletsome (Rybak and Renzulli, 1993); infusible platelet membranes, IPM (Graham et al., 2001); fibrinogen-coated albumin microcapsules, synthocyte (Levi et al., 1999); fibrinogen-bonded red blood cells (Agam and Livine, 1992); liposome-bearing fibrinogen (Casals et al., 2003); arginine–glycine–aspartic acid (RGD) peptide-bound red blood cells, thromboerythrocyte (Coller et al., 1992). Some of these make platelet-like aggregates, and others enhance platelet aggregation.

In the circulation, platelet aggregation is mediated by fibrinogen, which bridges adjacent platelets through integrin αIIbβIII (GPIIb/IIIa) on the platelet surface in an activation-dependent manner. We have developed platelet substitutes using liposome modified with dodecapeptide (HHLGGAKQAGDV, H12) as biodegradable carriers for the purpose of enhancing platelet aggregation. H12 peptide is a fibrinogen γ-chain carboxyl-terminal sequence (γ400–411) and recognizes specifically the active form of GPIIb/IIIa on the surface of activated platelets. Other sequences in fibrinogen have been designated as GPIIb/IIIa recognition sites: the RGD-based sequences ^{95}RGDF^{98} and ^{572}RGDS^{575} in the Aα-chain (Andrieux et al., 1989). Whereas, RGD-related peptides interact with many integrins expressed in various types of cells, H12 peptide

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has high specificity toward GPIIb/IIIa on platelets (Ruoslahti et al., 1996). In fact, H12-coated polymerized albumin and H12-coated polyethylene glycol-modified liposomes show specific interaction with activated platelets, augment platelet-mediated thrombus formation on collagen-immobilized surfaces under flow condition *in vitro*, and prolong hemostatic ability *in vivo* to correct bleeding time in a dose-dependent manner in thrombocytopenic rat and rabbit models (Okamura et al., 2005a,b, 2007).

To strengthen the hemostatic ability of the H12-coated liposome (H12-Lipo) as a platelet substitute, we exploited its drug delivery function by encapsulating adenosine diphosphate (ADP), potent platelet agonist, into the liposomes, referred to as H12-(ADP)Lipo. In fact, H12-(ADP)Lipo was more effective than H12-Lipo or liposome encapsulating ADP without surface modification (ADP)Lipo, for platelet aggregation *in vitro* and for hemostasis *in vivo* (Okamura et al., 2009). It is known that a large amount of fibrinogen (Fbg), approximately 200 mg/dL, is present in normal human blood (Halle et al., 1996), which would be expected to compete with H12 peptide for the binding to GPIIb/IIIa (Kloczewiak et al., 1984), as the affinity of Fbg for GPIIb/IIIa on platelets is known to be higher than that of H12 peptide (Timmons et al., 1984; Ruggeri et al., 1986). Therefore, the enhancing effect of H12-(ADP)Lipo on platelet aggregation and hemostasis means that H12-(ADP)Lipo overcomes the inhibition by Fbg and effectively binds to GPIIb/IIIa on platelets.

However, it is not yet clear how H12-(ADP)Lipo overcomes the inhibition by a large amount of Fbg. We thought that H12 density on liposomal surface greatly contributed to the binding ability to platelets. Therefore, in this study, we explored the relationship between the amount of H12 modification of liposomes and the platelet-binding ability of these liposomes, and evaluated the ability of H12-(ADP)Lipo to bind to platelets in the presence of Fbg at physiological concentration or more.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, and 1,5-dihexadecyl-N-succinyl-L-glutamate (DHSG, Chart 1) were purchased from Nippon Fine Chemical Co. Ltd. (Osaka, Japan) and 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N-[monomethoxypoly(ethyl-eneglycol)] (PEG-DSPE, 5.1 kDa) and MalPEG3400-DSPE were from NOF Co. Ltd. (Tokyo, Japan). Cys-coupled fibrinogen γ-chain dodecapeptide (C-HHLGGAKQAGDV, Cys-H12) was synthesized by GL Biochem. (Shanghai, China) on consignment contract. H12-MalPEG3400-DSPE was synthesized in our laboratory (Chart 1). Adenosine 5′-diphosphate sodium salt (ADP), prostaglandin E1 (PGE_1), fibrinogen from human plasma, poly-L-lysine hydrobromide, decaethylene glycol monododecyl ether ($C_{12}E_{10}$), and HEPES were purchased from Sigma (St. Louis, MO, USA). Thrombin receptor activator for peptide-6 amide trifluoroacetate salt (SFLLRN-amide, TRAP) was obtained from Bachem AG (Bubendorf, Switzerland). Anti-human fibrinogen, FITC-conjugated was obtained from Millipore (Victoria, Australia). 3,3′-Dioctadecyloxacarbocyanine perchlorate ($DiOC_{18}$) was purchased from Invitrogen Co. (Eugene, OR, USA). Sephadex G25 for gel permeation chromatography (GPC) came from GE Healthcare (Buckinghamshire, UK).

2.2. Preparation of non-modified and H12-modified liposomes (H12-Lipo)

DPPC (665 mg, 0.91 mmol), cholesterol (350 mg, 0.91 mmol), DHSG (126 mg, 181 μmol), and PEG-DSPE (31 mg, 5.5 μmol) were dissolved in benzene 25 mL, and it divided equally to five. H12-PEG-DSPE (50 mg, 9.2 μmol) dissolved in methanol 20 mL. 0, 0.6, 1.1, 2.2, 4.4 or 8.8 mL of H12-PEG-DSPE in methanol solution was mixed with the former five benzene solution to prepare DPPC/cholesterol/DHSG/PEG-DSPE/H12-PEG-DSPE liposomes (5/5/1/0.03/0, 0.02, 0.03, 0.06, 0.12, and 0.24 as molar ratio), which were then freeze-dried. The mixed lipids were hydrated with phosphate-buffered saline (PBS, pH 7.4) for 3 h at 25 °C, and extruded through membrane filters with 0.45 μm and 0.22 μm pores (Durapore; Millipore Co., Tokyo, Japan). The liposomes thus obtained were washed with PBS by centrifugation (100,000 × *g* for 30 min at 4 °C). $DiOC_{18}$ in DMSO (2 mM), a hydrophobic fluorescent compound, was added to the each liposome suspension, and incubated for 5 min at 25 °C. Unincorporated $DiOC_{18}$ and DMSO were removed by use of GPC.

2.3. Preparation of H12-modified ADP-encapsulated liposomes, H12-(ADP)Lipo

DPPC (665 mg, 0.91 mmol), cholesterol (350 mg, 0.91 mmol), DHSG (126 mg, 181 μmol), PEG-DSPE (31 mg, 5.5 μmol), and H12-PEG-DSPE (30 mg, 5.5 μmol) (5/5/1/0.03/0.03 as the molar ratio) were dissolved in 6.5 mL ethanol/methanol (6/7, v/v) and a partial of the solution was injected into an aqueous solution 40 mL to form liposomes. Then, the liposomal solution was sonicated at 60 °C, freeze–thawed by using liquid N_2, and freeze-dried. The dried liposomes were rehydrated with PBS containing 2 mM ADP with sonication at 60 °C and extruded through membrane filters having pore sizes of 0.45 and 0.22 μm. The remaining ADP was removed, and the liposomal suspension was concentrated by tangential flow filtration. A DMSO solution of $DiOC_{18}$ (2 mM) was added to the liposome suspension, and the suspensions were incubated at 25 °C for 5 min. The $DiOC_{18}$-labeled liposomes were collected by GPC.

2.4. Characterization of the liposomes

The particle size was measured by using a dynamic light-scattering method (FPAR-1000; Otsuka Electric Co. Ltd., Osaka, Japan). The zeta-potential of the liposomes was determined with an electrophoretic light-scattering apparatus (ELS-8000; Otsuka Electric Co. Ltd.). The amount of ADP in the liposomal suspension was measured as follows: liposomes were added to PBS containing 8% $C_{12}E_{10}$ and incubated at 50 °C for 5 min, and then sonicated for 10 min. ADP was measured by HPLC (Shimadzu Co., Kyoto, Japan)

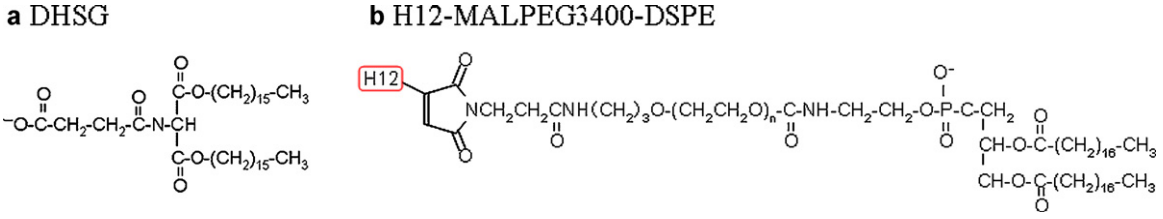

Chart 1. Structure of liposome contents. (a) 1,5-Dihexadecyl-N-succinyl-L-glutamate. (b) H12 is the fibrinogen γ chain (γ400–411) His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val.

and an ultra violet detector. The density of each component of liposomal lipids was measured by HPLC and a corona charged aerosol detector (CAD; ESA Bioscience Inc., Chelmsford, USA). $DiOC_{18}$ introduced into liposome was measured as follows: $DiOC_{18}$-labeled liposomes were added to isopropanol and incubated at 45 °C for 10 min. The fluorescence intensity of $DiOC_{18}$ eluted from the liposomes was measured by using a micro-plate reader (Multimode plate reader SPECTRAmaxM2; Molecular Devices, CA, USA).

Lamellarity, the average number of bilayer membranes of the liposomes, was calculated from the volume ratio of liposomes in the suspension. Liposomes were mixed with a poly(ethylene oxide) solution (MW = 100 kDa, final concentration, 12 mg/mL) and a poly-L-lysine hydrobromide solution (final concentration, 1.8 mg/mL). The volumes of the liposomal suspension, a poly(ethylene oxide) solution, and a poly-L-lysine hydrobromide solution were 120 μL, 40 μL and 3 μL, respectively. The liposomes were precipitated by hematocrit centrifugation (15,000 × *g* for 30 min at 25 °C) to measure their volume ratio. Lamellarity (*N*) was calculated by using Eq. (1); H12 density on the liposomal surface (D_{H12}), by using Eq. (2).

$$N = \frac{AV(C \times 10^{-3})R_{dil}N_A}{2SR \times 10^{-8}} \quad (1)$$

$$D_{H12} = \frac{V(C_{H12} \times 10^{-3})R_{dil}N_A}{2SRN \times 10^{-8}} \quad (2)$$

where the volume and surface area of single liposomes calculated from the average diameter are V (m^3) and S (m^2), the volume ratio of liposomes in the suspension and lipid concentration are R (%) and C (M), and the surface area of a lipid molecule is A (m^2). N_A is the Avogadro constant (6.0×10^{23}). R_{dil} is the dilution ratio of the liposomal suspension (120/163).

2.5. Flow cytometry for detection of Fbg binding to TRAP-activated platelets

Blood withdrawn from healthy volunteers was mixed with a 10% volume of 3.8% sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood (100 × *g* for 15 min at 25 °C). PRP was mixed with a 15% volume of acid–citrate–dextrose solution composed of 2.2% sodium citrate, 0.8% citric acid, and 2.2% glucose (ACD) containing 1 μM PGE_1. The suspension was centrifuged (2200 × *g* for 7 min at 25 °C), and the plasma was replaced with Ringer's-citrate–dextrose solution (RCD solution, composition: 0.76% citric acid, 0.090% glucose, 0.043% $MgCl_2$, 0.038% KCl, 0.60% NaCl; pH 6.5) containing 1 μM PGE_1. After the pellets had been resuspended in RCD solution, the suspension was centrifuged (2200 × *g* for 7 min at 25 °C); the concentrated platelets were then resuspended at 1.0×10^5 cells/μL in a modified HEPES–Tyrode buffer (137 mM NaCl, 0.42 mM NaH_2PO_4, 2.7 mM KCl, 12 mM $NaHCO_3$, 2 mM $MgCl_2$, 10 mM HEPES [N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid], and 0.1% glucose; pH 7.4). The platelet count was determined by using an automated hematology analyzer (K-4500, Sysmex Co., Kobe, Japan).

Anti-human fibrinogen, FITC-conjugated (FITC-Fbg) was added to the washed platelets (1.0×10^5 cells/μL). TRAP with various concentrations (final concentrations, 0–100 μM) were added to the suspension to activate the platelets, and the suspension was then incubated at 37 °C for 10 min before fixation with formaldehyde (final concentration, 1.8%, v/v). The mixture was incubated in darkness (15 min at 25 °C), and added to H–T buffer (1 mL). The platelets were gated to their characteristic forward versus side scatter, and 10,000 platelets were analyzed by using a FACSCalibur flow cytometer (Nippon Becton Dickinson Co., Tokyo, Japan). The platelet binding with the fibrinogen was quantified as the fraction of the fluorescent-positive platelets.

2.6. Flow cytometry of H12-Lipo binding to activated platelets

The washed platelets were prepared as described in Section 2.5 above. $DiOC_{18}$-labeled H12-Lipo (final concentration, 0.5 mg/mL) was added to the washed platelets (1.0×10^5 cells/μL). TRAP of various concentrations (final concentration, 0–50 μM) was added to the suspension to activate the platelets, and the suspension was then incubated at 37 °C for 10 min before fixation with formaldehyde. The mixture was incubated in darkness (15 min at 25 °C), and then added to H–T buffer (1 mL). The platelets were gated to their characteristic forward versus side scatter, and 10,000 platelets were analyzed by using the FACSCalibur flow cytometer. The platelet binding with the liposomes was quantified as the fraction of the fluorescent-positive platelets.

The mean fluorescence intensity (MFI) was calculated by using Eq. (3), and was the indicator of the relative amount of liposomes bound to a platelet cell. MFI was used as an index of the binding ability.

$$\text{MFI} = \frac{\sum(\text{FL1-H} \times \text{count})}{\text{total count}} \quad (3)$$

FL1-H, fluorescence intensity; count, number of cells with each fluorescence intensity; total count, detected number of all cells.

Average ($n = 3$) MFI of the non-stimulated group was subtracted from each MFI as background. Each $DiOC_{18}$ fluorescence was divided by the lipid amount (w/w, %).

2.7. Inhibitory effect of Fbg on H12-(ADP)Lipo binding to activated platelets

The washed platelets were prepared as described in Section 2.5. $DiOC_{18}$-labeled H12-(ADP)Lipo (final concentration, 0.5 mg/mL) and Fbg from human plasma (final concentration, 6 μM) were added to the washed platelets (1.0×10^5 cells/μL). TRAP (0–50 μM) was added to the suspension to activate the platelets, and the suspension was then incubated at 37 °C for 10 min. H12-(ADP)Lipo bound to the activated platelets was determined as described in Section 2.6.

In another experiment, $DiOC_{18}$-labeled H12-(ADP)Lipo and various concentrations of Fbg (0–60 μM) were added to the washed platelets, which were then activated with 30 μM TRAP for 10 min at 37 °C. The 50% inhibition concentration of Fbg for blocking the binding of H12-(ADP)Lipo (0.5 mg/mL as lipids) to the platelets was determined by using the analytical software Graph Pad Prism 5J. The MFI of the TRAP-added group was assumed to be 100% binding, and that without the TRAP-treated group was assumed to be 0% binding.

In this study, the final liposomal concentration was fixed at 0.5 mg/mL as lipids during incubation with platelets. Therefore, the calculated H12 concentration in the incubation mixture of H12-(ADP)Lipo was 2.3 μM. This value well correlated with the concentration of H12 peptide obtained from lipid analysis, which was about 2 μM (data not shown).

2.8. Statistical analysis

Statistical analysis was carried out by Student's *t*-test.

3. Results

3.1. Physicochemical characteristics of the liposomes

We prepared vacant liposomes without (plain-liposomes) or with various amounts of H12-PEG-DSPE (H12-Lipo), as well as H12-modified ADP-encapsulated liposomes, H12-(ADP)Lipo. The physicochemical characteristics of the various liposomes are

Table 1
Physicochemical characteristics of the liposomes.

	Plain-Lipo	H12-Lipo (0.006)	H12-Lipo (0.014)	H12-Lipo (0.036)	H12-Lipo (0.065)	H12-Lipo (0.175)	H12-(ADP)Lipo
Particle size (nm)	314.8	303.8	301.2	299.3	294.2	245.6	295.4
Polydispersity index	0.132	0.130	0.183	0.180	0.126	0.107	0.187
ζ-Potential (mV)	−12.26	−7.77	−5.46	−3.66	−4.72	−4.13	−7.70
ADP (μg/mL)	–	–	–	–	–	–	14.17
Molar ratio							
DPPC	4.975	4.972	4.845	4.959	5.137	5.341	4.836
Cholesterol	5.000	5.000	5.000	5.000	5.000	5.000	5.000
DHSG	0.929	0.910	0.906	0.910	0.940	0.987	0.937
PEG5000-DSPE	0.025	0.025	0.025	0.025	0.025	0.025	0.025
H12-PEG-DSPE	–	0.006	0.014	0.036	0.065	0.174	0.023
Lamellarity	1.20 ± 0.05	0.94 ± 0.02	0.95 ± 0.01	1.06 ± 0.01	1.01 ± 0.01	1.02 ± 0.02	0.97 ± 0.01
H12 density × 10^{15} (molecules/m^2)	0	1.32	2.93	8.15	13.8	35.9	4.91

DPPC/cholesterol/DHSG/PEG-DSPE with H12-PEG-DSPE liposomes (5/5/1/0.03 with 0 (plain-Lipo), 0.02, 0.03, 0.06, 0.12, and 0.24 as the molar ratio) was prepared, and the actual amount of each component after preparation was determined. The relative amount was expressed where the cholesterol content was assumed to be 5.000. Lamellarity is shown as the mean ± SD (n = 3).

shown in Table 1. The particle sizes were about 300 nm in diameter except for H12-Lipo (0.175), the diameter of which was about 250 nm. Lamellarity of all liposomes tested was calculated by using Eq. (1) shown in Section 2.4 and was about 1.0, indicating that these liposomes were unilamellar vesicles. The H12 density on the liposomal surface was calculated by using Eq. (2) described in Section 2.4 and N = 1 was applied.

The morphology of H12-(ADP)Lipo was observed by electron microscopy (Fig. 1): the liposomal size observed by TEM was almost the same as that measured by the dynamic light-scattering method, although the size was not homogeneous. Moreover, H12-(ADP)Lipo was mostly unilamellar or oligolamellar vesicles.

3.2. Effect of H12-modification of the liposomes on their binding to platelets

To evaluate the ability of H12-modified liposomes to bind to activated platelets, we firstly examined Fbg binding to platelets that had been activated with TRAP, a protease-activated receptor (PAR) 1 agonist and an activator of platelets and GPIIb/IIIa. The amount of FITC-Fbg bound to human platelets well correlated with the TRAP stimulation, and the increase in binding nearly reached its plateau at 30 μM TRAP (Fig. 2). It is known that Fbg binds to the activated GPIIb/IIIa on the platelet surface through the H12 peptide sequence, HHLGGAKQAGDV, located at the carboxyl-terminus of the fibrinogen γA chain (γ400–411), and through the RGD motifs presenting in a fibrinogen Aα chain (Aα95–97, Aα572–574). Fbg is present in the blood as a dimeric form, and the dimer has 2 H12 sites and 4 RGD sites as ligands for GPIIb/IIIa (Michael, 1990, 1992) suggesting that Fbg binds to platelets in a multivalent fashion.

Next, by FACS analysis we measured the ability of the $DiOC_{18}$-labeled liposomes bearing various amounts of H12 to bind to TRAP-activated platelets. The mean fluorescence intensity (MFI)

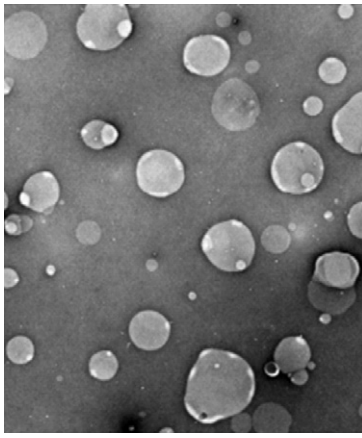

Fig. 1. TEM image of H12-(ADP)Lipo. This liposome sample was suspended on a transmission electron microscopic grid coated with collodion film, stained with phosphotungstic acid, and then examined with a Hitachi H-9000UHR type transmission electron microscope operated at 100 kV.

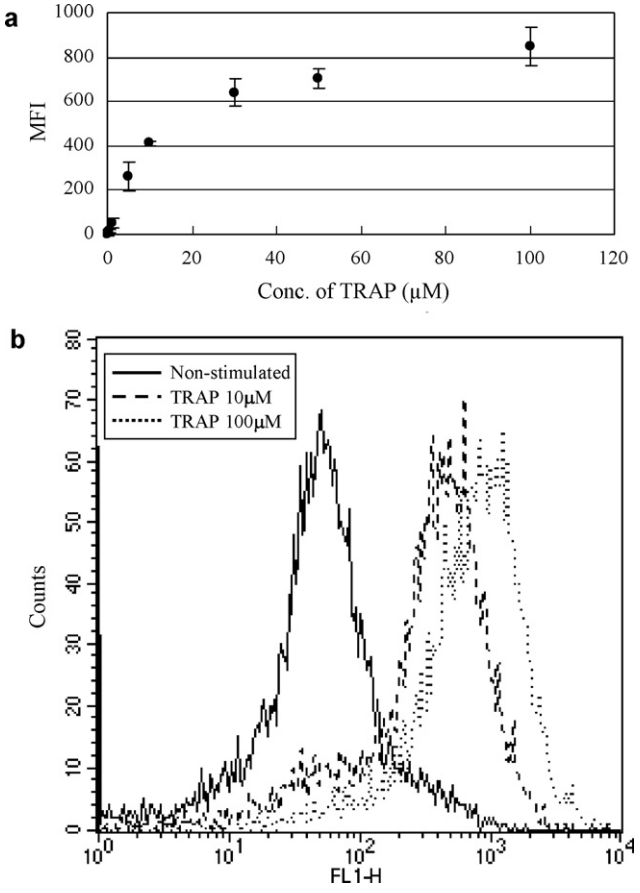

Fig. 2. TRAP-induced activation of GPIIb/IIIa on platelets. (a) Various concentrations of TRAP (0–100 μM) were added to washed platelets (platelet = 1.0×10^5 cells/μL, 80 μL) in the presence of FITC-Fbg, and incubated at 37 °C for 10 min before fixation with formaldehyde (1.8%, v/v). The mixture was incubated in the darkness (15 min at 25 °C), and mixed with H–T buffer (1 mL). The platelets were gated to their characteristic forward versus side scatter, and 10,000 platelets were analyzed by using a flow cytometer. FITC-Fbg binding to platelets was quantified as the fraction of fluorescent-positive platelets. Data are expressed as the mean ± SD (n = 3). (b) Each histogram represents the binding of FITC-Fbg to human platelets non-stimulated, stimulated by 10 μM TRAP or 100 μM TRAP.

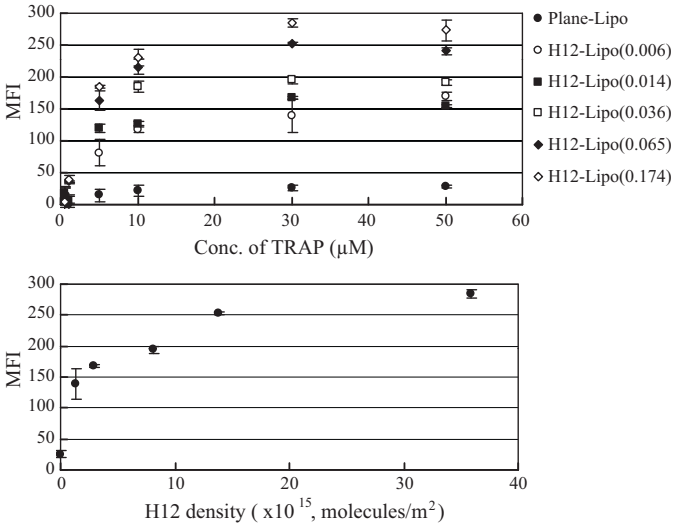

Fig. 3. Effect of modification of liposomes with H12 on their ability to bind to platelets. Liposomes modified with various amounts of H12 peptide were prepared. Each liposomal sample (0.5 mg/mL lipids) labeled with $DiOC_{18}$ was incubated with washed platelets (platelet = 1.0×10^5 cells/μL, 80 μL) at 37 °C for 10 min in the presence of various amounts of TRAP. Then the platelets were fixed with formaldehyde (1.8%, v/v), incubated in the darkness (15 min, 25 °C), and mixed with H–T buffer (1 mL). The platelet samples were then examined by flow cytometry. The liposomes bound to platelets were quantified as the fraction of fluorescent-positive platelets. Data are expressed as the mean ± SD ($n = 3$). (a) The relative binding of H12-Lipo to platelets activated with various concentrations of TRAP. The H12 molar ratios of each liposome to cholesterol (assumed to be 5) were 0 (●), 0.006 (○), 0.014 (■), 0.036 (□), 0.065 (♦) and 0.174 (◊), respectively. (b) The relative binding of liposomes with various H12 density to platelets activated with 30 μM TRAP.

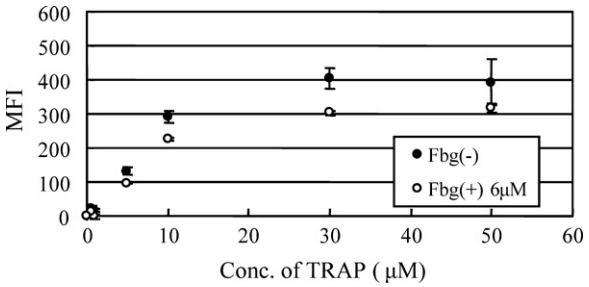

Fig. 4. H12-(ADP)Lipo binding to platelets in the presence of the physiological concentration of fibrinogen. H12-(ADP)Lipo labeled with $DiOC_{18}$ (0.5 mg/mL as lipids) and various concentrations of TRAP (0–50 μM) without (●) or with 6 μM Fbg (○) were added to washed platelets (1.0×10^5 cells/μL, 80 μL). The suspension was incubated at 37 °C for 10 min before fixation with formaldehyde (1.8%, v/v). Liposomal fluorescence associated with platelets was determined. Data are expressed as the mean ± SD ($n = 3$).

was used as an index of the binding ability. As a result, the binding of the liposomes to the platelets increased in a TRAP concentration-dependent manner and reached its plateau at the TRAP concentration of 30 μM (Fig. 3a). Fig. 3b shows the relationship between MFI and H12 density on the liposomal surface when the platelets were activated with 30 μM TRAP. The binding ability of the liposomes increased depending on the H12 density of the liposomes and reached its plateau at the calculated H12 density of 1.38×10^{16} molecules/m^2.

3.3. Inhibitory effect of fibrinogen on the binding of H12-(ADP)Lipo to platelets

The concentration of Fbg in normal human blood is about 200 mg/mL (5.9 μM) and Fbg is known to bind to activated GPIIb/IIIa on platelets (Halle et al., 1996) and would be expected to act as an inhibitor of H12-(ADP)Lipo binding to platelets. Therefore, by FACS analysis we evaluated the ability of H12-(ADP)Lipo (H12 density: 4.9×10^{15} molecules/m^2) to bind to activated platelets in the presence or absence of Fbg. The physiological concentration of Fbg (6 μM) inhibited the liposomal binding only partially at all TRAP concentrations tested (Fig. 4): the maximum inhibition (about 18.8%) was observed at 30 μM TRAP stimulation, where the MFI in the presence or absence of Fbg were 404 ± 32 and 304 ± 6, respectively. Furthermore, the inhibition ratios at 5 or 1 μM TRAP were 8.8% or 1.4%, respectively, suggesting that the effect of Fbg on liposomal binding to platelets was hardly observed in a low activation state of platelets. It is thought that only a small amount of activated GPIIb/IIIa is present on the surface of platelets in the low activation state.

To evaluate the specificity of H12-(ADP)Lipo binding to GPIIb/IIIa, we investigated the competitive inhibition of the liposomal binding to activated platelets in the presence of excess Fbg. The binding of H12-(ADP)Lipo to platelets was inhibited in the presence of excess concentrations of Fbg, and about 90% inhibition was observed at 60 μM Fbg (Fig. 5a). This finding suggests that H12-(ADP)Lipo bound to a specific site on activated platelets, that is, the same site for Fbg binding, namely, GPIIb/IIIa. The 50% inhibition concentration of Fbg for blocking the binding of H12-(ADP)Lipo (final concentration, 0.5 mg/mL) was calculated by using analytical software Graph Pad Prism 5J: Fig. 5b shows the fitting-curve of the inhibition. As a result, the 50% inhibition concentration of Fbg was 25.2 μM, about 4.3-fold higher than its blood level.

4. Discussion

The purpose of our study was to focus on the utility of H12-modified ADP-encapsulated liposomes, H12-(ADP)Lipo, as a hemostat. We previously reported that H12-(ADP)Lipo enhances the platelet aggregation in PRP and significantly reduces the bleeding times in thrombocytopenic rat and rabbit models and that H12-(ADP)Lipo is more efficient than H12-Lipo or (ADP)Lipo (Okamura et al., 2005a,b, 2007). It is thought that the hemostatic effect of H12-(ADP)Lipo is based on the following sequential events: binding to activated platelets, releasing ADP that further activates platelets to form a thrombus, and bridging platelets to make large aggregates for hemostasis.

The H12 peptide is derived from a fibrinogen γ chain carboxyl-terminal sequence and has the binding ability to activated GPIIb/IIIa on platelets (Michael, 1990, 1992). Therefore, the hemostasis induced by H12-(ADP)Lipo mimics the hemostasis with Fbg and platelets. In fact, platelet aggregation is induced by a large amount of Fbg and von Willebrand Factor (vWF) in blood (Takagi et al., 2002). Since H12 and Fbg share the same target, namely, activated GPIIb/IIIa, it is necessary that H12-(ADP)Lipo overcomes the competition with Fbg and binds to platelets for inducing enhanced hemostasis. However, the effect of Fbg on the binding of H12-(ADP)Lipo to platelets had not been evaluated previously. In this study, by using FACS analysis, we evaluated the effect of the presence of Fbg on the binding of H12-(ADP)Lipo to platelets.

It is known that GPIIb/IIIa is a resting form on non-stimulated platelets and is converted to its active form upon stimulation of platelets. The active GPIIb/IIIa exposes the Fbg-binding sites to which the H12 peptide binds (Du et al., 1991). Firstly, we examined the change in the activation level of platelets depending on the stimulation with TRAP, a platelet-activating agent. As a result, it was clear that GPIIb/IIIa activation depended on the TRAP concentration (Fig. 2). Next, we examined the influence of the H12 density on the liposomal surface under various stimulation conditions by

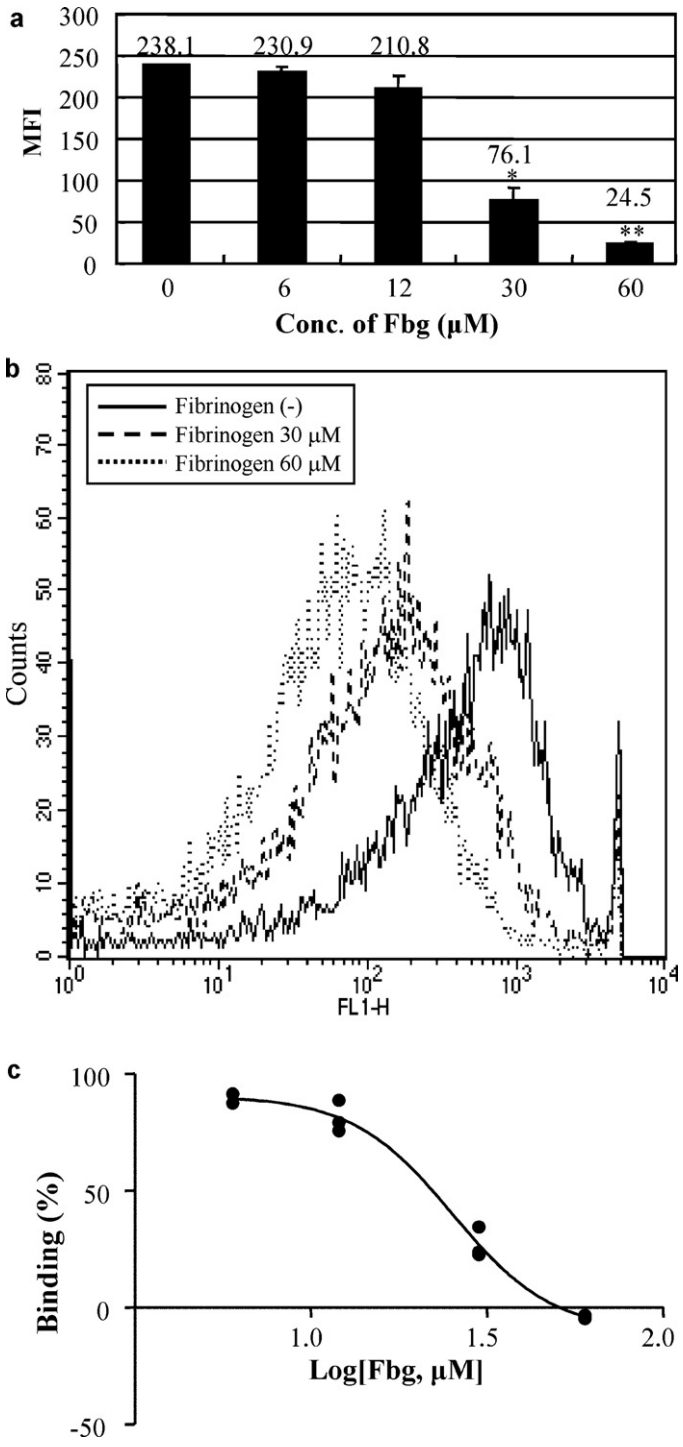

Fig. 5. Inhibitory effect of fibrinogen on the binding of H12-(ADP)Lipo to platelets. (a) H12-(ADP)Lipo labeled with $DiOC_{18}$ (0.5 mg/mL), 30 μM TRAP, and various concentrations of Fbg were added to washed platelets (1.0×10^5 cells/μL, 50 μL). Significant difference from Fbg 0 μM group are indicated by $^*P < 0.01$ and $^{**}P < 0.001$. The suspension was then incubated at 37 °C for 10 min before fixation with formaldehyde. Liposomes associated with platelets were analyzed fluorometrically by using flow cytometry. Data are expressed as the mean ± SD ($n = 3$). (b) Each histogram represents the binding of H12-(ADP)Lipo to human platelets stimulated by 30 μM TRAP with Fbg (30 μM) or Fbg (60 μM). (c) The curve for inhibition by Fbg of binding of H12-(ADP)Lipo to platelets was fitted by using the Graph Pad Prism 5J. The mean Fl of Fbg(−) TRAP-added group was assumed to be 100% binding; that without TRAP-treatment group, 0% binding.

varying the TRAP concentration. The ability of H12-Lipo to bind to the platelets increased depending on the H12 density of the liposomal surface and the TRAP concentration (Fig. 3a). At the low level of H12 density on the liposomes ($<2.9 \times 10^{15}$ molecules/m^2), the binding ability increased depending on the increase in H12 density, and it nearly reached the plateau at an H12 density of about 1.4×10^{16} molecules/m^2 (Fig. 3b).

On the other hand, GPIIb/IIIa, one of the most predominant glycoproteins on platelets, is present as approximately 5×10^4 molecules/platelet in healthy blood. Platelets are smaller than other cells, and the size of platelets is assumed to be 2 μm in diameter (Milton et al., 1985; Kuwahara et al., 2002). Therefore, if the platelet is assumed to be spherical, the density of GPIIb/IIIa on the platelet cell surface is 4.0×10^{15} molecules/m^2, although actual platelets were not spherical shape at the aggregated site.

Considering the data in Fig. 3, it seems that the binding ability of H12-Lipo is sensitively changed according to the H12 density when the H12 density on the liposome is lower than the GPIIb/IIIa density on the platelet surface (4.0×10^{15} molecules/m^2, when 30 μM TRAP is assumed to activate all GPIIb/IIIa molecules). On the contrary, when the H12 density on the liposomal surface becomes higher ($>1.4 \times 10^{16}$ molecules/m^2) than that of GPIIb/IIIa on the platelet, it seems that the change in the binding ability is not sensitive to the change in the H12 density. Taken together, the H12 density on the liposomal surface is an important factor for the binding of the liposomes to platelets, and the H12 peptide on the liposomal surface may act cooperatively in binding of the H12-Lipo to GPIIb/IIIa on the platelet.

The activation level of platelets at the site of a vascular injury may be dependent on the level of wounding. Therefore, we evaluated the binding ability of H12-(ADP)Lipo over a wide range of activation level of platelets (Fig. 4) in the presence of the physiological concentration of Fbg, namely, 6 μM. This concentration of Fbg did not suppress much the binding of the liposome to platelets because H12-(ADP)Lipo had a multivalent effect of H12 peptide enough for the inhibition effect of Fbg. Furthermore, this result suggested that H12-(ADP)Lipo may overcome the inhibition of Fbg over a wide range of platelet activation levels *in vivo*. The site of binding of H12-(ADP)Lipo to GPIIb/IIIa was confirmed to be the same as that of Fbg, since the binding was almost completely blocked by the high concentration of Fbg (Fig. 5a).

The 50% inhibition concentration of Fbg to block the binding of H12-(ADP)Lipo (final concentration, 0.5 mg/mL as lipids) to platelets was approximately 25 μM under the present experimental conditions (Fig. 5a). Since the concentration of H12 peptide in the reaction liquid was calculated to be about 2 μM, H12 peptide exposed on the surface of liposome was assumed to be about 1 μM in consideration of liposomal lamellarity (approximately 1.0): one-half of the H12 is assumed to be present in the outer leaflet of the lipid bilayer, and another one-half in the inner leaflet of the bilayer. Moreover, it was known that Fbg forms a dimer and that 1 molecule of Fbg has 2 H12 peptide sequences and 4 sites of RGD sequence (Michael, 1990, 1992). Therefore, the 50% inhibition of the liposomal binding was achieved with Fbg of which concentration is 25-fold excess of H12.

It is known that several peptides derived from amino acid sequence in Fbg, including the H12 peptide, competitively inhibit the binding of Fbg to platelets (Timmons et al., 1984; Gartner and Bennett, 1985; Plow et al., 1985). However, the affinity of these peptides for platelets is known to be less than one-tenth of that of native fibrinogen (Ruggeri et al., 1986). Therefore, it may be concluded that liposomalization of H12, which enabled multivalent and cooperative binding to the target molecules with high density of ligands, gave liposomes the binding ability to GPIIb/IIIa superior to Fbg overwhelmingly without losing the specificity of H12 to GPIIb/IIIa.

In the experiment on the ability of H12-(ADP)Lipo to bind to platelets in the presence of Fbg (Fig. 4), the concentrations of Fbg, H12-(ADP)Lipo and H12 peptide on the surface of liposome were about 3.6×10^{12} molecules/μL (6 μM), 4.0×10^{8} particles/μL (final concentration, 0.5 mg/mL as lipids) and 1.2×10^{12} molecules/μL (2 μM), respectively. The number of particles of H12-(ADP)Lipo was calculated by using the particle size and the data of each compositional lipid of the liposomes with the CAD device. Considering the values of the above-mentioned components, the number of H12 peptide molecules present on the surface of a single H12-(ADP)Lipo is calculated to be about 1400 molecules. In the reaction liquid, the number of Fbg molecules was approximately 9000-fold and 6.2-fold over the number of H12-(ADP)Lipo and H12 peptide chains, respectively, on the surface of the liposomes. These numbers mean that H12 molecules on the H12-(ADP)Lipo show far stronger affinity for platelets than does Fbg due to the cooperative effect by liposomalization and can overcome the influence of a large amount of Fbg in the blood.

5. Conclusions

GPIIb/IIIa-specific H12 peptides modified on the liposomal surface cooperatively and strongly bind to GPIIb/IIIa on the surface of activated platelets: the multivalent binding between H12 peptides and GPIIb/IIIa strengthened the binding of the liposomes to activated platelets. This strong binding could overcome the influence of the physiological concentration Fbg; therefore, H12-(ADP)Lipo may be considered as a candidate for a hemostat.

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