Pharmacokinetic Study of the Structural Components of Adenosine Diphosphate-Encapsulated Liposomes Coated with Fibrinogen γ-Chain Dodecapeptide as a Synthetic Platelet Substitute

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ABSTRACT

Fibrinogen γ-chain (dodecapeptide HHLGGAKQAGDV, H12)-coated, ADP-encapsulated liposomes [H12-(ADP)-liposomes] were developed as a synthetic platelet alternative that specifically accumulates at bleeding sites as the result of interactions with activated platelets via glycoprotein Ib/IIa and augments platelet aggregation by releasing ADP. The aim of this study is to characterize the pharmacokinetic properties of H12-(ADP)-liposomes and structural components in rats, and to predict the blood retention of H12-(ADP)-liposomes in humans. With use of H12-(ADP)-liposomes in which the encapsulated ADP and liposomal membrane cholesterol were radiolabeled with 14C and 3H, respectively, it was found that the time courses for the plasma concentration curves of 14C and 3H radioactivity showed that the H12-(ADP)-liposomes remained intact in the blood circulation for up to 24 hours after injection, and were mainly distributed to the liver and spleen. However, the 14C and 3H radioactivity of H12-(ADP)-liposomes disappeared from organs within 7 days after injection. The encapsulated ADP was metabolized to allantoin, which is the final metabolite of ADP in rodents, and was mainly eliminated in the urine, whereas the cholesterol was mainly eliminated in feces. In addition, the half-life of the H12-(ADP)-liposomes in humans was predicted to be approximately 96 hours from pharmacokinetic data obtained for mice, rats, and rabbits using an allometric equation. These results suggest that the H12-(ADP)-liposome has potential with proper pharmacokinetic and acceptable biodegradable properties as a synthetic platelet substitute.

Introduction

As the numbers of patients with hematologic malignancies and solid tumors increase, platelet transfusion represents one of the most essential prophylactic or therapeutic treatments, because these disorders induce severe thrombocytopenia caused by the intensive chemotherapy, surgical procedures, and radiotherapy. However, platelet transfusion introduces a variety of complications such as bacterial infection, allergic reaction, and acute lung injury. In addition, donated platelet for blood transfusions can only be stored for a period of 4 days in Japan and 5–7 days in the United States and Europe. This has become a serious concern in our aging society and a stable supply in an emergency situation such as disasters and pandemics needs to be on hand. To solve these problems, various platelet substitutes, which consist of materials derived from blood components, have been developed (Blajchman, 2003), such as solubilized platelet membrane protein conjugated liposomes (plateletosome) (Rybak and Renzulli, 1993), infusible platelet membranes (Graham et al., 2001), fibrinogen-coated albumin microcapsules (synthocyte) (Levi et al., 1999), red blood cells with bound fibrinogen (Agam and Livne, 1992), liposomes bearing fibrinogen (Casals et al., 2003), arginine-glycine-aspartic acid (RGD) peptide-bound red blood cells (thromboerythrocyte) (Coller et al., 1992), and fibrinogen-conjugated albumin polymers (Takeoka et al., 2001). However, these platelet substitutes have not yet been approved for clinical use.

ADP-encapsulated liposomes modified with a dodecapeptide (HHLGGAKQAGDV, H12) [H12-(ADP)-liposome] were developed as a new type of synthetic platelet alternative. The glycoprotein

ABREVIATIONS: CL, clearance; DHSG, 1,5-dihexadecyl-N-succinyl-L-glutamate; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine; GP, glycoprotein; H12, HHLGGAKQAGDV; H12-(ADP)-liposome, ADP-encapsulated liposomes modified with a dodecapeptide; HbV, hemoglobin vesicle; HPLC, high-performance liquid chromatography; ID, injected dose; MPS, mononuclear phagocyte system; PEG, polyethylene glycol; PEG-DSP, 2-diesteroyl-sn-glycero-3-phosphatidylethanolamine-N-[monomethoxy(polyethyleneglycol)]; RGD, arginine-glycine-aspartic acid; t1/2, half-life; Vdss, distribution volume.
(GP) IIb/IIIa, which is present on the platelet membranes, is converted from an inactive to an active form when platelets adhere to collagen that is exposed on sites of vascular injury (Takagi et al., 2002; Xiao et al., 2004), and platelet aggregation is mediated by fibrinogen by bridging adjacent platelets through GPIb/IIa in an activation-dependent manner in the circulation. Among several GPIb/IIa recognized sequence sites in fibrinogen such as the RGD-based sequences (RGDFK and RGDSD in the Aα chains) and H12 (HHLGGAKQAGDYH12) in the carboxy-terminus of the γ-chain (Kloczewiak et al., 1982, 1984; Hawiger et al., 1989), H12 is a specific binding site of the ligand for activated GPIb/IIa (Lam et al., 1987; Andrieux et al., 1989), whereas RGD-related peptides are nonspecific with respect to a wide variety of integrins from various cell types (Phillips et al., 1991). In addition, when ADP is released from activated platelets, it functions as a potent platelet agonist. Thus, these modifications to H12-(ADP)-liposomes enable them to specifically interact with activated platelets, resulting in platelet aggregation. In fact, H12-liposomes with polyethyleneglycol (PEG)-surface modification specificity accumulate at the site of an injury in vivo and were determined to shorten bleeding time in a dose-dependent manner in a thrombocytopenic rat and a rabbit model (Okamura et al., 2005, 2009, 2010a,b; Nishikawa et al., 2012). Therefore, these findings prompted us to conclude that H12-(ADP)-liposomes have considerable potential for use as an alternative for actual platelets in clinical settings.

Before new drugs are approved for clinical use, they are required to undergo a wide variety of evaluations, including physicochemical tests, preclinical studies, and clinical trials. As described above, preclinical studies of H12-(ADP)-liposomes have resulted in pharmacological evidence to indicate that they can be used as a platelet substitute (Okamura et al., 2005, 2009, 2010a,b; Nishikawa et al., 2012). However, information concerning pharmacokinetic properties is lacking, especially the disposition and retention of each component in tissues after injection. Our strategy for the development of H12-(ADP)-liposomes is based on the fact that not only better pharmacological effects but also acceptable biodegradable properties (no accumulation or retention) need to be documented. In addition, preclinical pharmacokinetic studies in various mammalian species are essential, because the results of such studies can be extrapolated to humans, allowing appropriate dosing regimens to be estimated in the case of humans.

In the present study, we report on an evaluation of the pharmacokinetic properties of the H12-(ADP)-liposomes and components thereof, from the standpoint of stability in the blood circulation and the metabolism and excretion of each component. For this purpose, we prepared H12-(ADP)-liposomes that were 14C, 3H double radio-labeled, in which the encapsulated ADP and membrane component (cholesterol) were labeled with 14C and 3H, respectively. Furthermore, we predicted some important pharmacokinetic parameters, especially retention in the blood circulation, in humans, based on data obtained in pharmacokinetic studies in mice, rats, and rabbits.

Materials and Methods

Reagents

Cholesterol and 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) were purchased from Nippon Fine Chemical (Osaka, Japan), and 2,3-dioleoyl-sn-glycero-3-phosphatidylethanolamine-N-[mono(2-hexadecanoyloxy)ethyl] (PEG-DSP-E, 5.1 Kda) was from NOF (Tokyo, Japan), 1,5-Dihexadecyl-N-succinyl-l-glutamate (DHSG) and H12-PEG-Glu2C18, in which the fibrinogen γ-chain dodecapeptide (C-HHLGGAKQAGDY, Cys-H12) was conjugated to the end of the PEG-lipids, were synthesized as previously reported (Okamura et al., 2005). Allantoin, uric acid, hypoxanthine, xanthine, and ADP were obtained from Sigma-Aldrich (St. Louis, MO).

Preparation of 14C, 3H Double-Labeled H12-(ADP)-Liposomes

First, 14C-labeled H12-(ADP)-liposomes were prepared under sterile conditions as previously reported, with minor modifications (Okamura et al., 2009). In brief, DPPC (1000 mg, 1.36 mmol), cholesterol (527 mg, 1.36 mmol), DHSG (189 mg, 272 μmol), PEG-DSP-E (52 mg, 9.0 μmol), and H12-PEG-Glu2C18 (47 mg, 9.0 μmol) were dissolved in t-butyl alcohol and then freeze-dried. The resulting mixed lipids were hydrated with phosphate-buffered saline (pH 7.4) containing ADP (1 mM) and [8-14C]ADP (1.85 MBq; Moravec Biochemicas Inc., La Brea, CA), and extruded through membrane filters (0.22 μm pore size, Durapore; Millipore, Tokyo, Japan). Liposomes were washed with phosphate-buffered saline by centrifugation (100,000×g, 30 minutes, 4°C), and the remaining ADP was eliminated by sephadex G25. The diameter and ζ potential of the 14C-labeled H12-(ADP)-liposomes used in this study are regulated at 250 ± 50 nm and −10 ± 0.9 mV, respectively. The 5–10% of added ADP was encapsulated in the inner space of the vesicle.

The 3H labeling of 14C-labeled H12-(ADP)-liposomes, to prepare 14C and 3H double-labeled H12-(ADP)-liposomes, was carried out according to a previous report (Taguchi et al., 2009). The 14C-labeled H12-(ADP)-liposomes (1 ml) were mixed with [1,2-3H(N)]cholesterol solution (10 μl), (PerkinElmer, Yokohama, Japan) and incubated for 12 hours at room temperature. 14C, 3H double-labeled H12-(ADP)-liposomes were filtered through a sterile filter to remove aggregates (450 nm pore size). Before being used in pharmacokinetic experiments, all of the samples were mixed with unlabeled H12-(ADP)-liposomes. To utilize the same procedure using H12-(ADP)-liposomes and [1,2-3H(N)]cholesterol, 3H-labeled H12-(ADP)-liposomes, which did not contain [8-14C]ADP, were prepared for the pharmacokinetic studies in mice and rabbits.

Animals

All animal experiments were undertaken in accordance with the guideline principle and procedure of Kumamoto University for the care and use of laboratory animals. Experiments were carried out with male ddY mice (28–30 g body weight; Japan SLC, Inc., Shizuoka, Japan), male Sprague-Dawley rats (180–210 g body weight; Kyudo Co., Kumamoto, Japan), and male New Zealand White rabbits (2.0–2.2 kg body weight; Bioteck Co., Saga, Japan). All animals were maintained under conventional housing conditions, with food and water ad libitum in a temperature-controlled room with a 12-hour dark/light cycle.

Pharmacokinetic Studies

Administration and Collecting Blood and Organs in Rats. Twenty-four Sprague-Dawley rats were anesthetized with diethyl ether and received a single injection of 14C, 3H-labeled H12-(ADP)-liposomes [10 mg lipids/kg (n = 16), 20 mg lipids/kg (n = 4), and 40 mg lipids/kg (n = 4)]. In all rat groups, four rats were selected to undergo the plasma concentration test. Under ether anesthesia, approximately 200 μl blood samples in all administration groups were collected from tail vein at multiple time points after the injection of 14C, 3H-labeled H12-(ADP)-liposomes (3, 10, and 30 minutes, and 1, 2, 3, 6, 12, 24, 48 and 168 hours) and the plasma was separated by centrifugation (3000g, 5 minutes). After collecting the last blood sample (168 hours), the rats were euthanized for excision of organs (kidney, liver, spleen, lung, and heart). Urine and feces were collected at fixed intervals in a metabolic cage. In addition, the four rats were sacrificed and organs were collected at 2, 6, and 24 hours after an injection of 14C, 3H-labeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg.

Administration and Collection of Blood and Organs in Mice and Rabbits. Twenty-eight ddY mice received a single injection of 14C-labeled H12-(ADP)-liposomes (10 mg lipids/kg) in the tail vein under ether anesthesia. At each time after the injection of 14C-labeled H12-(ADP)-liposomes (3 and 30 minutes, and 1, 3, 6, 12, and 24 hours), four mice were anesthetized with ether and blood was collected from the inferior vena cava, and plasma was obtained by centrifugation (3000g, 5 minutes). Four New Zealand White rabbits received a single injection of 3H-labeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg. The blood was collected from the auricular veins at each time after injection (3, 10, and 30 minutes, and...
Measurement of $^{14}$C and $^3$H Radioactivity. Plasma samples were solubilized in a mixture of Soluene-350 (PerkinElmer) and isopropanol alcohol (at a ratio of 1/1) for 24 h at 50°C. The organ samples were rinsed with saline, minced, and solubilized in Soluene-350 for 24 hours at 50°C. Urine and feces were also weighed and solubilized in Soluene-350. All samples were decolorized by treatment with a hydrogen peroxide solution after treatment of Soluene-350 or isopropanol alcohol. The $^{14}$C $^3$H radioactivity was determined by liquid scintillation counting (LSC-5121; Aloka, Tokyo, Japan) with Hionic-Fluor (PerkinElmer).

Analysis of Metabolites of Encapsulated ADP. ADP metabolites in urine were determined by high-performance liquid chromatography (HPLC), as described previously (George et al., 2006). A part of the urine obtained in the pharmacokinetic study in rats was used for this analysis, and aliquots of urine samples (2.5 ml) were mixed with 200 μl 10% sulfuric acid. Just before the analysis, the urine samples were centrifuged and filtered through a Disomic-25c (0.2 μm pore size; ADVANTEC, Tokyo, Japan) and diluted 10-fold with water after adjusting the pH to 7 with 0.01 N sodium hydroxide and 0.01 N sulfuric acid. A standard solution containing ADP, allantoin, uric acid, hypoxanthine, and xanthine was prepared as reported in a previous study (George et al., 2006).

The HPLC system consisted of a Waters 2695 pump (Waters, Milford, MA), a Waters 2487 detector (Waters) operated at 220 nm. LC analyses were achieved with a 250 × 4 mm, 5 μm LiChrospher 100 RP-18 end-capped column (LiChroCART 250-4; Merck, Darmstadt, Germany). Furthermore, each ADP metabolite separated by HPLC was collected by a fraction collector (CHF121SA; ADVANTEC) and $^{14}$C radioactivity was determined by liquid scintillation counting with Hionic-Fluor.

Interspecies Scaling of Pharmacokinetic Parameters

Allometric relationships between various pharmacokinetic parameters (P) and body weight (W) were plotted on a log-log scale. Linear regression of the logarithmic values was calculated using the least-squares method using Eq. 1 (Boxenbaum, 1984).

$$P = a \cdot W^b$$

P is the parameter of interest [distribution volume (V_diss) or clearance (CL)], W is the body weight (kg), and α and β are the coefficient and exponent of the allometric equation, respectively. The average body weights of 0.034 kg (mouse), 0.242 kg (rat), 2.08 kg (rabbit), and 70 kg (human) were used for prediction of V_diss and CL for human. After predicting of V_diss and CL for humans (70 kg) using Eq. 1, the half-life for human was estimated.

Data Analysis

A noncompartmental model was used for the pharmacokinetic analysis. Each parameter, including half-life ($t_{1/2}$, h), mean residence time (h), area under the concentration-time curve ($h \cdot \%$ of dose/ml), CL (ml/h), and V_diss (ml), was calculated using the moment analysis program available in Microsoft Excel (Microsoft Corporation, Redmond, WA) (Yamakawa et al., 2013). Data are shown as means ± S.D. for the indicated number of animals.

Results

Pharmacokinetics of H12-(ADP)-Liposome Components in Rats. To investigate the pharmacokinetics of each component of the H12-(ADP)-liposomes, $^{14}$C, $^3$H-labeled H12-(ADP)-liposomes, in which the encapsulated ADP was labeled with $^{14}$C and the membrane component (cholesterol) was labeled with $^3$H, were prepared (Fig. 1A). As shown in Fig. 1B and Table 1, the plasma concentration curves and pharmacokinetic parameters for $^{14}$C radioactivity and $^3$H radioactivity were similar. These data indicate that the H12-(ADP)-liposomes remained intact in the blood circulation for periods of up to 24 hours after injection in rats.

Moreover, we evaluated the tissue distribution of both the encapsulated ADP and membrane component (cholesterol) of the H12-(ADP)-liposomes. Figure 2 shows the tissue distribution in organs at 2, 6, and 24 hours after the administration of $^{14}$C, $^3$H-labeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg to rats. Among these organs, the majority of both the $^{14}$C and $^3$H radioactivity of the H12-(ADP)-liposomes were distributed in the liver and spleen. However, both the $^{14}$C and $^3$H radioactivity of the H12-(ADP)-liposomes were eliminated from each organ, and the activity essentially disappeared within 7 days after injection (unpublished data). These data indicate that the H12-(ADP)-liposomes are mainly distributed to the liver and spleen, but the retention in these organs is negligible.

To identify the excretion pathway of the H12-(ADP)-liposomes, the levels of $^{14}$C and $^3$H in urine and feces were measured (Fig. 3, A and B). The $^{14}$C was excreted mainly in the urine [80.4% ± 4.9% of the injected dose (ID) at 7 days after injection], but was low in feces (7.6% ± 2.7% of ID at 7 day after injection). On the other hand, the majority of the $^3$H was excreted in the feces (74.2% ± 5.7% of ID at 7 days after injection), and excretion into the urine was essentially nil. In addition, as shown in Fig. 3C, it is well known that, in rodents, endogenous ADP is ultimately metabolized to allantoin and excreted. Thus, we qualitatively determined the fate of the encapsulated ADP of the H12-(ADP)-liposomes using an HPLC method. Figure 3D shows the separated peaks for ADP and its metabolites in the standard solution and in a urine sample 6 hours after the administration of the H12-(ADP)-liposomes to a rat. Furthermore, to exclude the effect of endogenous ADP and its metabolites, we measured the $^{14}$C radioactivity of each peak that had been separated by HPLC. As a result, almost all of the $^{14}$C radioactivity was detected in the peak corresponding to allantoin, which is the final metabolite of ADP in rodents, in the urine sample (Table 2).

These results indicate that more than 75% of each structural component of the H12-(ADP)-liposome is excreted from the body within 7 days after injection, and the encapsulated ADP and membrane component (cholesterol) derived from H12-(ADP)-liposomes were metabolized to final metabolites and excreted into the urine and feces, respectively.

Dose-Dependence of H12-(ADP)-Liposomes Pharmacokinetics. Figure 4 shows the time courses for the plasma concentration for the $^{14}$C, $^3$H-labeled H12-(ADP)-liposomes administered to rats at doses of 10, 20, and 40 mg lipids/kg. No significant difference was found in the plasma concentration curve or pharmacokinetic parameters among all groups (Fig. 4, A and B). In fact, a linear relationship between the administration dose and the area under the concentration-time curve was found, the values for which were calculated based on the lipids concentration (Fig. 4C). These data indicate that the disposition of the H12-(ADP)-liposomes is linear for a dose of 40 mg lipids/kg.

Moreover, the tissue distribution of both the encapsulated ADP and the membrane lipids component (cholesterol) of the $^{14}$C, $^3$H-labeled H12-(ADP)-liposomes was evaluated at 7 days after the injection of H12-(ADP)-liposomes at doses of 10, 20, 40 mg lipids/kg. The level of $^{14}$C and $^3$H radioactivity was nearly undetectable in the observed organs (kidney, liver, spleen, lung, and heart) (unpublished data). In addition, the radioactive $^{14}$C was excreted mainly in the urine (80.4% ± 4.9%, 52.1% ± 3.6%, and 58.4% ± 7.1% of ID at 7 days after the injection at doses of 10, 20, and 40 mg lipids/kg, respectively), but was low in feces (7.6% ± 2.7%, 6.5% ± 2.9%, and 2.5% ± 1.9% of ID at 7 days after the injection at doses of 10, 20, and 40 mg lipids/kg, respectively). On the other hand, the majority of the radioactive $^3$H was excreted in the feces (74.2% ± 5.7%, 98.9% ± 14.9%, and 70.6% ± 6.2% of ID at 7 days after the injection at doses of 10, 20, and 40 mg lipids/kg, respectively), and a small portion of the $^3$H radioactivity was...
excreted into the urine. These data indicate that more than 75% of H12-(ADP)-liposomes are eliminated within 7 days after injection and retention in the body can be limited to detect at a dose of up to 40 mg lipids/kg.

**Pharmacokinetics of the H12-(ADP)-Liposomes in Mice and Rabbits.** To calculate the pharmacokinetic parameters of the H12-(ADP)-liposomes in mice and rabbits, the 3H-labeled H12-(ADP)-liposomes were administered to mice and rabbits at a dose of 10 mg lipids/kg. According to the pharmacokinetic parameters calculated from the plasma concentration curve, the CL and Vdss of the 3H-labeled H12-(ADP)-liposomes in mice were 0.54 ± 0.12 ml/h and 3.81 ± 0.35 ml, respectively, whereas the values in the case of rabbits were 23.5 ± 2.8 ml/h and 827 ± 163 ml, respectively (Supplemental Table 1).

**Prediction of Pharmacokinetics of the H12-(ADP)-Liposomes in Humans.** To predict the pharmacokinetics in humans, we examined the allometric relationship between Vdss and body weight (Fig. 5A) and CL and body weight (Fig. 5B) in mice, rats, and rabbits using the results summarized in Table 1 and Supplemental Table 1. As shown in Fig. 5, a good correlation in both relationships was observed. Furthermore, we calculated the half-life, based on extrapolation, of the H12-(ADP)-liposomes that were administered at a dose of 10 mg lipids/kg in humans to be approximately 96 hours.

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>10 mg lipid/kg</th>
<th>20 mg lipid/kg</th>
<th>40 mg lipid/kg</th>
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<tr>
<td></td>
<td>3H</td>
<td>14C</td>
<td>3H</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>8.18±0.77</td>
<td>8.21±1.01</td>
<td>7.48±0.56</td>
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<tr>
<td>MRT (h)</td>
<td>10.2±1.18</td>
<td>10.4±1.46</td>
<td>9.20±0.51</td>
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<tr>
<td>AUC (h · % of dose/ml)</td>
<td>58.4±6.45</td>
<td>54.2±10.1</td>
<td>54.0±1.97</td>
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<tr>
<td>CL (ml/h)</td>
<td>1.73±0.18</td>
<td>1.89±0.32</td>
<td>1.85±0.07</td>
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<tr>
<td>Vdss (ml)</td>
<td>17.7±3.49</td>
<td>19.5±3.61</td>
<td>17.0±0.58</td>
</tr>
</tbody>
</table>

AUC, area under the concentration-time curve; MRT, mean residence time.
In the present study, the pharmacokinetic properties of H12-(ADP)-liposomes and structural components thereof, including the encapsulated ADP and membrane components (cholesterol), were characterized. The findings confirmed that the product has proper pharmacological functions and acceptable biodegradable properties (little retention). This leads to the conclusion that the H12-(ADP)-liposomes have the potential for use as a synthetic platelet substitute from the viewpoint of the pharmacokinetic properties in rodents.

We encapsulated ADP into H12 coated liposomes to strengthen the hemostatic ability of the H12 coated liposome as a platelet substitute, because this physiologically relevant platelet agonist is stored in dense granules and released upon cellular activation, and then functions to reinforce or maintain platelet aggregation through corresponding platelet nucleotide receptors P2Y1 and P2Y12. Thus, the stable encapsulation of ADP in liposomes permits them to function at sites of vascular injuries.

The findings herein clearly show that, for up to 24 hours after injection in rats, the plasma concentration curves for 14C-, radiolabeled H12-(ADP)-liposome exhibited similar behaviors (Fig. 1), indicating that the H12-(ADP)-liposomes circulate in the bloodstream without any leakage of ADP. In addition, we also realized that the nonliposomal ADP was immediately eliminated from blood (unpublished data), because ADP released into blood was metabolized by leukocytes, erythrocytes, and endothelial cells (Marcus et al., 2003; Heptinstall et al., 2005). This means that ADP encapsulated in the vesicle has advantages that are not only specific delivery ADP to injury site but also improvement of the blood retention of ADP. Previous in vivo hemostatic studies of H12-(ADP)-liposomes using a rat model with busulphan-induced thrombocytopenia (platelet counts; 1.9 ± 0.2 × 10^5 μl⁻¹) clearly showed that the tail vein bleeding times of thrombocytopenic rats after an infusion of H12-(ADP)-liposomes (10 mg lipids/kg) were significantly reduced compared with that of controls [H12-liposome (10 mg lipids/kg) and ADP-liposome (10 mg lipids/kg)] (Okamura et al., 2009). Furthermore, the specific accumulation of H12-(Iopamidol)-liposomes at the injury site at the rat tail vein and jugular vein were identified using an explore Locus CT system (Okamura et al., 2009, 2010a). These results indicate that the H12-(ADP)-liposomes circulate in the bloodstream in a stable form until reaching the site of a vascular injury, and successfully augments hemostatic effects.

Retention in the blood is also an important factor in the evaluation of the hemostatic effects of H12-(ADP)-liposomes, because if the systemic half-life of the H12-(ADP)-liposome is too short, it cannot effectively function as a platelet substitute. From the viewpoint of future clinical applications, an allometric prediction of human pharmacokinetics based on data obtained from animal studies—so-called “animal scale-up”—is important for the determination of optimal doses and intervals (Izumi et al., 1996). In fact, we successfully predicted the blood retention properties of hemoglobin vesicles (HbV), the liposomal characteristics of which have similar characteristics in terms of liposomal structure to H12-(ADP)-liposomes.
was accomplished using an allometric equation that is generally applied in animal scale-up studies to extrapolate the half-life of pharmaceuticals in humans. In the present study, we showed that the predicted half-life of H12-(ADP)-liposomes in humans would be approximately 96 hours (Fig. 5) using the above approach. The results obtained for a single-dose pharmacokinetic study of recombinant factor VIIa (rFVIIa), which is widely used as a hemostatic agent in clinical settings, showed that its half-life was 2–3 hours in patients with hemophilia (Lindley et al., 1994). These results indicate that H12-(ADP)-liposomes would be expected to adequately function as a hemostatic agent in the treatment of massive bleeding in humans.

Since H12-(ADP)-liposomes were developed as a synthetic platelet substitute, it is necessary to characterize the biodegradable properties of these particles, such as the determination of their metabolism and excretion pathways. Liposomes are generally captured and degraded by mononuclear phagocyte system (MPS) in the liver and spleen, such as by Kupffer cells and splenic macrophages (Kiwada et al., 1998). As expected, more than 10% of initial dose of the H12-(ADP)-liposomes were distributed to the liver and spleen (Fig. 2), which is in good agreement with a previous in vivo study using HbV (Sakai et al., 2001, 2004). In addition, an in vitro finding also reported that the specific uptake and degradation of HbV were observed only in macrophage cells but not in parenchymal and endothelial cells in the liver (Taguchi et al., 2009). Furthermore, linear pharmacokinetics were found for the H12-(ADP)-liposomes within the dose of 40 mg lipids/kg (Fig. 4). These results strongly suggest that the majority of the H12-(ADP)-liposomes are also scavenged and degraded by the MPS, such as by Kupffer cells or splenic macrophages, and that this process was not saturated at a dose of 40 mg lipids/kg. However, it was observed the different amount of 3H and 14C distribution in liver and spleen (Fig. 2). This was similar to our previous finding using HbV that inner hemoglobin was rapidly eliminated from organs to urine and outer lipid component (cholesterol) was delayed to eliminate from organs to feces (Taguchi et al., 2009). Therefore, the different elimination pathway would be related to the retention in liver and spleen. Further study will be needed in this point.

The findings herein also showed that most of the ADP in H12-(ADP)-liposomes was mainly metabolized to allantoin and excreted into the urine within 7 days after the injection of the 14C, 3H-labeled H12-(ADP)-liposomes (Fig. 3). It is well known that uric acid is the final metabolite of purines, such as adenosine 3',5'-phosphate, in mammals. On the other hand, the principal metabolite of exogenous cyclic nucleotides in the rat is allantoin, and not uric acid (Coulson, 1976). Furthermore, another study showed that, in rats, hepatic uricase

<table>
<thead>
<tr>
<th>Time Course</th>
<th>Allantoin</th>
<th>ADP</th>
<th>Uric Acid</th>
<th>Hypoxanthine</th>
<th>Xanthine</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 h</td>
<td>89.7 ± 12.2</td>
<td>1.3 ± 1.2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>1 day</td>
<td>78.7 ± 13.3</td>
<td>16.0 ± 15.8</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>3 day</td>
<td>71.8 ± 15.9</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>11.2 ± 10.5</td>
</tr>
<tr>
<td>5 day</td>
<td>75.0 ± 20.8</td>
<td>N.D.</td>
<td>3.9 ± 3.4</td>
<td>N.D.</td>
<td>6.7 ± 5.9</td>
</tr>
</tbody>
</table>

N.D., not determined.

Fig. 4. Dose-dependent plasma concentration curve of (A) 3H- and (B) 14C-radiolabeled H12-(ADP)-liposome after intravenous injection at doses of 10, 20, and 40 mg lipids/kg to rats. Each point represents the mean ± S.D. (n = 4). (C) Relationship between the dose of H12-(ADP)-liposome and the area under the blood concentration-time curve. The linear regression of logarithmic values was calculated using the least-squares method (y = 98.33x+124.98, r² = 1)

TABLE 2
Time course for the percentage of total detected 14C radioactivity of ADP and metabolites derived from 14C-ADP in urine after intravenous injection of 3H- and 14C-radiolabeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg to rats

Each value represents the mean ± S.D. (n = 4).
than 100% at higher doses, it was suggested that a part of encapsulated HbV, using HbV labeled with \(^{3}H\)cholesterol after an injection of

It is also possible that phospholipids in H12-(ADP)-liposome are also metabolized and excreted in the same manner as the other liposome components, as mentioned above.

From the standpoint of biodegradable properties, it is also important to realize the possibility that H12-(ADP)-liposomes and components might accumulate in tissues, because it is well known that cholesterol is a risk factor for several diseases, including arteriosclerosis and hyperlipidemia. The findings reported herein indicate that both H12-(ADP)-liposomes and components derived from them disappeared from the bloodstream and organs within the 7 days after the injection of the H12-(ADP)-liposomes, indicating that H12-(ADP)-liposomes and components derived from them possess low accumulative properties. Therefore, H12-(ADP)-liposomes contain the appropriate components and have the potential for use as a synthetic platelet substitute, because they possess acceptable biodegradable properties.

Based on the present findings, we provide the first demonstration to show that the disposition of H12-(ADP)-liposomes and components derived from them occurs as follows. After being systemically administered, the H12-(ADP)-liposomes are stable and circulate in an intact form in the circulation. As a result, some of the H12-(ADP)-liposomes would be specifically recruited at an injury site and would exert a pharmacological action, whereas the rest mainly are distributed to the liver and spleen, where they are degraded by the MPS. Finally, the encapsulated ADP and membrane components are eliminated mainly to the urine and feces, respectively, as final metabolites. In addition, our pharmacokinetic study, using different animal species, enabled us to predict that the half-life of H12-(ADP)-liposomes in humans is 96 hours. The above findings provide usable information for the development of the H12-(ADP)-liposomes for use as a platelet substitute.

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Authorship Contributions

Participated in research design: Taguchi, Otagiri, Maruyama.
Conducted experiments: Taguchi, Ujihira, Ogaki, Fujiyama, Doi.
Contributed new reagents or analytic tools: Ikeda, Handa.
Performed data analysis: Taguchi, Ujihira, Watanabe.
Wrote or contributed to the writing of the manuscript: Taguchi, Okamura, Takeoka, Handa, Otagiri, Maruyama.

References

Supplemental table 1

The pharmacokinetic parameters of 
H12-(ADP)-liposome after intravenous injection of 
3H radiolabeled H12-(ADP)-liposome at a dose of 10 mg lipids/kg to mice or after intravenous injection 3H radiolabeled H12-(ADP)-liposome at a dose of 10 mg lipid/kg to rabbits.

t\(_{1/2}\): half-life, MRT: mean residence time, AUC: area under the concentration-time curve, CL: clearance, V\(_{\text{dss}}\): distribution volume

<table>
<thead>
<tr>
<th></th>
<th>mice</th>
<th>rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(_{1/2}) (hr)</td>
<td>6.38 ± 0.81</td>
<td>28.6 ± 4.47</td>
</tr>
<tr>
<td>MRT (hr)</td>
<td>7.27 ± 1.15</td>
<td>37.1 ± 5.6</td>
</tr>
<tr>
<td>AUC (hr (*) % of dose/mL)</td>
<td>194 ± 47.1</td>
<td>4.53 ± 0.48</td>
</tr>
<tr>
<td>CL (mL/hr)</td>
<td>0.54 ± 0.12</td>
<td>22.2 ± 2.13</td>
</tr>
<tr>
<td>V(_{\text{dss}}) (mL)</td>
<td>3.81 ± 0.35</td>
<td>827 ± 163</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. (n=4).

*Drug Metabolism and Disposition*

Pharmacokinetic study of the structural components of adenosine diphosphate-encapsulated liposomes coated with fibrinogen \(\gamma\)-chain dodecapeptide as a synthetic platelet substitute

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