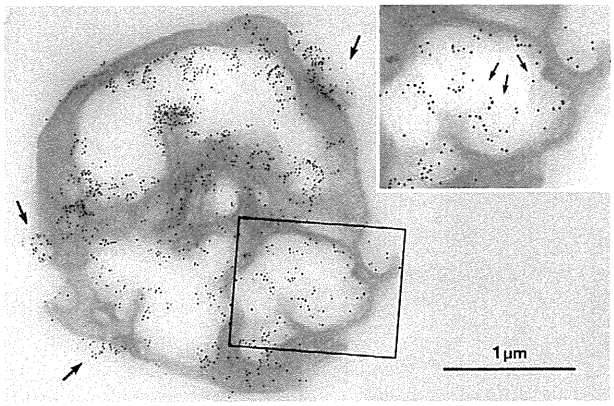

Fig. 4. Localization of H12-liposomes in the platelets activated by thrombin without stirring for 60 sec. The spheroidal platelet has several large-sized swollen OCS. Gold particles representing H12 are distributed not only on the exterior surface (arrows) but also in the lumen of the swollen OCS in the platelet. (Inset) At high-magnification, electron-lucent liposomes labeled with gold particles are visible along the membrane of the large-sized swollen OCS in the platelet (arrows).

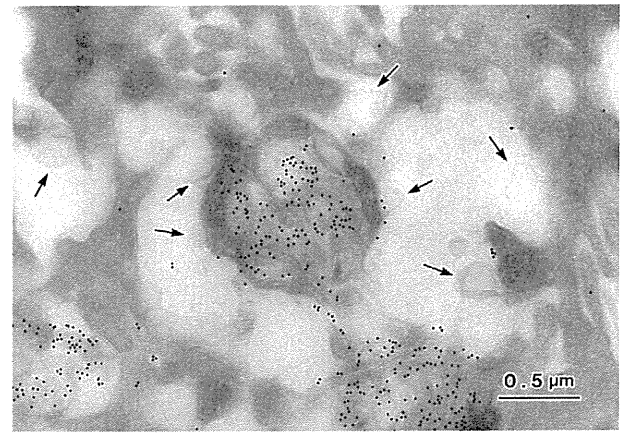

Fig. 6. Localization of fibrinogen in the platelet aggregates induced by thrombin for 60 sec. Many H12-liposomes with unit membranes are present in the electron-transparent areas between adherent platelets in the aggregates (arrows). Gold particles representing fibrinogen are densely distributed in the swollen or fused α-granules in the platelets discrete from the H12-liposomes.

and H12-liposomes was stirred for 60 sec after the addition of thrombin, extensive aggregates of platelets had formed (Fig. 5). The aggregates contained many electron-transparent areas between adherent platelets, ranging in diameter from around 1 μm to several μm, in which many H12-liposomes were identified (Fig. 5 inset). Although the ultrastructural appearance of the electron-transparent areas resembled that of the large-sized swollen OCS containing the liposomes in non-aggregated and activated platelets, their size was greater than that of the OCS. On the other hand, the localization of H12-liposomes in platelet aggregates differed from that of fibrinogen, as detected by immunogold staining (Fig. 6). Fibrinogen released from α-granules in platelets was densely localized in regions where granules appeared to be fused and at sites of discharge of the granule contents.

Inhibitory effect of antagonists to GPIIb/IIIa on the formation of the large-sized swollen OCS in platelets incubated with H12-liposomes after thrombin-induced activation

To assess the mechanism of formation of the large-sized swollen OCS in platelets incubated with H12-liposomes after thrombin-induced activation, liposome-unbound H12 and several antagonists to GPIIb/IIIa were subjected to ultrastructural analysis (Fig. 7). The control platelets in the mixture with H12-liposomes changed their form to spheroids and contained several large-sized swollen OCS 5 min after the addition of thrombin (Fig. 7A). In contrast, when the mixture of platelets and H12-liposomes was pre-incubated with liposome-unbound H12 (Fig. 7B), EDTA (Fig. 7C) or the anti-GPIIb/IIIa antibody PAC-1 (Fig. 7D) for 60 sec at 37 °C, the formation of the large-sized swollen OCS at 5 min after the addition of thrombin was inhibited completely. Especially in the platelets pre-incubated with liposome-unbound H12, not only the formation of the large-sized swollen OCS but also the release reaction of granules was inhibited after the addition of thrombin.

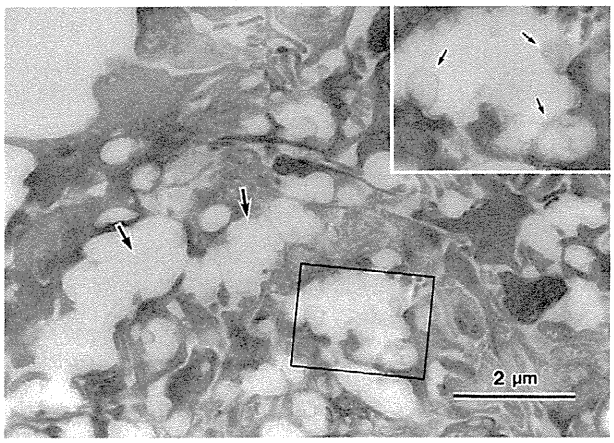

Fig. 5. Localization of H12-liposomes in the platelet aggregates induced by thrombin for 60 sec. Large aggregates have formed and many platelets have been degranulated. Electron-transparent areas between adherent platelets are widespread in the aggregates (arrows). (Inset) At high-magnification, H12-liposomes can be observed on the membrane of platelets in the electron-transparent areas (arrows).

Discussion

We have previously confirmed that H12-coated microparticles such as liposomes, latex, and nanosheets show specific interaction with activated platelets and augmented effects on platelet thrombus formation onto collagen-immobilized surfaces under flow conditions *in vitro*, and prolonged hemostatic ability *in vivo* to correct bleeding time in a dose-dependent manner in a thrombocytopenic rat model [11,16,21–23]. These findings indicated that H12 on the surface of the particles reproduced the ability to bind GPIIb/IIIa on the activated platelet, leading to adhesion and aggregation at the site of vascular injury [33]. Our purpose in this study was to visualize the specific interaction between human platelets and H12-liposomes during thrombin-induced activation using flow cytometry and electron microscopy.

Our flow cytometry results suggest that many H12-liposomes should be associated with the surface of the thrombin-activated platelets, and that this should be visible through electron microscopy. In contrast to our expectation, however, we could not identify H12-liposomes associated with platelets before and after the addition of thrombin in the sections of Epon-embedded sample using conventional electron microscopy. Instead, we observed the formation of large-sized swollen OCS, 1 μm in diameter or larger, in the spheroidal platelets from 60 sec to 5 min after the addition of thrombin. Furthermore, hazy and/or formless contents were present in the lumen of the OCS. Swollen OCS is observed commonly during the platelet release reaction; its size is usually about 0.5 μm in diameter [32,34,35]. It is well known that actin assembly induced by an increase of intracellular $[Ca^{2+}]$ and activation of protein kinase C in response to several agonists results in the platelet release reaction as follows: α-granules fuse with each other and the membrane of the OCS, resulting in the formation of the swollen OCS, and causing the intra-granule contents to be compressed so as to flow through the lumen of the OCS

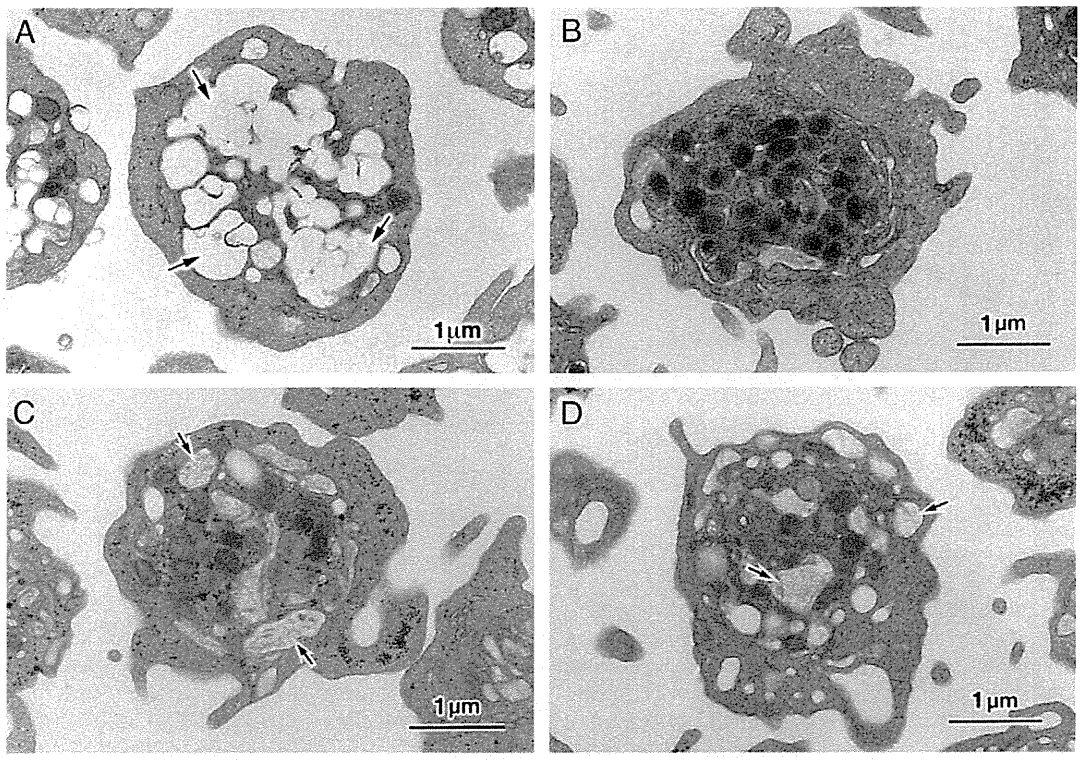

Fig. 7. Effect of antagonists to GPIIb/IIIa on the formation of the large-sized swollen OCS in platelets incubated with H12-liposomes after thrombin-induced activation. The mixture of platelets with H12-liposomes was pre-incubated with liposome-unbound H12, EDTA or the anti-GPIIb/IIIa antibody PAC-1 for 60 sec at 37 °C, activated with thrombin for 5 min without stirring, fixed and viewed by conventional electron microscopy. (A) Control platelets in the mixture with H12-liposomes appear as spheroid forms with several large-sized swollen OCS at 5 min after the addition of thrombin (arrows). (B) When the mixture of platelets and H12-liposomes was pre-incubated with liposome-unbound H12, the platelets have spheroid forms with pseudopodia, and the centralization of granules can be seen after thrombin activation, though the swollen OCS cannot. (C) The platelets pre-incubated with EDTA appear as spheroid forms similar to H12-pre-incubated cells after thrombin activation. Some α-granules are fusing with the membrane of the OCS, resulting in the release reaction of granules in the platelets. On the other hand, the lumen size of the OCS is relatively normal, and there is no large-scale swelling (arrows). (D) After thrombin activation, the platelets pre-incubated with PAC-1 are striking similar in shape to the EDTA-pre-incubated ones. The release of storage granules is observed in the platelets (arrows), but the large-sized swollen OCS is not.

to the exterior of the platelets. Moreover, recent studies have suggested that soluble NSF attachment protein receptors (SNARE) proteins such as syntaxin, SNAP-23 and VAMP and Rab proteins participate in the α-granule fusion and the formation of swollen OCS [36,37]. In the present study, using ultrathin frozen sections and immunogold electron microscopy, we have succeeded in visualizing H12-liposomes localized not only on the surface membrane but also in the lumen of the swollen OCS in the platelets from 60 sec to 5 min after the addition of thrombin. The swollen OCS containing H12-liposomes in the platelets of ultrathin frozen sections expanded greatly, similar to the platelet OCS in the sections of Epon-embedded sample. We also observed that the large-sized swollen OCS failed to form in the thrombin-activated platelets with control liposomes. Taken together, these observations lead to the conclusion that the large-sized swollen OCS is formed after specific interaction between platelets and H12-liposomes by the following pathway: H12-liposomes bind to the surfaces of spheroidal platelets after thrombin stimulation, and a fraction of the liposomes redistribute into the lumen of the OCS in the platelets, resulting in the formation of the large-sized swollen OCS in the cells. We confirmed that the formation of the large-sized swollen OCS was a GPIIb/IIIa-mediated interaction, because it was completely inhibited by pre-incubation with liposome-unbound H12, EDTA or the anti-GPIIb/IIIa antibody PAC1. However, the physiological role of the redistribution of H12-liposomes from the platelet surface to the OCS and the formation of the large-sized OCS remains unknown, although several investigators have reported that platelets have the ability to take up small particles such as cationized ferritin, latex, bacteria and viruses [38–40] into the lumen of the OCS. Some electron microscopic studies using cytochemical methods have also demonstrated that these small particles were eventually internalized into the platelet cytoplasm from the OCS by phagocytosis [38,40]. Whether the above-mentioned redistribution of H12-liposomes participates in platelet phagocytosis remains to be determined. The hazy and/or formless contents present in the lumen of the large-sized swollen OCS seemed to be the wreckage of the H12-liposome, which had been dissolved and broken down by the ethyl alcohol used for dehydration during sample preparation.

In the thrombin-induced aggregates, H12-liposomes were found to localize in the electron-transparent areas between adherent platelets in the aggregates. The large-sized swollen OCS containing H12-liposomes observed in non-aggregated platelets may be formed in each platelet even in the aggregates. Although it may appear that the electron-transparent areas were just as small as the OCS, we could not identify the OCS containing H12-liposomes in the aggregated platelets due to difficulties in distinguishing between the demarcated boundaries of each platelet in the aggregates. At any rate, these findings indicate that H12-liposomes bind to stimulated platelets and are incorporated between adherent platelets in the aggregates. The process of platelet aggregation is known to be regulated by the activation of GPIIb/IIIa and its subsequent binding of fibrinogen or other adhesive proteins [24–30]. When washed human platelets in the presence of exogenous fibrinogen were stimulated by ADP or thrombin, fibrinogen was found to localize between entire adherent platelets in the aggregates [35,41]. In the present study, although the localization of H12-liposomes differed from that of fibrinogen released from α-granules in the thrombin-induced aggregates, the localization of liposomes seems to resemble that of the exogenous fibrinogen in the ADP- or thrombin-induced aggregation, suggesting that H12-liposomes behave like fibrinogen by bridging adjacent platelets through GPIIb/IIIa activation after the addition of thrombin,

leading to the formation of the aggregates consisting of platelets and the liposomes. H12-liposomes have two advantages over fibrinogen in terms of their participation in the platelet aggregates. The first is that the modified H12 on the surface of liposomes is specifically bound to the activated GPIIb/IIIa on the surface of the platelets. Several sequences in fibrinogen have been designated as GPIIb/IIIa recognition sites, including two RGD-based sequences in the Aα chains and H12 in the carboxy-terminus of the γ-chain [27–29]. Selection of the H12 peptide was based on general observations that the interaction of H12 is highly specific to GPIIb/IIIa, whereas RGD-related peptides are promiscuous with many integrins from various cell types [30]. Thus, it is assumed that the binding ability of H12-liposomes to activated GPIIb/IIIa on the platelet surface is stronger than that of fibrinogen. The second advantage of H12-liposomes is that they have a diameter of about 0.3 μm. It is assumed that due to their large size compared to that of fibrinogen molecules, H12-liposomes are capable of forming large aggregates in combination with only a small number of platelets. In fact, even in a thrombocytopenic rat model, H12-liposomes reduced bleeding time in a dose-dependent manner, suggesting that the liposomes interact with small numbers of platelets and form big aggregates at sites of vascular injury [22,23].

In conclusion, we have clarified the interaction between platelets and H12-liposomes during thrombin-induced activation using flow cytometry and electron microscopy. In a previous study, Okamura et al. succeeded in visualizing the specific accumulation of H12-liposomes at a site of vascular injury using iopamidol encapsulation and computed tomography observation, and undertook semiquantitative analyses of the H12-liposomes accumulated at the injured site [33]. They also generated schematic images of the accumulation mechanism of H12-liposomes at endothelial injury sites. Our present finding, namely, that H12-liposomes were localized abundantly between adherent platelets in the thrombin-induced aggregates, strongly supports the theory that the liposomes could participate in hemostasis by accumulating specifically in platelet aggregates at the site of bleeding. H12-liposomes appear to require endogenous platelets to work, and thus may not be sufficiently effective when administered to patients with significant thrombocytopenia. Yet H12-liposomes may be useful for the treatment of bleeding in patients with qualitative platelet disorders, such as storage pool deficiency. Further study is needed under conditions simulating various clinical settings to establish potential indications in platelet transfusion.

Disclosure of conflict of interests

The authors state that they have no conflict of interest.

Acknowledgements

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Shear-dependent suppression of platelet thrombus formation by phosphodiesterase 3 inhibition requires low levels of concomitant Gs-coupled receptor stimulation

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Summary

Phosphodiesterase (PDE)3 inhibitors exert potent antiplatelet effects through maintaining elevated intracellular cyclic adenosine monophosphate levels, but do not prolong bleeding time. To resolve this discrepancy, we hypothesised that PDE3 inhibitors effectively suppress shear-induced platelet thrombus formation initiated by the interaction of the platelet receptor GPIb/V/IX with its ligand, von Willebrand factor (VWF), since arterial thrombosis is more dependent on shear stress as compared with haemostatic plug formation. To test the hypothesis, we compared the *in vitro* effects of K-134 (a PDE3 inhibitor), tirofiban (a GPIIb/IIIa inhibitor) and acetylsalicylic acid (ASA) on ristocetin-induced platelet aggregation and platelet thrombus formation on VWF or collagen surfaces under flow conditions. K-134 inhibited GPIIb/IIIa-dependent platelet aggregation to the same extent as tirofiban and more potently than ASA. Likewise, K-134 and tirofiban effectively inhibited stable platelet thrombus formation (platelet firm adhesion and subsequent aggregation) on the VWF or collagen surface under high shear, but ASA only inhibited aggregation. Notably, inhibition by K-134 became evident only when a low concentration of PGE1 was present. These inhibitors did not block shear-induced initial platelet contact with VWF via GPIb/V/IX. In contrast, under low shear, the inhibitory effects of K-134 on platelet aggregation on the collagen surface were lower than tirofiban or ASA. The observed shear-dependent suppression of platelet thrombus formation by PDE3 inhibitor in the presence of low levels of adenylate cyclase stimulator may contribute to high therapeutic benefit with low risk of bleeding.

Keywords

Phosphodiesterase 3 inhibitor, GPIb/V/IX, VWF, shear-induced platelet thrombus formation, PGE1

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Introduction

In platelets, cyclic adenosine monophosphate (cAMP) is a versatile negative regulator of key signalling pathways including Ca^{2+} mobilisation and integrin αIIbβ3 (glycoprotein (GP)IIb/IIIa) activation, virtually through serine/threonine phosphorylation by the cAMP-dependent protein kinase (PK)A. The cAMP is synthesised from adenosine triphosphate (ATP) by adenylate cyclase (AC), activated by Gs-coupled receptor stimulation with endogenous agonists such as prostaglandin (PG)I2 (also known as prostacyclin) or adenosine, and is degraded to 5'-AMP by cyclic guanosine monophosphate (cGMP)-inhibited cAMP phosphodiesterase (PDE)3. As a result, cAMP concentrations in platelets are regulated by the activity balance between AC and PDE3 (1). In fact, a prominent functional synergy exists *in vitro* and *ex vivo* between AC stimulation (e.g. by the PGI2 analogue PGE1) and PDE3 inhibition (e.g. by the PDE3 inhibitor cilostazol) to suppress platelet activation (1).

Cilostazol is the only PDE3 inhibitor to date approved for clinical use to manage intermittent claudication in patients with peripheral arterial disease (PAD) (2), and has been shown to inhibit agonist-induced human platelet aggregation *ex vivo* as effectively as the cyclooxygenase inhibitor acetylsalicylic acid (ASA) and the P2Y12 inhibitor clopidogrel (3). Although use of ASA or clopidogrel is complicated by an increased risk of bleeding, cilostazol does not prolong human bleeding time (3, 4), and the risk of haemorrhage associated with treatment is quite low (5, 6). To clarify this discrepancy, we hypothesised that PDE3 inhibitors suppress platelet thrombus formation in a shear-dependent manner, since pathological thrombus at injured arterioles or stenosed arteries is more dependent on high shear stress than physiological haemostatic plug formation. Thrombus formation on von Willebrand factor (VWF) and collagen surfaces under high shear requires association of platelet GPIb/V/IX with the A1 domain of VWF (7, 8), and GPIb/V/IX engagement itself activates GPIIb/IIIa independently of other receptors (9). Conversely, under low shear, direct platelet binding to collagen via GPVI, and platelet-to-platelet crosslinking via GPIIb/IIIa and fibrinogen are functionally significant in thrombus formation on collagen (10, 11), whereas GPIb/V/IX is not necessarily required (8).

Therefore, to test the aforementioned hypothesis, we explored the effect of PDE3 inhibition both on platelet aggregation induced by ristocetin, a non-physiological inducer of VWF binding to GPIbα and on platelet thrombus formation on a VWF or collagen surface under flow conditions at wall shear rates of 150 s^{-1} (low shear) or 1,500 s^{-1} (high shear) by utilising parallel plate flow chambers *in vitro*. Cilostazol is not appropriate for *in vitro* experiments to deduce its clinical effect because its active metabolites also play major roles in its pharmacological effects on human (12). Cilostazol neither increases cAMP levels of human platelets nor inhibits shear stress-induced platelet aggregation at therapeutic concentration in the absence of Gs stimulator *in vitro* (13). Moreover, the specificity of cilostazol is less selective for PDE3 isozyme (14). Hence, to specifically evaluate the effect of PDE3 inhibition on platelet function *in vitro*, we used a more potent and selective PDE3 inhibitor, K-134 (6-(3-(3-cyclopropyl-3-(2-hydroxycyclohexyl)ureido)propoxy)-2(1H)-quinolinone, also known as OPC-33509), which is a cilostazol analogue but is not a pro-drug (14, 15). While IC_{50} of cilostazol towards PDE2, PDE3A and PDE5 are 45.2, 0.20 and 4.4 μM, respectively, those of K-134 are >300, 0.10 and 12.1 μM, respectively (14). Here, we demonstrated that the K-134 effectively suppressed *in vitro* platelet thrombus formation under flow conditions in a more shear-dependent manner than ASA or the GPIIb/IIIa inhibitor tirofiban, and the effect of K-134 was apparent only in the presence of a low concentration of the AC stimulator PGE1. We propose that our data indicate a mechanism whereby PDE3 inhibition exhibits efficient antiplatelet effects on arterial thrombosis with a minimal impact on primary haemostasis.

Materials and methods

Antiplatelet reagents

The PDE3 inhibitors K-134 and cilostazol were obtained from Kowa (Tokyo, Japan). The GPIIb/IIIa inhibitor tirofiban was purchased from Toronto Research Chemicals (Toronto, Canada). The cyclooxygenase inhibitor ASA, Arg-Gly-Asp-Ser (RGDS) peptides (inhibitors of integrins with RGD binding sites), and forskolin (an AC activator) were from Sigma-Aldrich (St Louis, MO, USA). These reagents were dissolved in dimethyl sulfoxide (DMSO). PGE1 was purchased from Cayman Chemical (Ann Arbor, MI, USA) and dissolved in ethanol (EtOH). An inhibitory mouse monoclonal antibody against human GPIbα, GUR83–35, was obtained from Takara Bio (Shiga, Japan), and the isotype-matched control mouse IgG1 was purchased from SIGMA-Aldrich. The selection of K-134 concentrations is based on the phase-I study and non-clinical study, in which K-134 treatment with a maximal serum concentration of 1–5 μM showed antiplatelet effects on human *ex vivo* and beneficial effects in a rat model of thrombosis or ischaemia (manuscript in preparation).

Blood sampling

After obtaining informed consent according to the Declaration of Helsinki, blood was collected from the antecubital vein of healthy, medication-free volunteers through a 21-gauge needle and was anticoagulated with D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride (PPACK; Calbiochem, San Diego, CA, USA) (final concentration (fc), 40 μM) or a 10% volume of 3.8% (w/v) sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation (100 *g*, 15 minutes [min], 22°C) of blood, and the platelet count was adjusted to 2.0 $\times 10^5$/μl with platelet-poor plasma prepared by centrifugation (2,200 *g*, 10 min, 22°C) for agonist-induced platelet aggregation assay. The platelet concentration was determined using an automated haematology analyzer (K-4500; Sysmex, Kobe, Japan).

Agonist-induced platelet aggregation

PPACK-anticoagulated PRP was incubated with antiplatelet agents with or without 6 nM PGE1 at 37°C for 2 min and stimulated with adenosine diphosphate (ADP) (fc, 10 μM; MC Medical, Tokyo, Japan) or collagen (fc, 1.75–2.75 μg/ml; Chronolog, Havertown, PA, USA). ADP- or collagen-induced platelet aggregation was quantified by measuring maximum aggregation rate (MAR; percent of maximal light transmittance) within 5 min after addition of trigger using an aggregometer (Hema Tracer T-638; Nico Bioscience, Tokyo, Japan) (n=3). All volunteers' PRP samples showed similar dose-response curves for ADP (EC_{50}: about 5 μM, MAR induced by 10 μM ADP: 55–65%). The concentration of collagen was adjusted to give the EC_{85}. Ristocetin-induced platelet aggregation (RIPA) was performed by adding ristocetin solution (fc, 1.5 mg/ml; Sigma-Aldrich) to citrated PRP after incubation with antiplatelet agents at 37°C for 5 min. The effects of antiplatelet agents on RIPA were evaluated by aggregation rates at 10 min after addition of trigger. All volunteers' PRP samples (n=4) showed similar dose-response curves for ristocetin (EC_{50}: about 1.2 mg/ml, aggregation rate at 10 min: > 90%).

Preparation of thrombogenic substrate-coated surfaces

Human VWF (10 μg/ml; purified from plasma as previously described [16]) and type I collagen derived from porcine tendon (30 μg/ml; Cellmatrix Type I-A, Nitta Gelatin, Osaka, Japan) were prepared in Dulbecco's phosphate-buffered saline (PBS). Glass coverslips (No. 5, diameter 24 mm, thickness 0.5 mm; Matsunami Glass, Osaka, Japan) were immersed in VWF (22°C, 2.5 h) or collagen (4°C, 12 h) solution, carefully rinsed with PBS, and then blocked with bovine serum albumin (20 mg/ml; Sigma-Aldrich) in PBS (22°C, >2 h). After additional rinsing with PBS, coverslips were assembled into a parallel plate flow chamber just before perfusion experiments.

Perfusion experiments

PPACK-anticoagulated whole blood (5 ml) was incubated with a fluorescent marker ($DiOC_6$; Molecular Probes, Eugene, OR, USA) and antiplatelet agents at 37°C for 5 min, then aspirated using a roller pump (Minipulse 3; Gilson, Villiers Le Bel, France) and perfused over thrombogenic substrate-coated glass coverslips at a wall shear rate of 150 or 1,500 s^{-1} in a recirculating chamber (circulation cycle, 180 s or 18 s, respectively) mounted on an inverted epifluorescence microscope (Eclipse TE300; Nikon, Tokyo, Japan) equipped with a charge-coupled device camera (C2400–80V; Hamamatsu Photonics, Hamamatsu, Japan). These experiments were performed at 37°C and recorded on S-VHS videotape using a video cassette recorder (BR-S662; Victor, Tokyo, Japan), and digital images were captured using an Argus-50 image processor (Hamamatsu Photonics) with fixed contrast and brightness. The percentage of total area covered with platelets (designated as surface coverage) and the mean size of surface-bound platelet aggregates (designated as average platelet aggregate size) were calculated using the Argus-50 software. Surface coverage reflects platelet adhesion to the thrombogenic substrate-coated surface (2-dimensional thrombus growth), while average platelet aggregate size represents the degree of platelet aggregation (3-dimensional thrombus growth) on the surface (17, 18). We chose threshold values (grayness level from 0–255, where 0 is black) of 50 for surface coverage and 200 for average platelet aggregate size, to eliminate background fluorescence and fluorescence of single platelets that do not aggregate, respectively.

Measurement of cAMP and cGMP

Citrated PRP was pre-incubated with K-134 with or without 6 nM PGE1 at 37°C for 5 min, and simulated with ristocetin (fc, 1.5 mg/ml) for 10 min under stirring. Platelet intracellular cAMP and cGMP levels were analysed using an Amersham cAMP/cGMP Biotrak Enzymeimmunoassay system (GE Healthcare, Buckinghamshire, UK) and were presented as a concentration of cAMP or cGMP pmol/10^8 platelets.

Statistical analysis

Statistical analyses were performed using SAS Preclinical Package version 5.0 software (SAS Institute Japan, Tokyo, Japan).

Results

Effects of PDE3 inhibitors on RIPA

First, we compared the inhibitory effects of the PDE3 inhibitors cilostazol and K-134 with other antiplatelet agents on RIPA *in vitro*.

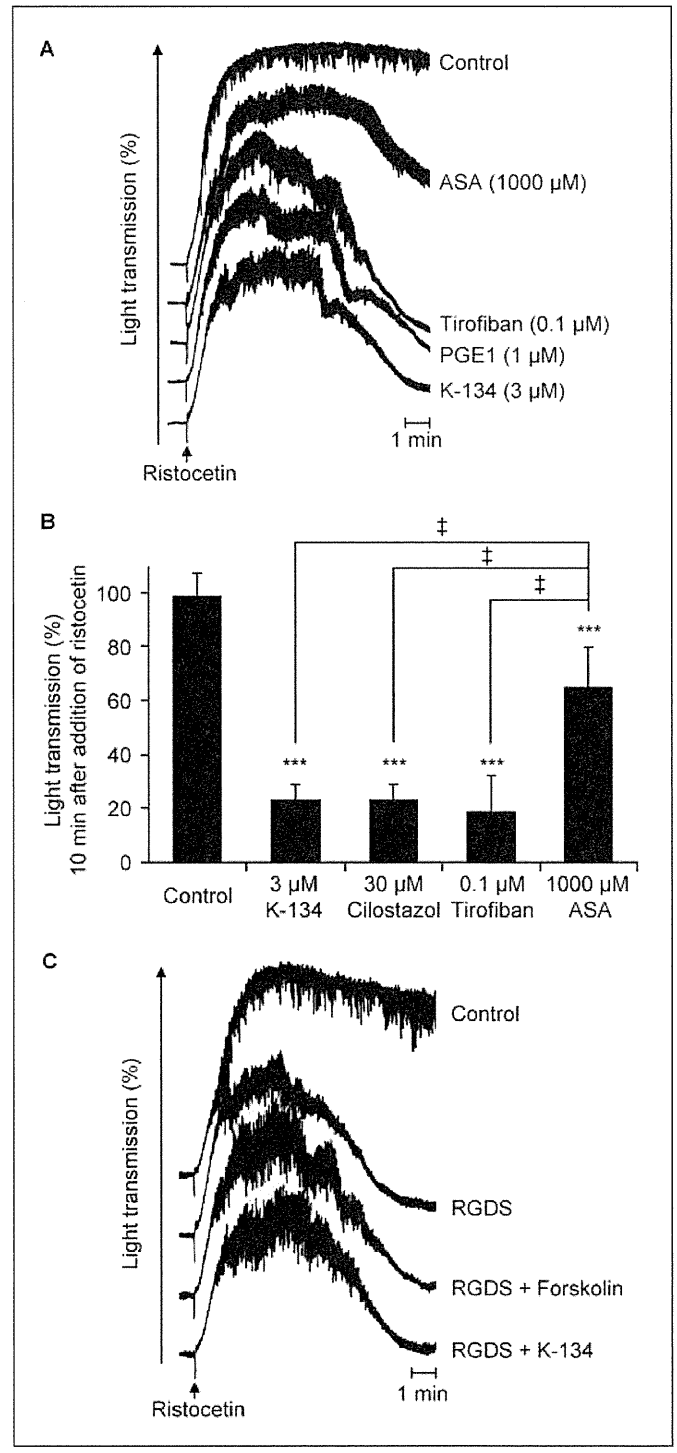

Figure 1: Effects on ristocetin-induced human platelet aggregation. Citrated PRP was preincubated with DMSO (control) or each antiplatelet agent, and stimulated with ristocetin. A, B) Inhibitory effects of agents were estimated by measuring platelet aggregation rate (%) at 10 min after stimulation (n=5). ***p<0.001 vs. control, ‡p<0.001 vs. ASA group (Tukey's test). C) The effects of 10 μM forskolin and 10 μM K-134 on ristocetin-induced platelet agglutination were evaluated in the presence of 1 μM RGDS peptides. The agglutination and aggregation curves are representatives of five experiments. Values are mean ± SD.

IC50 (μM)	Ristocetin	Ristocetin (+PGE1)	ADP	ADP (+PGE1)	Collagen	Collagen (+PGE1)
K-134	2.2 (2.0–2.4)	0.77 (0.47–1.1)	6.1 (4.4–8.3)	0.95 (0.76–1.2)	0.74 (0.55–1.0)	0.24 (0.18–0.33)
Cilostazol	22 (18–25)	8.3 (4.8–12)	32 (24–42)	7.3 (6.5–8.2)	6.4 (5.5–7.5)	1.6 (1.4–1.8)
Tirofiban	0.045 (0.011–0.072)	0.061 (0.049–0.072)	0.028 (0.018–0.042)	0.027 (0.021–0.034)	0.022 (0.019–0.026)	0.016 (0.012–0.022)
ASA	>1000	>1000	>1000	>1000	59 (39–91)	55 (40–73)

Table 1: Effects of PDE3 inhibitors on ristocetin-, ADP- or collagen-induced platelet aggregation. Calculated half-maximal inhibitory concentration (IC_{50}) values are expressed as the mean from 3–4 human volunteers. Values in parentheses indicate 95% confidence intervals.

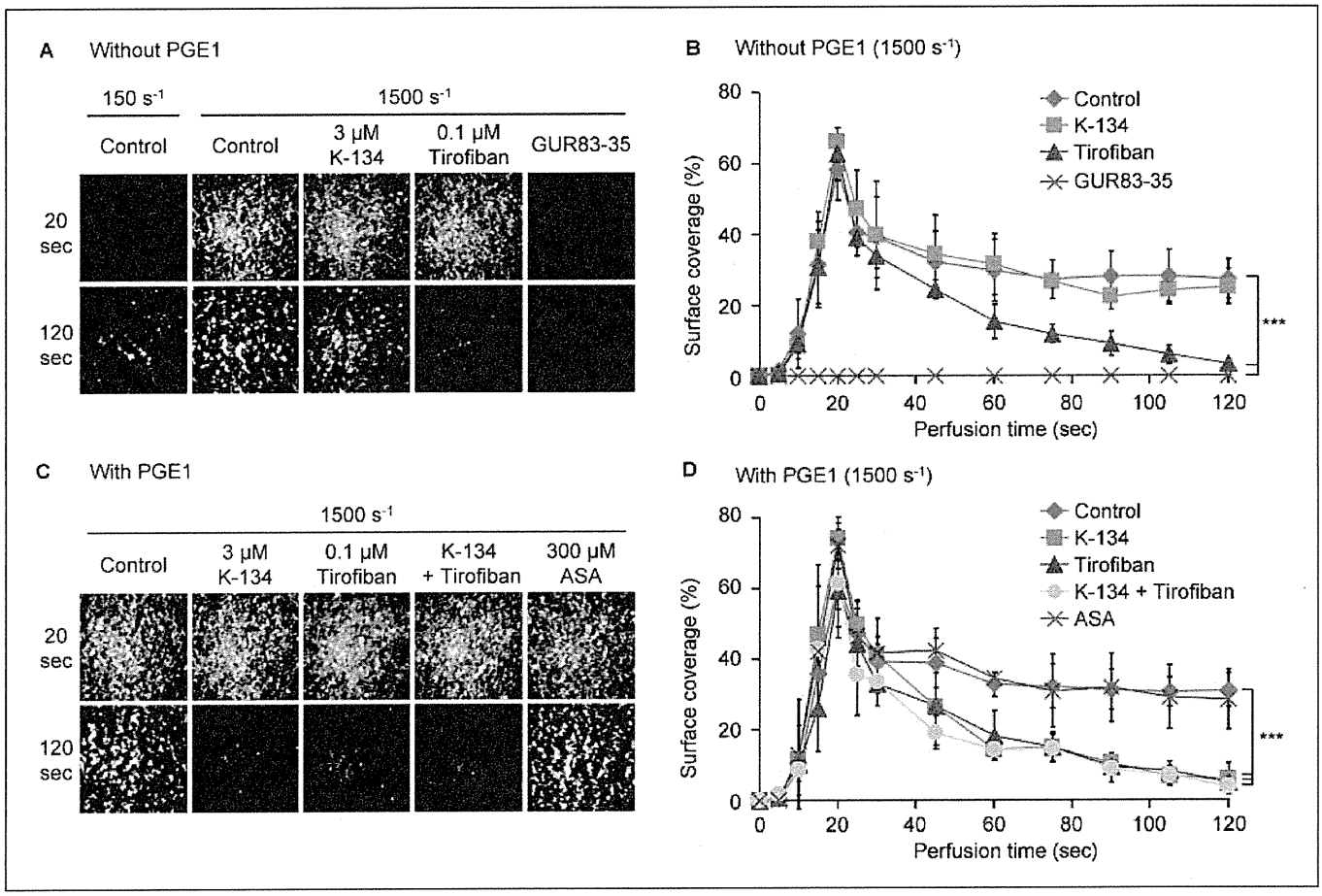

Figure 2: Effects on thrombus formation on a VWF surface under flow. PACK-anticoagulated blood was incubated with DiOC6 for platelet visualisation and DMSO (control) or each antiplatelet agent, and perfused over VWF-immobilised glass coverslips in a parallel plate flow chamber at a wall shear rate of 150 s^{-1} (A) or 1,500 s^{-1} (A-D) in the absence (A, B) or presence (C, D) of 6 nM PGE1. To confirm the GPIbα-dependency of platelet binding to VWF, 15 μg/ml GUR83–35 was used as a GPIbα blocking antibody. The effect of each inhibitor on platelet initial accumulation via GPIbα and on subsequent platelet aggregation was evaluated by measuring surface coverage at 0–120 s after initiation of perfusion. A, C) Representative images, corresponding to a 220 × 220 μm area at 20 or 120 s after initiation of perfusion. ***p<0.001 vs. control (Tukey's test was performed at 120 s). Values are mean ± SD (n=6 for control and n=3 for others).

In effect, 3 μM K-134 and 30 μM cilostazol significantly (p<0.001) inhibited RIPA (evaluated at 10 min after addition of trigger) to the same extent as 0.1 μM tirofiban and 1 μM PGE1, and more potently than 1,000 μM ASA (▶Fig. 1A, B). To explore the effects of PDE3 inhibitor on GPIIb/IIIa-independent platelet agglutination mediated through direct interaction between VWF and GPIb/V/IX, PRP was incubated with an integrin-antagonist peptide, RGDS, before ristocetin stimulation. Under such conditions, K-134 caused no change in agglutination extent and the same was true with PGE1 (not shown) and forskolin, which also increases in-

tracellular cAMP levels via direct AC activation (▶Fig. 1C). It was found that GUR83–35, a monoclonal antibody against GPIbα completely suppressed the agglutination phenomenon (not shown). These results indicate that an intracellular cAMP level heightened by PDE3 inhibition or AC activation is a crucial down-regulator for platelet aggregation via VWF-GPIb/V/IX-mediated signals, but not for VWF-GPIb/V/IX interaction itself that is independent of GPIIb/IIIa.

Next, we examined the synergistic effects of concomitant stimulation of AC on the observed PDE3 inhibition of RIPA. Inducible Gs-coupled receptor stimulation by vascular wall-derived PGI2 or adenosine is thought to be pathologically relevant in the circulation to defend against thrombosis (19, 20). Therefore, to mimic the *in vivo* situation *in vitro*, we conducted aggregation study in the presence of a very low concentration (6 nM) of PGE1. Our preliminary experiments demonstrated that 6 nM PGE1 was close to the optimal concentration that could elevate cAMP levels in the presence of PDE3 inhibitor, but did not affect agonist-induced platelet aggregation in the absence of PDE3 inhibitor (not shown). Indeed, the inhibitory effects of PDE3 inhibitors on not only ADP- or collagen- but also ristocetin-induced platelet aggregation were readily augmented in the presence of 6 nM PGE1 (▶Table 1). In contrast, the effects of tirofiban and ASA were unaffected by PGE1. Taken together, these results indicate that similar to other signals stimulated by ADP and collagen under stirring conditions, the GPIb/V/IX signal elicited by VWF binding is a cAMP-sensitive pathway.

PDE3 inhibition prevents stable platelet thrombus formation on a VWF surface under flow

To further clarify the effect of PDE3 inhibition on VWF-GPIb/V/IX-mediated platelet activation under physiological conditions, PPACK-anticoagulated blood was perfused over VWF coated onto glass coverslips. Under arterial flow conditions at a high wall shear rate of 1,500 s^{-1}, platelets exhibited rapid and progressive attachment onto the VWF surface with a peak surface coverage of ~70% at 20 s after perfusion (▶Fig. 2A, B). Platelets then started to detach over time (possibly due to the unstable platelet adherence induced by recirculating small platelet aggregates), and stable thrombus formation (surface coverage ~30%) was finally constructed by 120 s. In contrast, at a venous wall shear rate of 150 s^{-1}, platelet thrombus tended to grow slowly on the VWF surface with surface coverage of about 10% even at 120 s (▶Fig. 2A), and was not completely abolished by the previous addition of GUR83–35 (not shown). The effect of K-134 on platelet thrombus formation was studied under arterial flow conditions (1,500 s^{-1}). Unexpectedly, K-134 at a therapeutic concentration of 3 μM did not affect the whole profile of thrombus formation, while tirofiban showed an apparent inhibitory effect on the second phase of thrombus formation (at 20–120 s). Although tirofiban did not affect the first phase of platelet contact at 0–20 s, GUR83–35 completely blocked this platelet interaction (▶Fig. 2A, B) but the isotype-matched control mouse IgG1 did not show any effect (not shown), indicating that the first phase of the interaction involves GPIIb/IIIa-independent initial contact of platelets onto VWF via GPIb/V/IX (▶Fig. 2A, B). Prominently enough, although 6 nM PGE1 itself had no inhibitory effect on platelet thrombus formation at such low concentration, K-134 showed a potent inhibitory effect on the second phase of platelet interaction in the presence of 6 nM PGE1 (▶Fig. 2C, D). The extent of inhibition by K-134 resembled that by tirofiban and no synergic effect from both inhibitors was observed. In contrast, ASA showed no inhibitory effect under these experimental conditions. These results of perfusion experiments thus indicate that under physiological flow conditions, antiplatelet effects by PDE3 inhibition are mediated by elevated cAMP to efficiently block the initial signalling of the VWF-GPIb/V/IX pathway and subsequent autocrine activation signals, and the inhibitory effect is only significant in the presence of concomitant low level stimulation of AC.

PDE3 inhibition suppresses platelet thrombus formation on a collagen surface in a shear-dependent manner

To evaluate the effect of PDE3 inhibition on platelet thrombus formation mediated simultaneously by both collagen and VWF, we performed perfusion experiments over glass coverslips coated with type I collagen fibrils, to which plasma-derived VWF was expected to adsorb via the A3 domain. Indeed, we observed that platelet thrombus formation on the collagen surface under high shear (1,500 s^{-1}) was completely blocked by anti-GPIbα antibody

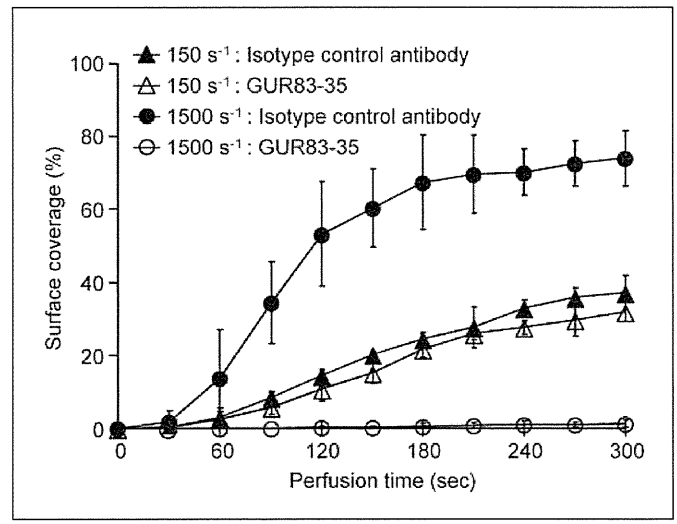

Figure 3: Effect of GPIbα blocking on thrombus formation on a collagen surface under flow. Blood treated as described in the legend for Figure 2 was perfused over collagen-immobilised glass coverslips. Effects of GPIbα blocking antibody, GUR83–35 (15 μg/ml) on thrombus formation at a wall shear rate of 150 or 1,500 s^{-1} was evaluated by measuring surface coverage. Values are mean ± SD (n=3).

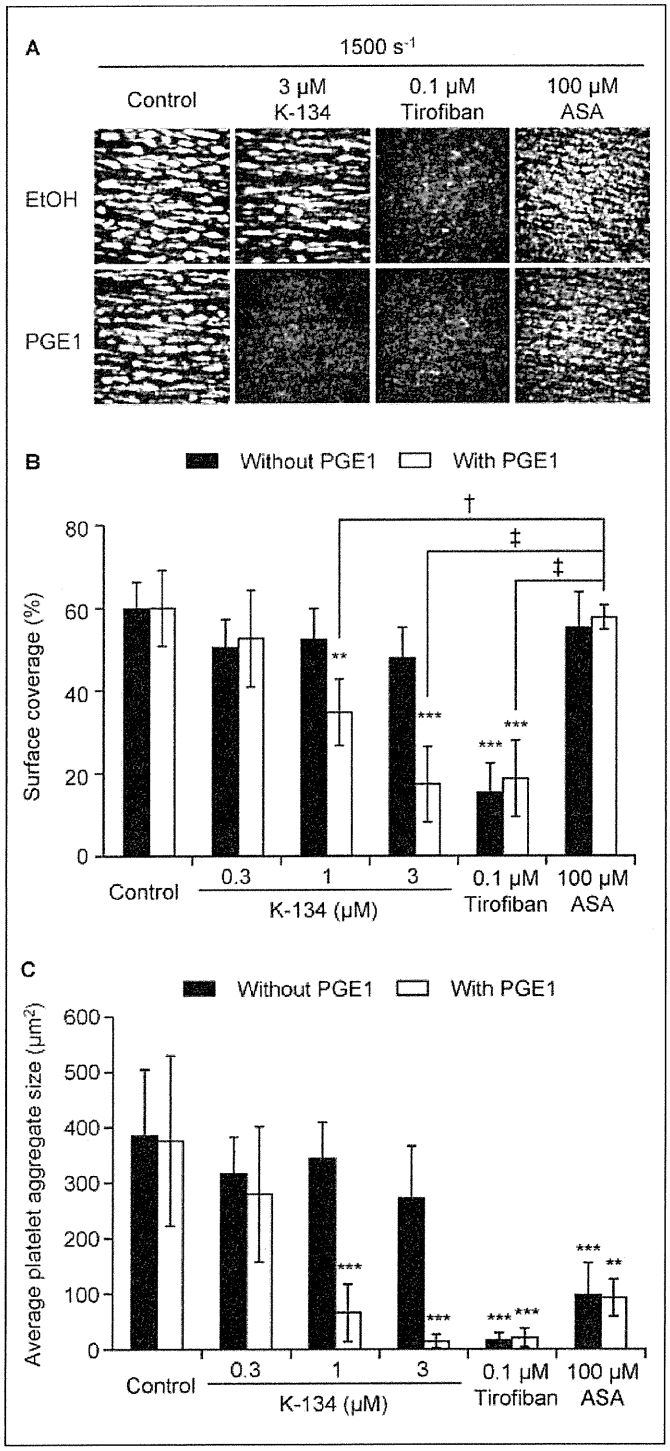

Figure 4: Effects on thrombus formation on a collagen surface under high shear. Blood treated as described in the legend for Figure 2 was perfused over collagen-immobilised glass coverslips at a wall shear rate of 1,500 s^{-1}. A) Representative images corresponding to a 340 x 340 μm area, captured at 270 s after initiation of perfusion. Effects on platelet adhesion and aggregation were evaluated by measuring surface coverage (B) and average platelet aggregate size (C), respectively. **p<0.01, ***p<0.001 vs. control. †p<0.05, ‡p<0.001 vs. ASA with PGE1 group (Tukey's test). Values are mean ± SD (n=8 for control, n=4 for others).

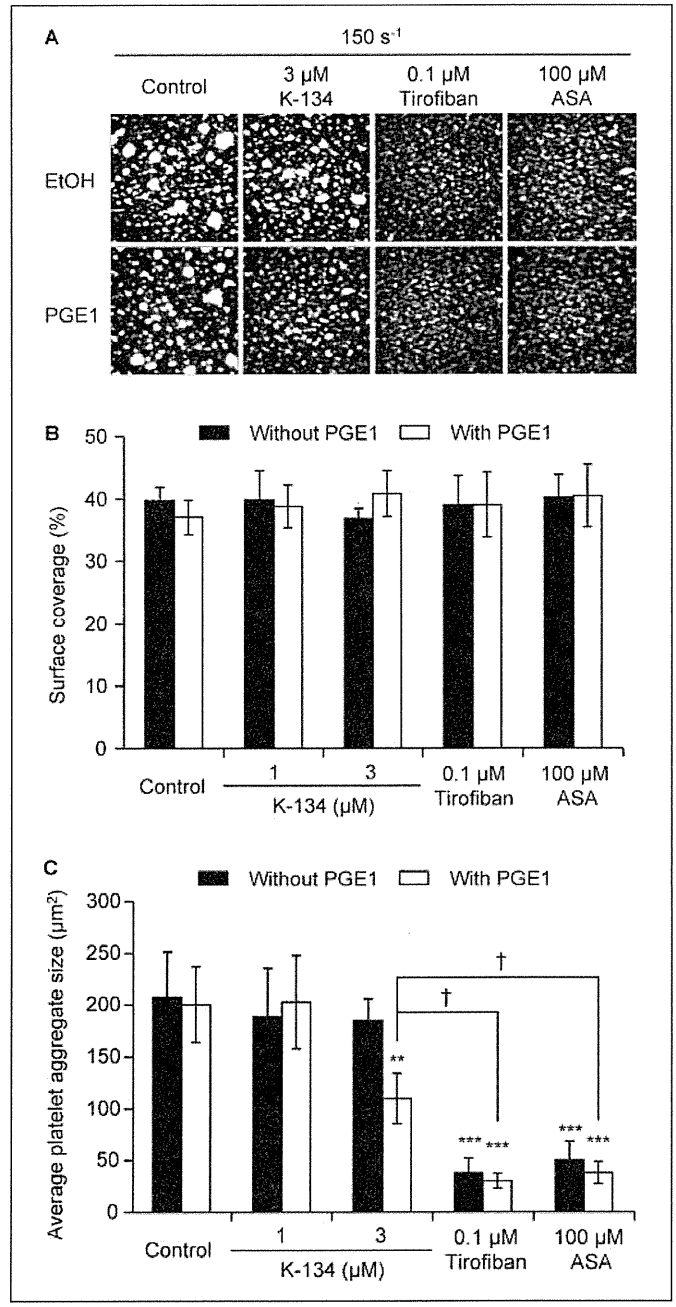

Figure 5: Effects on thrombus formation on a collagen surface under low shear. Blood treated as described in the legend for Figure 2 was perfused over collagen-immobilised glass coverslips at a wall shear rate of 150 s^{-1}. A) Figure shows representative images, corresponding to a 340 x 340 μm area, captured at 270 s after initiation of perfusion. Effects on platelet adhesion and aggregation were evaluated by measuring surface coverage (B) and average platelet aggregate size (C), respectively. **p<0.01, ***p<0.001 vs. control, †p<0.05 vs. 3 μM K-134 with PGE1 group (Tukey's test). Values are mean ± SD (n=4).

GUR83–35, while the antibody was without effect on thrombus formation under low shear (150 s^{-1}) (▶Fig. 3). Consistent with previous findings (8, 11), these results indicate that platelet adhesion and subsequent aggregation onto the collagen surface

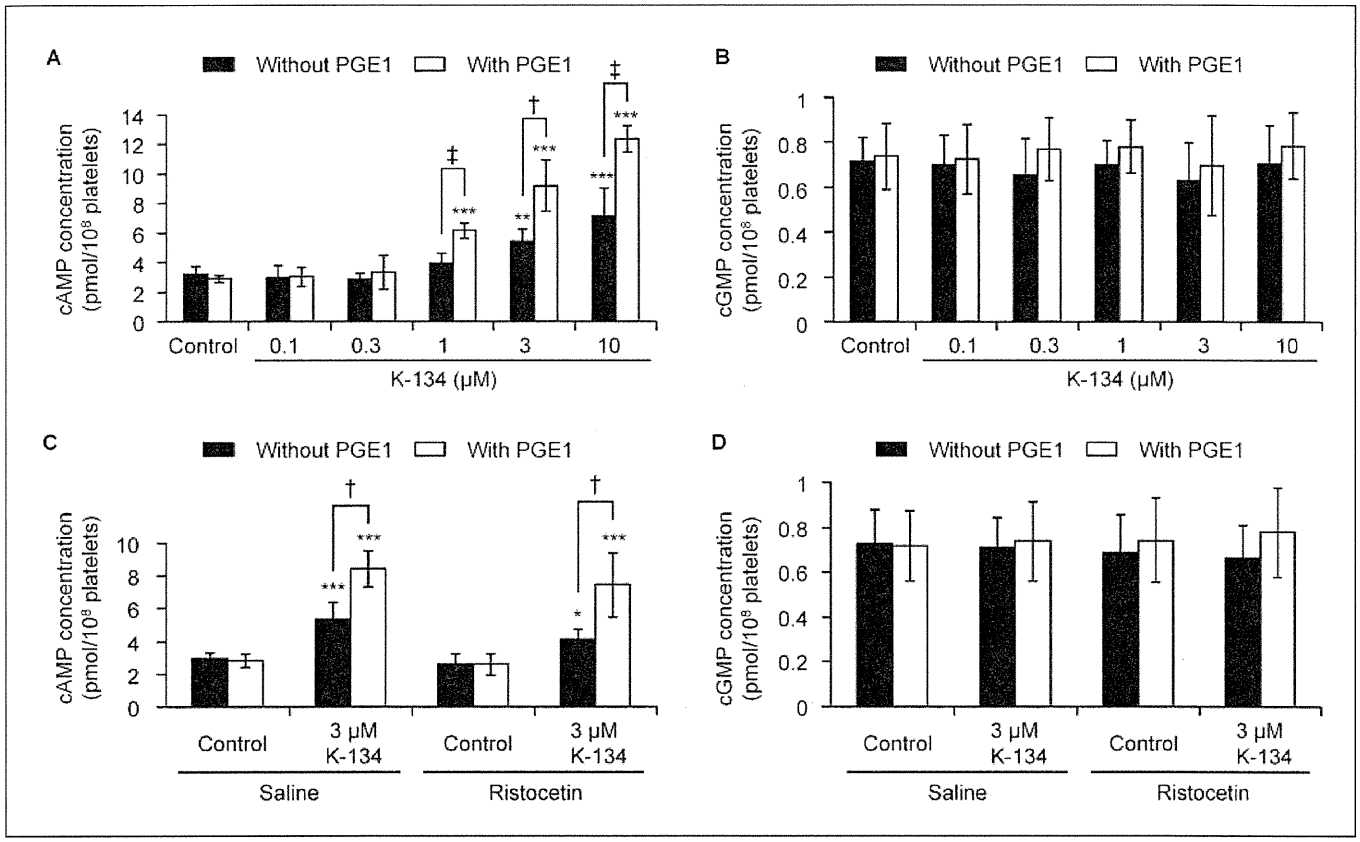

Figure 6: Effects on platelet cAMP and cGMP levels. Citrated PRP was pretreated with DMSO (control) or K-134 with or without 6 nM PGE1 for 5 min, and incubated with ristocetin or vehicle control (saline) for 10 min under stirring. A, B) platelet cAMP and cGMP levels after K-134 pretreatment (n=5). **p<0.01, ***p<0.001 vs. control (Dunnett's test), †p<0.01, ‡p<0.001 vs. absence of PGE1 (t-test). C, D) platelet cAMP and cGMP levels after ristocetin-stimulation (n=5). *p<0.05, ***p<0.001 vs. control (Tukey's test). Values are mean ± SD.

under flow conditions is mediated through VWF-GPIb/V/IX engagement in a shear rate-dependent manner. We then tested the effects of antiplatelet agents on platelet thrombus formation on the collagen surface under flow conditions. Under high shear, K-134 at least at a therapeutic concentration (~3 μM) was found to be inhibitory only when a low concentration of PGE1 (6 nM) was present (▶Fig. 4A). In fact, K-134 readily suppressed irreversible platelet adhesion (surface coverage at 270 s) and stable thrombus formation by platelet aggregation (average platelet aggregate size at 270 s) on the collagen surface in a dose-dependent manner in the presence of PGE1 (▶Fig. 4B, C). Conversely, tirofiban showed potent inhibitory effects on both parameters regardless of the presence or absence of PGE1, whereas ASA was without effects on surface coverage, but effectively (with a weaker effect than K-134 or tirofiban) inhibited platelet aggregate size. In contrast, under low shear, these antiplatelet agents including K-134 did not show any inhibitory effect on platelet surface coverage even in the presence of PGE1 (▶Fig. 5A, B). Of note, however, was the finding that these agents exhibited significant inhibitory effects on platelet aggregate size and the effect of K-134 became apparent only in the presence of PGE1 (▶Fig. 5A, C). Also, compared with results obtained under high shear (▶Fig. 4), the inhibitory effects of K-134 under low shear were significantly weaker than those of 0.1 μM tirofiban or 100 μM ASA ($p<0.05$).

Taken together, these results indicate that shear-dependent platelet thrombus formation *in vitro* initiated by GPIb/V/IX signalling is sensitive to cAMP-mediated regulatory pathways elicited by PDE3 inhibition in the presence of a low concentration of AC stimulator PGE1.

PDE3 inhibition increases platelet intracellular cAMP levels but not cGMP

To ascertain if the inhibitory effect of K-134 are mediated through cAMP or an alternative mechanism, we measured the intracellular cAMP and cGMP levels of platelets treated with K-134. Indeed, K-134 dose-dependently increased platelet cAMP levels but not cGMP, and the effect was potentiated by a low concentration of PGE1 (▶Fig. 6A, B). Furthermore, the cAMP-elevating effect of K-134 was also maintained in platelets activated by ristocetin under stirring conditions, while K-134 had no effect on cGMP levels (▶Fig. 6C, D).

Discussion

The GPIb/V/IX engagement with VWF immobilised on the subendothelial collagen surface under arterial flow conditions not only mediates platelet recruitment, but also initiates cellular activation leading to integrin-dependent firm platelet adhesion and aggregation at the site of vascular injury (8, 11). In fact, our findings (▶ Figs. 1, 2) indicated that the observed suppression of VWF-mediated platelet thrombus formation by PDE3 inhibitors was due to efficient blocking of VWF-induced GPIb/V/IX signalling by elevated cAMP, but was not attributable to the down-regulation of GPIb/V/IX binding affinity with the ligand led by PKA-induced phosphorylation of the GPIbα subunit intracellular domain, which is contrary to previously reported study (21). Consistent with our finding, Mazzucato et al. (18, 22) reported that the magnitude of platelet translocation velocity on immobilised VWF in the presence of cAMP elevating reagents appeared to be the same as that obtained by GPIIb/IIIa blocking in similar perfusion experiments, while raising cAMP levels achieved complete inhibition of intracellular Ca^{2+} elevation and GPIIb/IIIa-mediated stable adhesion and aggregation.

PDE3 inhibitor reduced stable platelet thrombus formation on the collagen surface under high shear in the presence of PGE1 more potently than ASA, but had much lower inhibitory effects under low shear than tirofiban or ASA (▶ Figs. 4, 5). There is a possible simple explanation as to why PDE3 inhibitor interfered with thrombus formation on the collagen surface in a more shear-dependent manner than tirofiban and ASA: while the secretion-independent signal mediated by VWF bound onto collagen is very sensitive to cAMP, that by collagen through GP VI is resistant to cAMP. In fact, PGE1, but not ADP receptor antagonists and ASA, could obliterate VWF-GPIb/V/IX-mediated initial intracytoplasmic Ca^{2+} oscillation (18). Moreover, cAMP analog strongly blocked GPIIb/IIIa activation induced by the dimeric VWF A1 domain (through GPIb/V/IX) in the presence of inhibitors of autocrine signalling through ADP and thromboxane A_2 (TXA_2) receptors (9). In addition, Shaun P. Jackson's group reported that platelet adhesion to VWF under high shear (at 600 and 1,800 s^{-1}) was not dependent on either ADP or TXA_2 (23). A primary collagen receptor of platelets is GPVI, which activates c-Src and Syk kinases leading to full cellular activation. In fact, tyrosine phosphorylation of these kinases induced by an anti-GPVI antibody or collagen was not abrogated with PGI2 treatment (24). Also, marked elevation of cAMP by forskolin reportedly did not inhibit collagen-induced and secretion-independent signalling events including protein-tyrosine phosphorylation, polyphosphoinositide liberation and granular secretion (25). In contrast, GP IV-mediated platelet aggregation (which is secretion-dependent) was completely inhibited by cAMP-elevating agents (24). Thus our findings were consistent with the fact that stable platelet thrombus formation on collagen under high shear is dependent on GPIb/V/IX signalling triggered by VWF and that under low shear that is mediated by GP VI signalling triggered by collagen itself (8, 10, 23).

Gs stimulation with PGE1 or adenosine and PDE3 inhibition synergistically accumulate cAMP to suppress platelet activation (1, 20). In fact, our results indicated that the observed antiplatelet effects of K-134 are most likely to be due to cAMP rather than to cGMP elevation (▶ Fig. 6). Although K-134 alone potently inhibited VWF-dependent platelet aggregation induced by ristocetin (▶ Fig. 1A, B), the inhibitory effect was readily enhanced by a low level of Gs stimulation with 6 nM PGE1 (▶ Table 1). Notably enough, however, an absolute requirement of PGE1 was observed with K-134 at the therapeutic concentrations needed to inhibit VWF-dependent platelet thrombus formation under flow conditions (▶ Figs. 2, 4). This discrepancy may be due to difference in threshold level of cAMP required to block intracellular signalling: one is ADP- and TXA_2-dependent GPIIb/IIIa activation pathway downstream of VWF-GPIb/V/IX interaction induced by ristocetin (aggregometer studies) and the other is secretion-independent integrin activation pathway induced by shear (flow chamber studies) ([23, 26] and ▶ Figs. 1, 2, 6). Thus, our *in vitro* findings obtained under physiological flow conditions raised the notion that VWF-induced platelet thrombus formation may be efficiently blocked by PDE3 inhibitor under *in vivo* conditions where the endogenous Gs stimulators (PGI2 and adenosine) are up-regulated. As a matter of fact, considerable amounts of PGI2 were reportedly produced locally in response to mural platelet thrombus formation on de-endothelialised arterial wall (19). In addition, adenosine was released in hypoxic tissues during ischaemia and exerted ischaemic preconditioning effects (27, 28). Since both of these substances have very short biological lives *in vitro*, the stable PGI2 analogue, PGE1 may instead mimic the *in vivo* supporting effects on PDE3 inhibitor-driven suppression of VWF-dependent platelet thrombus formation under arterial shear conditions. Meanwhile, we speculated that there is no feasibility of clinical use of combined PDE3 inhibitor and Gs stimulator, since long-term administration of Gs stimulator leads to decreased sensitivity of human platelets to the drug (29).

What is known about this topic?

- Phosphodiesterase (PDE)3 inhibitor, cilostazol inhibits agonist-induced human platelet aggregation *ex vivo* but does not prolong bleeding time, and the risk of haemorrhage associated with treatment is quite low.
- Cilostazol neither increases cAMP levels of human platelets nor inhibits shear stress-induced platelet aggregation at therapeutic concentration in the absence of Gs stimulator *in vitro*.
- Cilostazol is not appropriate for *in vitro* experiments, because it is converted into several active metabolites *in vivo*.

What does this paper add?

- We investigated the anti-platelet mechanism of a more potent and selective PDE3 inhibitor, K-134, which is a cilostazol analog but not a pro-drug.
- Therapeutic concentrations of K-134 plus a low concentration of Gs stimulator synergistically increased platelet cAMP levels and strongly suppressed high shear-dependent platelet thrombus formation initiated by interactions between GPIb/V/IX and VWF but had much lower inhibitory effects under low shear than GPIIb/IIIa inhibitor or cyclooxygenase inhibitor.

Unlike tirofiban and ASA, PDE3 inhibitor does not prolong human bleeding time, even under repeated dosing, but exhibits significant inhibitory effects on agonist-induced platelet aggregation *ex vivo* (3, 4). Considering the observed high shear stress- and Gs stimulation-dependent antiplatelet effects of PDE3 inhibitor *in vitro* (▶Figs. 4, 5), it may exert *in vivo* antiplatelet activity in a shear-dependent manner when concomitant stimulation of Gs-coupled receptors is induced at the site of arterial thrombosis and ischaemia. In contrast, tirofiban and ASA inhibit platelet aggregation regardless of shear and Gs stimulation, and thus may affect physiological haemostatic plug formation. To further understand the mechanism of their low risk of bleeding, we need to explore how the local concentrations of endogenous Gs stimulators are regulated at sites of vascular perturbation.

Care should be taken when the results of our *in vitro* perfusion experiments at a high shear rate of 1,500 s^{-1} are interpreted into the pathological conditions *in vivo*, since the regions of arterial stenosis are exposed to much higher shear rates ranging from 1,000 up to 10,000 s^{-1} (11). Nonetheless, considering the rebound phenomenon of Gs-stimulator (29), PDE3 may be a better drug-target to elevate platelet cAMP levels for the treatment of PAD (2) or secondary prevention of cerebral infarction (6) that needs long-term drug administration.

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Conflict of Interest

The study was supported in part by a research grant from Kowa Company, Ltd. (Tokyo, Japan). H.Y. was an employee of Kowa Company, Ltd., Japan.

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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.

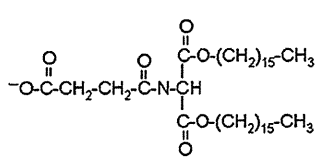
2.3. Preparation of H12-modified ADP-encapsulated liposomes, H12-(ADP)Lipo

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)

a DHSG



b H12-MALPEG3400-DSPE

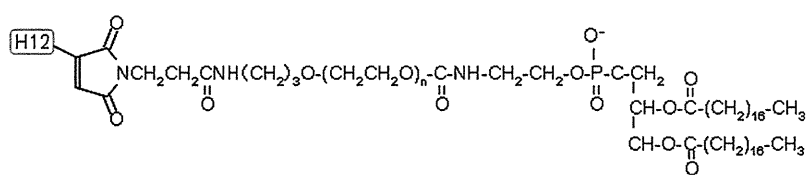


Chart 1. Structure of liposome contents. (a) 1,5-Dihexadecyl-N-succinyl-L-glutamate. (b) H12 is the fibrinogen γ chain (γ400–411) His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val.

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)

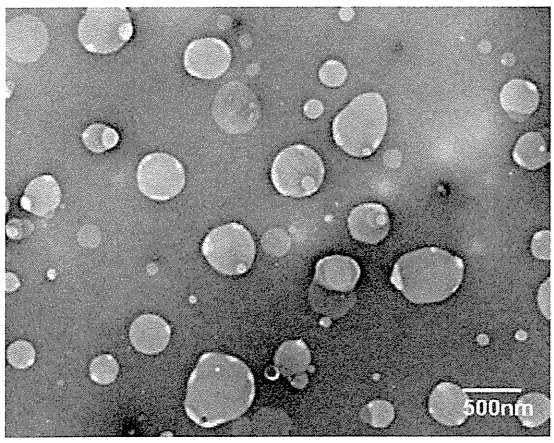


Fig. 1. TEM image of H12-(ADP)Lipo. This liposome sample was suspended on a transmission electron microscopic grid coated with collodion film, stained with phosphotungstic acid, and then examined with a Hitachi H-9000UHR type transmission electron microscope operated at 100 kV.

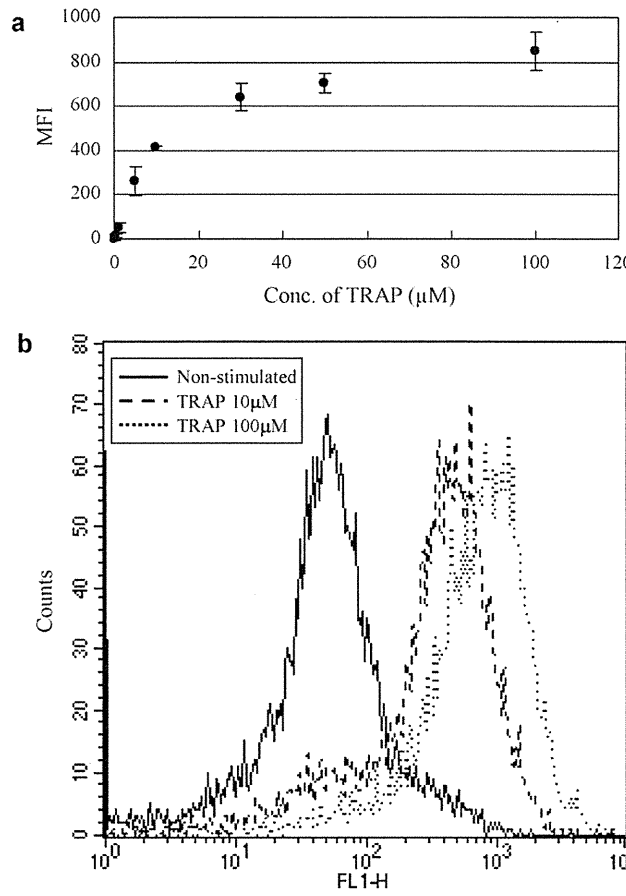


Fig. 2. TRAP-induced activation of GPIIb/IIIa on platelets. (a) Various concentrations of TRAP (0–100 μM) were added to washed platelets (platelet = 1.0×10^5 cells/μL, 80 μL) in the presence of FITC-Fbg, and incubated at 37 °C for 10 min before fixation with formaldehyde (1.8%, v/v). The mixture was 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.

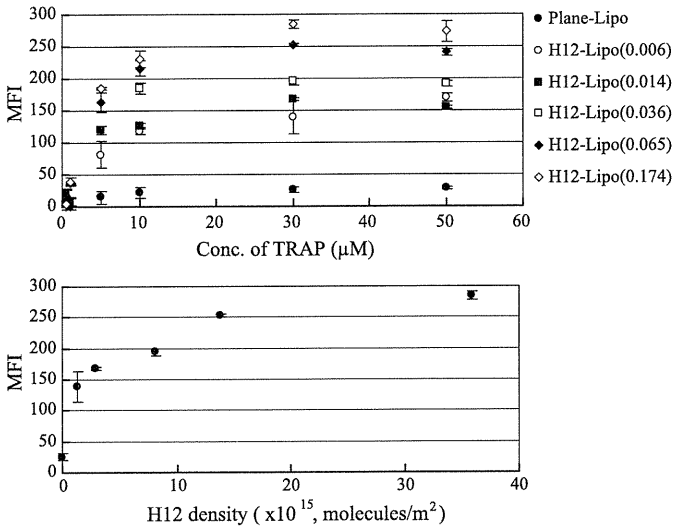


Fig. 3. Effect of modification of liposomes with H12 on their ability to bind to platelets. Liposomes modified with various amounts of H12 peptide were prepared. Each liposomal sample (0.5 mg/mL lipids) labeled with $DiOC_{18}$ was incubated with washed platelets (platelet = 1.0×10^5 cells/μL, 80 μL) 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.

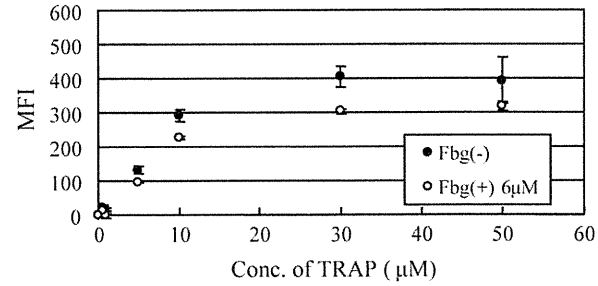


Fig. 4. H12-(ADP)Lipo binding to platelets in the presence of the physiological concentration of fibrinogen. H12-(ADP)Lipo labeled with $DiOC_{18}$ (0.5 mg/mL as lipids) and various concentrations of TRAP (0–50 μM) without (●) or with 6 μM Fbg (○) were added to washed platelets (1.0×10^5 cells/μL, 80 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

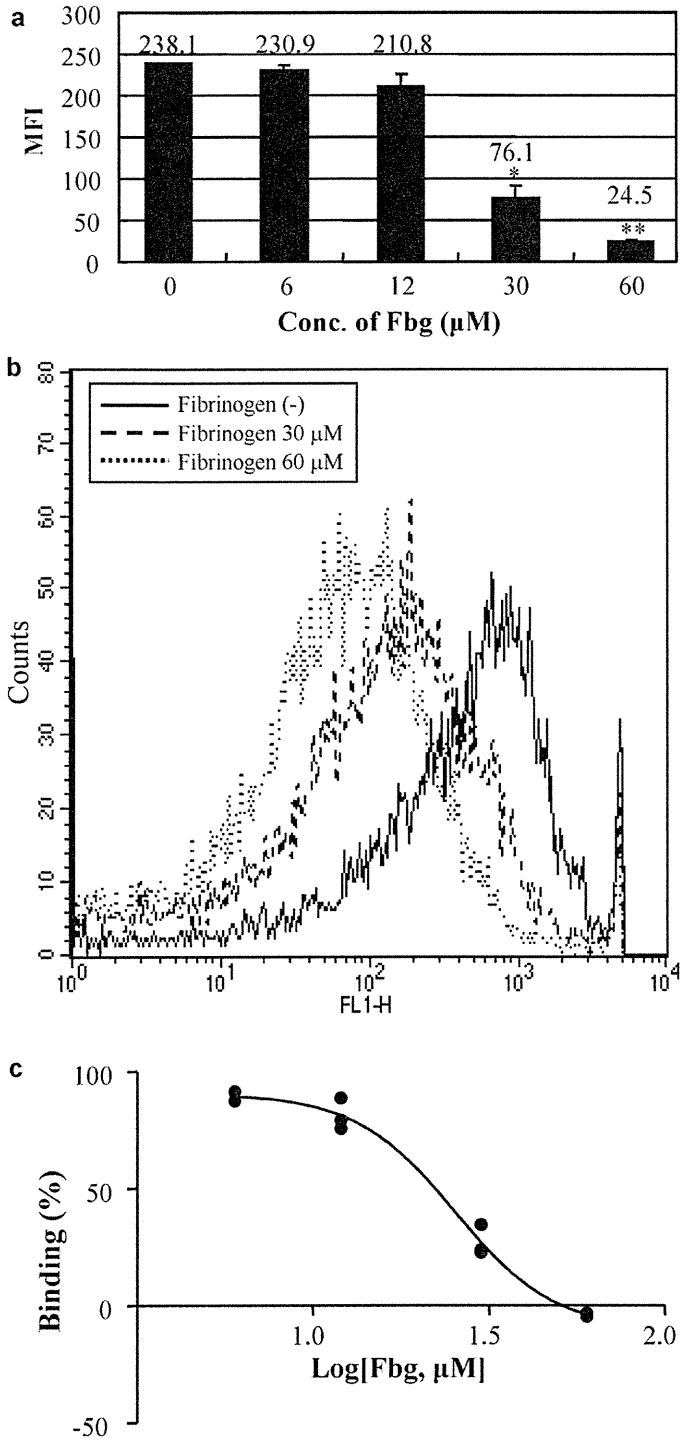


Fig. 5. Inhibitory effect of fibrinogen on the binding of H12-(ADP)Lipo to platelets. (a) H12-(ADP)Lipo labeled with $DiOC_{18}$ (0.5 mg/mL), 30 μM TRAP, and various concentrations of Fbg were added to washed platelets (1.0×10^5 cells/μL, 50 μL). Significant difference from Fbg 0 μM group are indicated 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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