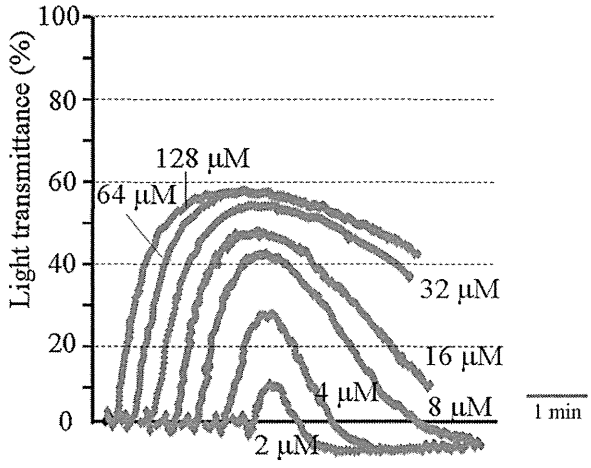

Fig. 3. ADP-induced platelet aggregation. Aggregation measurements using rat PRP (platelet = 2.0×10^5 cells/μL), obtained from F344 male rats, in the presence of several concentrations of ADP solution (final concentration, 2–128 μM). The light transmittance was measured with an aggregometer. The light transmittance of PPP was assumed to be 100%; and that of PRP before the addition of ADP, 0%.

them and thus inhibit platelet aggregation. Fig. 3 shows the concentration dependence of ADP to induce the aggregation of rat PRP, as assessed by using an aggregometer. The transient aggregation of rat PRP increased in an ADP concentration-dependent manner and reached its plateau at 64 μM ADP. Then we examined the inhibitory effect of human-H12 or rat-H12 on platelet aggregation. In this experiment, the concentration of ADP was set at 20 μM in consideration of the sensitivity of aggregation. Both human-H12 and rat-H12 inhibited the aggregation of rat PRP in a dose-dependent manner, and no significant difference in the inhibitory effect was observed between them (Fig. 4): the IC_{50} values of human-H12 and rat-H12 were 0.350 ± 0.148 μM and 0.377 ± 0.155 μM, respectively.

4. Discussion

H12 is a promising peptide for hemostasis or for a probe to deliver hemostatic agents to activated platelets. We previously developed ADP-encapsulated liposomes modified with human-H12 (H12-(ADP)Lipo; Okamura et al., 2005, 2009, 2010a,b). H12-(ADP)Lipo not only accelerates the aggregation of platelets by bridging them, but also further activates the platelets. In fact, H12-modified liposomal ADP, H12-(ADP)Lipo, actually enhances platelet aggregation in human PRP and significantly reduces the bleeding time in thrombocytopenic rat and rabbit models (Okamura et al., 2005, 2009, 2010a,b). Moreover, the binding ability of these H12-modified liposomes toward platelets steeply increases depending on H12 density on the liposomal surface. However, the experiments using human-H12, especially *in vivo* experiments, were done in rats or rabbits, the H12 peptides of which are different from the sequence of human-H12: HHMG-GSKQVGDM (rat-H12) and FHMGGAKQAGDV (rabbit-F12). Four and 2 amino acid residues are different from the human-H12 sequence, respectively (Gene Bank, XM_002716891.1; Peter and Verhallen, 1991). In light of these species differences, in this present study we examined whether such differences would affect the ability of these peptides (human-H12 and rat-H12) to bind to rat platelets.

Collagen and ADP are generally used for the activation of platelets, and are known to induce strongly the aggregation of platelets in PRP. However, it is unknown how much of the population of GPIIb/IIIa is in its active form to allow binding to the H12 peptide. In this study, we compared the affinity of human-H12 and rat-H12 for activated GPIIb/IIIa on rat platelets by use of flow cytometry; and, therefore, extremely strong activating agents

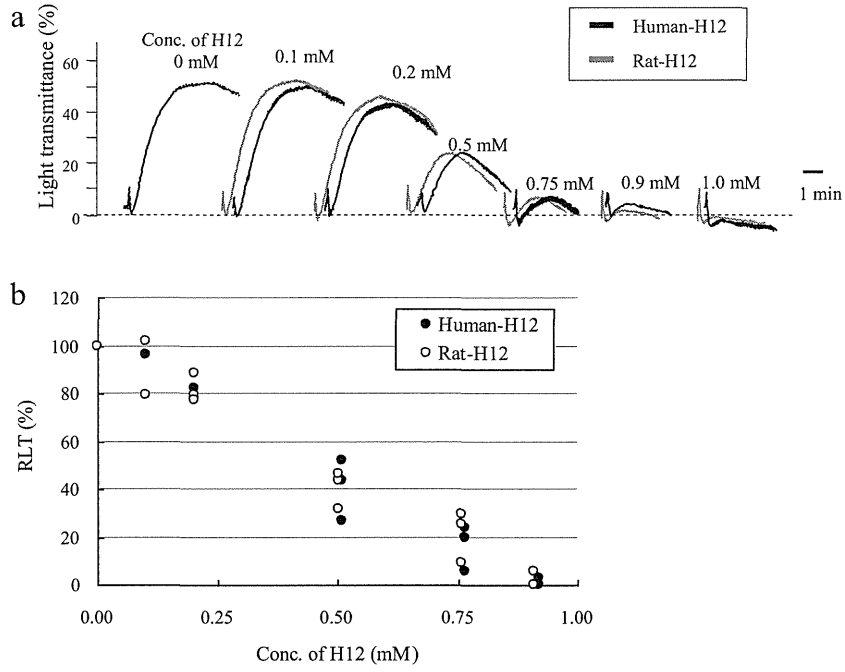

Fig. 4. Inhibitory effect of H12 peptide on the aggregation of rat PRP. (a) Aggregation of rat PRP (2×10^5 platelets/μL) obtained from F344 male rats in the presence of several concentrations of human-H12 (black line) or rat-H12 (gray line) was determined. ADP solution (20 μM as a final concentration) was added to the PRP containing each H12 (0.1–1.0 mM), and light transmittance was measured by using an aggregometer. The light transmittance of PPP was assumed to be 100%, and that of PRP before the addition of H12 and ADP was assumed to be 0%. (b) Effect of H12 on light transmittance change in rat PRP. Relative light transmission (RLT) percentages were obtained from the peak of the transmittance curve: The light transmittance without H12 was assumed to be 100%. The 50% inhibitory concentration of each H12 toward aggregation of rat PRP (IC_{50}) was determined by using the analytical software Graph Pad Prism 5J.

were firstly examined. As a result, PMA was revealed to be the most potent activator of GPIIb/IIIa on rat platelets among the agents tested.

Interestingly, U46619 and ADP did not show any significant activation of GPIIb/IIIa when the binding of FITC-Fbg was monitored. This result suggests that ADP activated only a small population of GPIIb/IIIa molecules, although this population was sufficient for inducing platelet aggregation. In fact, micromolar concentrations of ADP sufficed for platelet activation (Fig. 3).

Next, we used PMA as an activator of GPIIb/IIIa on rat platelets, and determined by flow cytometry the dissociation constant of human-H12 and rat-H12 for dissociation of the H12's from GPIIb/IIIa on rat platelets. The K_d values of human-H12 and rat-H12 were almost equal (Fig. 2). Furthermore, we evaluated the inhibitory effect of each H12 on the aggregation of rat PRP by using an aggregometer. As a result, human-H12 and rat-H12 showed almost equal inhibitory activity (Fig. 4). These results suggest that there was not a big difference in the ability of human-H12 and rat-H12 to bind to activated rat platelets.

Considering the peptide sequence of human-H12 and rat-H12, neither peptide has a proline residue which might have distorted the peptide structure in these sequences. On the other hand, each peptide has glycine residues, which break the secondary structure of the peptide, in the same positions. Therefore, it may be assumed that the molecular mobility of both peptides is almost the same. Moreover, the difference in the electric charge between the 2 peptides is only a little, because both peptides have the charged amino acid residues at the same positions. The hydrophobicity of these peptides was calculated based on the partition coefficient between water and octanol and the distribution energy to the lipoidal barrier (Wimley et al., 1996; Wimley and White, 1996). As a result, the ΔG (kcal/mol)/residue of membrane/octanol of human-H12 and rat-H12 were calculated to be 0.38/1.12 and 0.37/1.07, respectively. This result suggests that human-H12 and rat-H12 have similar characteristics, such as molecular mobility, electric charge, and hydrophilicity, even though 4 out of the 12 amino acid residues are different. These results may explain the similar ability of human-H12 and rat-H12 to bind to GPIIb/IIIa on rat platelets.

On the other hand, rabbit-H12 has 83% similarity to human-H12 at the amino acid sequence level. The calculated ΔG (kcal/mol)/residue of membrane/octanol of rabbit-H12, FHMGGAKQAGDV, was 0.25/0.95. Therefore, the hydrophilicity of rabbit-H12 may be considered to be almost the same as that of human-H12, as in the case of rat-H12. It is known that human-H12 binds to GPIIb at the positions of GPIIb from 294 to 314, TLGAVEILDSYYQRLHRLRGE (Poncz et al., 1987), on platelets. The sequences of GPIIb of rat and rabbit at the position corresponding to the human-H12 binding site are TLGAVEILDSYYQTLHRLHGE (Gene Bank, XM_0010815132) and TMGAVEILDSYFYRLHRLQGE (Gene Bank, AAD51954.1), respectively. We calculated the ΔG (kcal/mol)/residue of membrane/octanol of human-GPIIb, rat-GPIIb, and rabbit-GPIIb to be 0.25/0.71, 0.23/0.66, and 0.18/0.57, respectively. Therefore, in terms of hydrophobicity, the GPIIb of these 3 species is also very similar.

In conclusion, our data suggest that there was not much species difference, at least among the reactivity of human-H12 and rat-H12 to rat platelets. Also, the results of the rat experiments allow us to predict the practical use of H12 as a platelet-aggregation enhancer in humans, such as H12-(ADP)Lipo.

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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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Running title: Disposition of H12-(ADP)-liposomes

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Abbreviation: RGD, arginine-glycine-aspartic acid; **ADP**, adenosine diphosphate; **H12**, HHLGGAKQAGDV; **H12-(ADP)-liposome**, ADP-encapsulated liposomes modified with a dodecapeptide; **GP**, glycoprotein; **PEG**, polyethyleneglycol; **DPPC**, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine; **PEG-DSPE**, 2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-N-[monomethoxypoly(ethylenegly

col)]; **DHSG**, 1,5-Dihexadecyl-*N*-succinyl-L-glutamate; **HPLC**, high performance liquid chromatography; **ID**, injected dose; **HbV**, hemoglobin-vesicles; **MPS**, mononuclear phagocyte system.

Abstract

Fibrinogen γ-chain (dodecapeptide HHLGGAKQAGDV, H12)-coated, adenosine-diphosphate (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 GPIIb/IIIa 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 radiolabled with ^{14}C and ^{3}H, respectively, it was found that the time courses for the plasma concentration curves of ^{14}C and ^{3}H radioactivity showed that the H12-(ADP)-liposomes remained intact in the blood circulation for up to 24 h after injection, and were mainly distributed to the liver and spleen. However, the ^{14}C and ^{3}H radioactivity of H12-(ADP)-liposomes disappeared from organs within 7 day after injection. The encapsuated ADP was metabolized to allantoin, which is the final metabolite of ADP in rodents, and was mainly eliminated in the urine, while the cholesterol were mainly eliminated in feces. In addition, the half-life of the H12-(ADP)-liposomes in humans was predicted to be approximately 18 hrs from pharmacokinetic data obtained for mice, rats and rabbits using an allometric equation. These results suggest that H12-(ADP)-liposome has a potential with proper pharmacokinetic and acceptable biodegradable properties as 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 can introduce 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 USA 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 (Plateletsome) (Rybak and Renzulli, 1993), infusible platelet membranes (IPM) (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) peptidebound 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.

Adenosine diphosphate (ADP)-encapsulated liposomes modified with a dodecapeptide (HHLGGAKQAGDV, H12) (H12-(ADP)-liposome) was developed as a new type of synthetic platelet alternative. The glycoprotein (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 GPIIb/IIIa in an activation-dependent manner in the circulation. Among several GPIIb/IIIa recognized sequence sites in fibrinogen such as the RGD-based sequences (^{95}RGDF^{98} and ^{572}RGDS^{575} in the Aα chains) and H12 (^{400}HHLGGAKQAGDV^{411}) in the carboxy-terminus of the γ-chain (Kloczewiak et al., 1982; Kloczewiak et al., 1984; Hawiger et al., 1989), H12 is a specific binding site of the ligand for activated GPIIb/IIIa (Lam et al., 1987; Andrieux et al., 1989), whereas RGD related peptides are non-specific 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 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 specifically 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; Okamura et al., 2009; Okamura et al., 2010a; Okamura et al., 2010b; 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, pre-clinical studies and clinical trials. As described above, pre-clinical 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; Okamura et al., 2009; Okamura et al., 2010a; Okamura

et al., 2010b; Nishikawa et al., 2012). However, information concerning pharmacokinetic properties is lacking, especially the disposition and accumulation of each component in tissues after injection. Our strategy for the development of H12-(ADP)-liposome is based on the fact that, not only better pharmacological effects, but also acceptable biodegradable properties (no accumulation) need to be documented. In addition, pre-clinical pharmacokinetic studies in various mammalian species are essential, as 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 ^{14}C, ^{3}H double radiolabeled, in which the encapsulated ADP and membrane component (cholesterol) were labeled with ^{14}C and ^{3}H, 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-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-N-[monomethoxypoly(ethyleneglycol)] (PEG-DSPE, 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-HHLGGAKQAGDV, Cys-H12) was conjugated to the end of the PEG-lipids, were synthesized as previous reported (Okamura et al., 2005). Allantoin, uric acid, hypoxantine, xanthine and ADP were obtained from Sigma-Aldrich (St Louis, MO, USA).

Preparation of ^{14}C, ^{3}H double labled H12-(ADP)-liposomes

Firstly, ^{14}C 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-DSPE (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), which partly contains [8-^{14}C]ADP (Moravec Biochemicas, Inc., USA), and extruded through membrane filters (pore size, 0.22 μm; Durapore®; Millipore, Tokyo, Japan). Liposomes were washed with phosphate-buffered saline by centrifugation (100000 g, 30 min, 4°C), and the remaining ADP was eliminated by sephadexG25. The diameter and Zeta-potential of the ^{14}C labeled H12-(ADP)-liposomes used in this study are regulated

at 250 ± 50 nm and -10 ± 0.9 mV, respectively.

The ^{3}H labeling of ^{14}C labeled H12-(ADP)-liposomes, to prepare ^{14}C and ^{3}H double labeled H12-(ADP)-liposomes was carried out according to a previous report (Taguchi et al., 2009). The ^{14}C labeled H12-(ADP)-liposomes (1 mL) was mixed with [1,2-^{3}H(N)]-cholesterol solution (10 μL), (PerkinElmer, Yokohama, Japan) and incubated for 12 hrs at room temperature. ^{14}C, ^{3}H labeled H12-(ADP)-liposomes were filtered through a sterilile filter to remove aggregates (pore size, 450 nm). Before being used in pharmacokinetic experiments, all of the samples were mixed with unlabeled H12-(ADP)-liposomes. To employ the same procedure using H12-(ADP)-liposomes and [1,2-^{3}H(N)]-cholesterol, ^{3}H labeled H12-(ADP)-liposomes, which did not contain [8-^{14}C]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 (SD) rats (180-210 g body weight; Kyudou Co. Kumamoto, Japan) and male New Zealand White (NZW) rabbits (2.0-2.2 kg body weight; Biotek 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-hrs dark/light cycle.

Pharmacokinetic studies

Administration and collecting blood and organs in rats

Twenty-four SD rats were anesthetized with diethyl ether and received a single injection of ^{14}C, ^{3}H 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, blood samples were collected from all administration groups at multiple time points after the injection of the ^{14}C, ^{3}H labeled H12-(ADP)-liposomes (3, 10, 30 min, 1, 2, 3, 6, 12, 24, 48 and 168 hrs) and the plasma was separated by centrifugation (3000 g, 5 min). After collecting the last blood sample (168 hrs), the rats were sacrificed 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, 24 hrs after an injection of ^{14}C, ^{3}H 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 were received a single injection of ^{3}H labeled H12-(ADP)-liposomes (10 mg lipids/kg) in the tail vain under ether anesthesia. At each time after the injection of ^{3}H labeled H12-(ADP)-liposomes (3, 30 min, 1, 3, 6, 12, 24 hrs), four mice were anesthetized with ether and blood was collected from the inferior vena cava, and plasma was obtained by centrifugation (3000 g, 5 min).

Four NZW rabbits were received a single injection of ^{3}H 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, 30 min, 1, 2, 12, 24, 36, 48, 72 hrs), and plasma was obtained by centrifugation (3000 g, 5 min).

Measurement of ^{14}C and ^{3}H radioactivity

Plasma samples were solubilized in a mixture of Soluene-350 (Perkin Elmer, Yokohama, Japan) and isopropyl alcohol (at a ratio of 1/1) for 24 hrs at 50°C, and decolorized by treatment with a hydrogen peroxide solution. The organ samples were rinsed with saline, minced, and solubilized in Soluene-350 for 24 hrs at 50°C. Urine and feces were also weighed and solubilized in Soluene-350. The ^{14}C, ^{3}H radioactivity was determined by liquid scintillation counting (LSC-5121, Aloka, Tokyo, Japan) with Hionic Flour (Perkin Elmer, Yokohama, Japan).

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 of 10% sulphuric acid. Just before the analysis, the urine samples were centrifuged and filtered through a Dismic-25cs (ADVANTEC, Tokyo, Japan, 0.2 、μm pore size) and diluted ten-fold with water after adjusting the pH to 7 with 0.01N sodium hydroxide and 0.01N sulphuric acid. A standard solution containing ADP, allantoin, uric acid, hypoxantine and xanthine was prepared as reported in a previous study (George et al., 2006). The HPLC system consisted of a Waters 2695 pump (Waters, Massachusetts, USA), a Waters 2487 detector (Waters, Massachusetts, USA) operated at 220 nm. LC analyses were achieved with a 250 × 4 mm, 5 μm LiChrospher® 100 RP-18 endcapped column (LiChroCART® 250-4, Merck, Darmstadt, Germany). Furthermore, each ADP metabolite separated by HPLC was collected by a fraction collector (CHF121SA, ADVANTEC, Tokyo, Japan) and ^{14}C radioactivity was determined by liquid scintillation counting with Hionic Flour.

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) to obtain the coefficient (α) and exponent (β) values (Boxenbaum, 1984).

$$P = \alpha \cdot W^{\beta} \qquad (1)$$

To calculate pharmacokinetic parameters, such as distribution volume (V_{dss}) and clearance (CL) for humans using Eq. (1), the total blood volume of humans was assumed to be 7% of body weight (Sou et al., 2005). After predicting of V_{dss} and CL for humans using Eq. (1), the half-life for human was estimated.

Data Analysis

Pharmacokinetic analyses proceeded based on a two-compartment model, and were calculated by fitting using MULTI. Data are shown as means ± SD for the indicated number of animals.

Results

Pharmacokinetics of H12-(ADP)-liposome components in rats

In order 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 (Figure 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 structure of the H12-(ADP)-liposomes remained intact in the blood circulation for periods of up to 24 hrs 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 hrs 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 (data not shown). These data indicate that the H12-(ADP)-liposomes are mainly distributed to the liver and spleen, but the accumulation in these organs is negligible.

In order 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. 3A 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 Figure 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 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 each structural component of the H12-(ADP)-liposome is nearly completely 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-dependency 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 (Figure 4A and B). In fact, a linear relationship between the administration dose and the area under the concentration-time curve (AUC) was found, the values for which were calculated based on the lipids

concentration (Figure 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 a dose 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) (data not shown). In addition, the radioactive ^{14}C was excreted mainly in the urine (80.4±4.9 %, 52.1±3.6 %, 58.4±7.1 % of ID at 7 days after the injection at a dose of 10, 20, 40 mg lipids/kg, respectively), but was low in feces (7.6±2.7 %, 6.5±2.9 %, 2.5±1.9 % of ID at 7 days after the injection at a dose of 10, 20, 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 %, 70.6±6.2 % of ID at 7 days after the injection at a dose of 10, 20, 40 mg lipids/kg, respectively), and small portion of the ^{3}H radioactivity was excreted into the urine. These data indicate that H12-(ADP)-liposomes are nearly completely eliminated within 7 days after injection and little accumulation in the body can be detected 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 ^{3}H 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 V_{dss} of the ^{3}H labeled H12-(ADP)-liposomes in mice were 0.54±0.12 mL/hr and 3.81±0.35 mL, respectively, while the values in the case of rabbits were 23.5±2.8 mL/hr and 827±163 mL,

respectively (Supplemental Table 1).

Prediction of pharmacokinetics of the H12-(ADP)-liposomes in human.

To predict the pharmacokinetics in humans, we examined the allometric relationship between V_{dss} and body weight (Fig. 5A) and CL and body weight (Fig. 5B) in mice, rats, rabbits using the results summarized in Table 1 and supplemental Table 1. As shown in Figure 5A and B, 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 18 hrs.

Discussion

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 accumulation). 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, 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 h after injection in rats, the plasma concentration curves for ^{14}C, ^{3}H radiolabeled H12-(ADP)-liposome exhibited similar behaviors (Fig.1), indicating that the H12-(ADP)-liposomes circulate in the bloodstream without any leakage of ADP. Previous in vivo hemostatic studies of H12-(ADP)-liposomes using a rat model with busulphan-induced thrombocytopenia (platelet counts; $1.9 \pm 0.2 \times 10^5$ 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 to 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