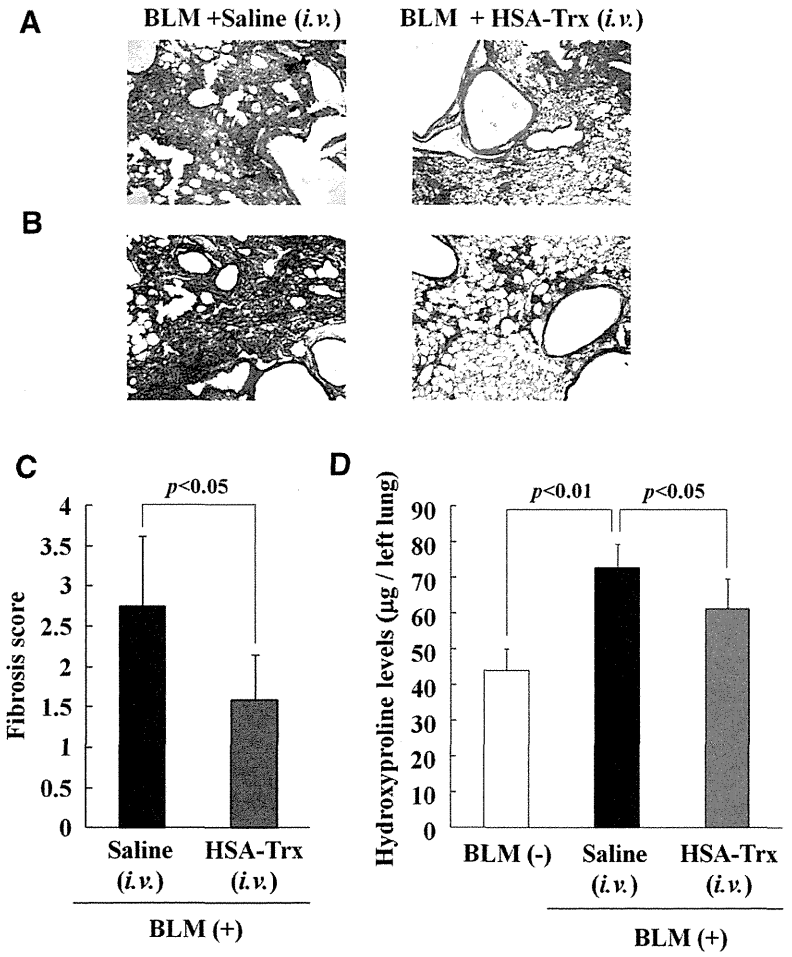

Fig. 9. Effects of postadministration of HSA-Trx (every 1 week) against BLM-induced pulmonary fibrosis 1 day after BLM treatment. (A and B) Sections of pulmonary tissue were prepared 14 days after BLM administration and subjected to histopathological examination ([A] H&E and [B] Masson's trichrome staining). (C) Fibrosis score was evaluated as the quantity of the section positively stained for collagen and displaying alveolar wall thickening. (D) Hydroxyproline levels in lung were determined 14 days after BLM administration. Each bar represents the mean ± S.D. (C, $n = 4$; D, $n = 5$).

Because weekly administration of HSA-Trx produced a better suppressing effect on pulmonary fibrosis than the fortnightly administration of HSA-Trx (Fig. 2), the ROS-scavenging action of HSA-Trx administered after the 7 days of BLM treatment may inhibit this vicious circle.

In addition to its antioxidative action, Trx has anti-inflammatory characteristics, including its ability to suppress the migration and/or infiltration and extravascular leakage of inflammatory cells (Nakamura et al., 2001). Therefore, it is highly possible that the anti-inflammatory actions of Trx are involved in the therapeutic effects of HSA-Trx against BLM-induced lung injury. In fact, HSA-Trx suppressed the pulmonary infiltration of inflammatory cells at days 1 and 3 of the administration of BLM (Fig. 5). The anti-inflammatory effects of Trx can be attributed mainly to suppression of the production of cytokines and chemokines. We also found that HSA-Trx significantly decreased the lung concentrations of these cytokines in mice with BLM-induced lung disorders (Fig. 6, A and B). Although the mechanism of suppression by Trx on the production of these cytokines remains somewhat unclear, an interesting observation was recently reported that Trx exhibited its anti-inflammatory effect by suppressing the expression and activity of MIF (Tamaki et al., 2006; Son et al., 2009). MIF promotes the production of TNF-α, IL-6, and other cytokines, as well as ROS, such as nitric oxide and superoxide anions (Baugh and Bucala, 2002). MIF is expressed at increased levels in a broad range of oxidative stress situations and inflammation-related pathologic conditions, such as sepsis, rheumatoid arthritis, inflammatory bowel disease, and lung disorders, including BLM-induced pulmonary fibrosis (Tanino et al., 2002). Furthermore, the administration of a MIF-neutralizing antibody has been shown to improve survival rates by significantly suppressing the pulmonary infiltration of inflammatory cells and the development of lung disorders (Tanino et al., 2002). It is noteworthy that HSA-Trx was also able to significantly suppress MIF expression induced by BLM administration (Fig. 6, C and D). Taking all these findings into consideration, the inactivation of MIF by HSA-Trx may also involve the anti-inflammatory effects of HSA-Trx observed in this study.

The findings of this study indicate that the progression of lung injury in a BLM-induced lung disorder mice model could be suppressed by the pre- or postadministration of HSA-Trx, demonstrating its potential as a novel therapeutic agent for the treatment of IPF, acute interstitial pneumonia, or drug-induced lung diseases. HSA-Trx may function as a long acting antioxidative and anti-inflammatory modulator. In recent years, acute exacerbation of IPF due to conditions, such as a cold or influenza, along with its progression, have contributed to the mortality among patients with IPF, and preventing acute exacerbation has been a key strategy for the treatment of the disease. Because HSA-Trx suppressed lung disorders, even when administered after BLM treatment, it is speculated that HSA-Trx may be effective in the prevention and treatment of the acute exacerbation associated with IPF.

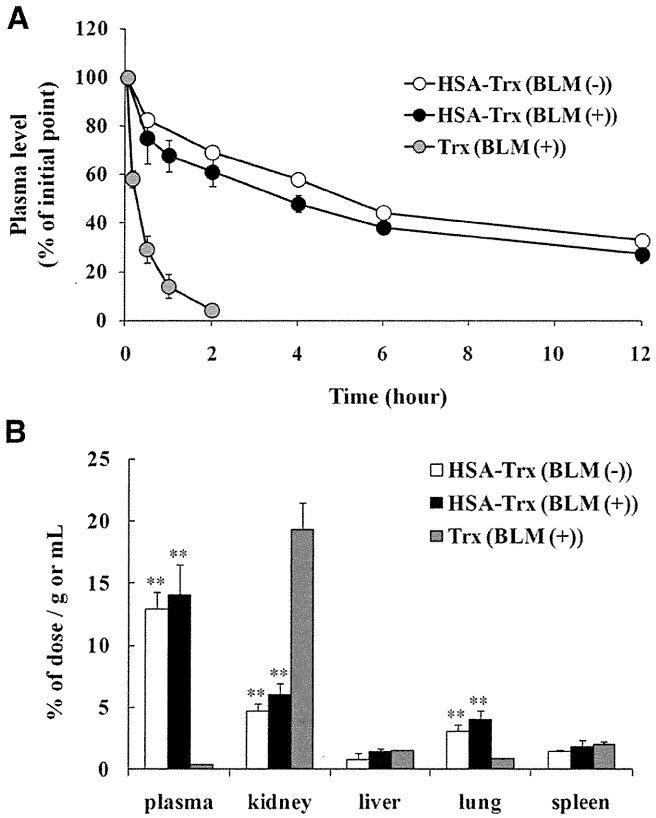

Fig. 10. Pharmacokinetic profiles of HSA-Trx and Trx and in BLM mice. (A) Plasma levels of ^{125}I-labeled HSA-Trx and Trx after intravenous administration into the tail vein of BLM and normal mice. Each point represents mean ± S.D. (n = 3). (B) Tissue distribution profile of HSA-Trx and Trx in kidney, liver, lung, and spleen at 2 hours after intravenous administration into the tail vein of BLM and normal mice. Each bar represents the mean ± S.D. (n = 3). $^{**}P < 0.01$ versus Trx [BLM(+)].

Although HSA-Trx exhibited similar blood retention levels as HSA in the BLM-induced lung disorders animal model, it is speculated to have a therapeutic effect in patients with IPF with a possibility of fortnightly or monthly administrations, considering the fact that the half-life of HSA in humans is approximately 20 days (Peters, 1985). Although further investigations related to establishing the treatment regimen need to be performed, a reduced administration frequency and stable therapeutic effect of HSA-Trx would make the treatment more convenient, safe, and cost-effective and improve the health-related quality of life for patients with IPF. The BLM animal model is widely used in the assessment of potential antifibrotic agents. However, the aspect of slow and irreversible progression of IPF in patients is not reproduced in the BLM model. It would be interesting to see whether this therapeutic could reverse or slow progression of fibrosis in the clinical setting as opposed to preventing the response evoked by BLM.

Authorship Contributions

Participated in research design: R. Tanaka, Watanabe, Kodama, Chuang, Ishima, Hamasaki, K. Tanaka, Mizushima, Otagiri, Maruyama.

Conducted experiments: R. Tanaka, Watanabe, Kodama, Ishima.

Contributed new reagents or analytic tools: R. Tanaka, Watanabe, Kodama.

Performed data analysis: R. Tanaka, Watanabe, Kodama.

Wrote or contributed to writing of the manuscript: R. Tanaka, Watanabe, Chuang, Otagiri, Maruyama.

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Address correspondence to: Dr. Toru Maruyama, Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: tomaru@gpo.kumamoto-u.ac.jp

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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[S]

Kazuaki Taguchi, Hayato Ujihira, Shigeru Ogaki, Hiroshi Watanabe, Atsushi Fujiyama, Mami Doi, Yosuke Okamura, Shinji Takeoka, Yasuo Ikeda, Makoto Handa, Masaki Otagiri, and Toru Maruyama

Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences (K.T., H.U., S.O., H.W., M.O., T.M.), and Center for Clinical Pharmaceutical Sciences (H.W., T.M.), Kumamoto University, Kumamoto, Japan; Department of Life Science and Medical Bioscience, Graduate School of Advanced Science and Engineering, Waseda University, Tokyo, Japan (A.F., M.D., S.T., Y.I); Institute of Innovative Science and Technology, Tokai University, Tokyo, Japan (Y.O.); Department of Transfusion Medicine & Cell Therapy, Keio University, Tokyo, Japan (M.H.); and Faculty of Pharmaceutical Sciences and DDS Research Institute, Sojo University, Kumamoto, Japan (M.O.)

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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 IIb/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 radiolabeled 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 hours 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 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 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 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 (plateletsome) (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

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ABBREVIATIONS: 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, polyethyleneglycol; PEG-DSPE, 2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[monomethoxypoly(ethyleneglycol)]; RGD, arginine-glycine-aspartic acid; $t_{1/2}$, half-life; V_{dss}, 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 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, 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 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 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, 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 ^{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, hypoxanthine, xanthine, and ADP were obtained from Sigma-Aldrich (St. Louis, MO).

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

First, ^{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) and [8-^{14}C]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 sephadexG25. The diameter and ζ 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 5–10% of added ADP was encapsulated in the inner space of the vesicle.

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) were mixed with [1,2-^{3}H(*N*)]cholesterol solution (10 μl), (PerkinElmer, Yokohama, Japan) and incubated for 12 hours at room temperature. ^{14}C, ^{3}H-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-^{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 rats (180–210 g body weight; Kyudou Co., Kumamoto, Japan), and male New Zealand White 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-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 ^{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, approximately 200 μl blood samples in all administration groups were collected from tail vein at multiple time points after the injection of the ^{14}C, ^{3}H-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 (3000*g*, 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 ^{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 received a single injection of ^{3}H-labeled H12-(ADP)-liposomes (10 mg lipids/kg) in the tail vein under ether anesthesia. At each time after the injection of ^{3}H-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 (3000*g*, 5 minutes).

Four New Zealand White rabbits 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, and 30 minutes, and

1, 2, 12, 24, 36, 48, and 72 hours), and plasma was obtained by centrifugation (3000*g*, 5 minutes).

Measurement of ^{14}C and ^{3}H Radioactivity. Plasma samples were solubilized in a mixture of Soluene-350 (PerkinElmer) and isopropyl 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 isopropyl 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 Dismic-25cs (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).

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

P is the parameter of interest [distribution volume (V_{dss}) 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_{dss} and CL for human. After predicting of V_{dss} 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 · % of dose/ml), CL (ml/h), and V_{dss} (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 structure of 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

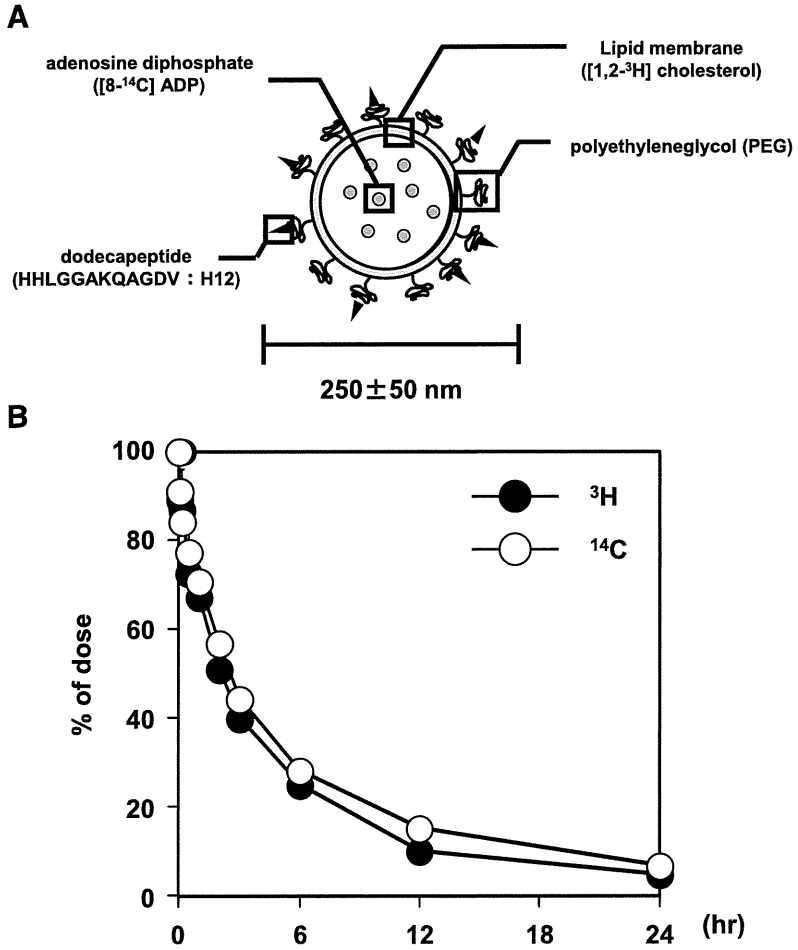

Fig. 1. (A) Structure and regiospecifically ^{3}H- and ^{14}C-radiolabeled H12-(ADP)-liposome. (B) Time course for the plasma concentration of ^{3}H and ^{14}C radiolabeled H12-(ADP)-liposome after intravenous injection at a dose of 10 mg lipids/kg to rats. Each point represents the mean ± S.D. (n = 4).

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 ^{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/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 V_{dss} 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

The pharmacokinetic parameters of inner ADP ([8-^{14}C]ADP) and outer lipid membranes ([1,2-$^{3}H(N)$]cholesterol) derived from ^{3}H- and ^{14}C-radiolabeled H12-(ADP)-liposomes after an intravenous injection at doses of 10, 20, and 40 mg lipids/kg to rats

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

Parameter	10 mg lipid/kg		20 mg lipid/kg		40 mg lipid/kg	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
$t_{1/2}$ (h)	8.18 ± 0.77	8.21 ± 1.01	7.48 ± 0.56	7.63 ± 0.82	6.34 ± 0.53	7.94 ± 1.10
MRT (h)	10.2 ± 1.18	10.4 ± 1.46	9.20 ± 0.51	10.1 ± 1.00	7.65 ± 0.27	10.7 ± 0.97
AUC (h · % of dose/ml)	58.4 ± 6.45	54.2 ± 10.1	54.0 ± 1.97	67.8 ± 3.86	56.0 ± 1.94	84.4 ± 5.68
CL (ml/h)	1.73 ± 0.18	1.89 ± 0.32	1.85 ± 0.07	1.48 ± 0.08	1.79 ± 0.06	1.19 ± 0.07
V_{dss} (ml)	17.7 ± 3.49	19.5 ± 3.61	17.0 ± 0.58	14.8 ± 0.64	13.7 ± 0.58	12.6 ± 0.98

AUC, area under the concentration-time curve; MRT, mean residence time.

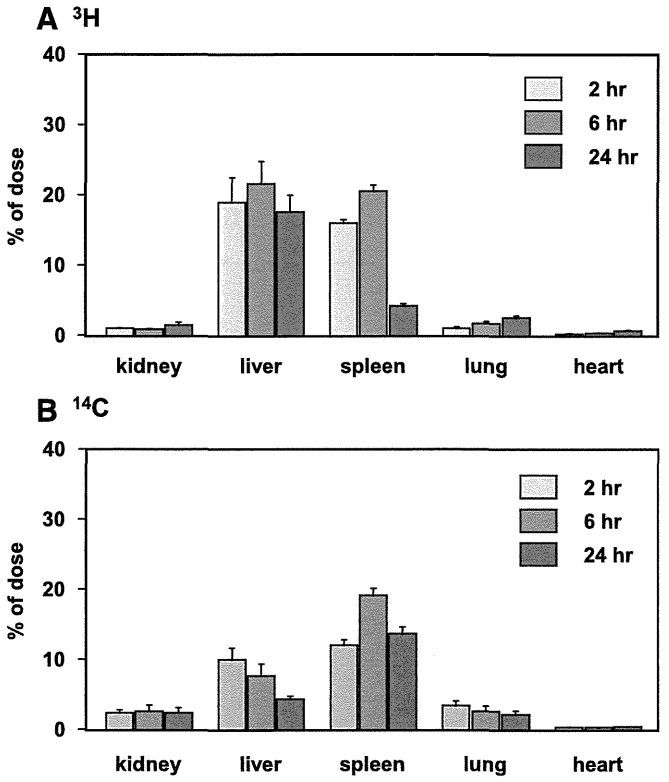

Fig. 2. The tissue distribution of ^{3}H (A) and ^{14}C radioactivity (B) at 2, 6, and 24 hours after an intravenous injection of ^{3}H- and ^{14}C-radiolabeled H12-(ADP)-liposome at a dose of 10 mg lipids/kg to rats. Each point represents the mean ± S.D. (n = 4).

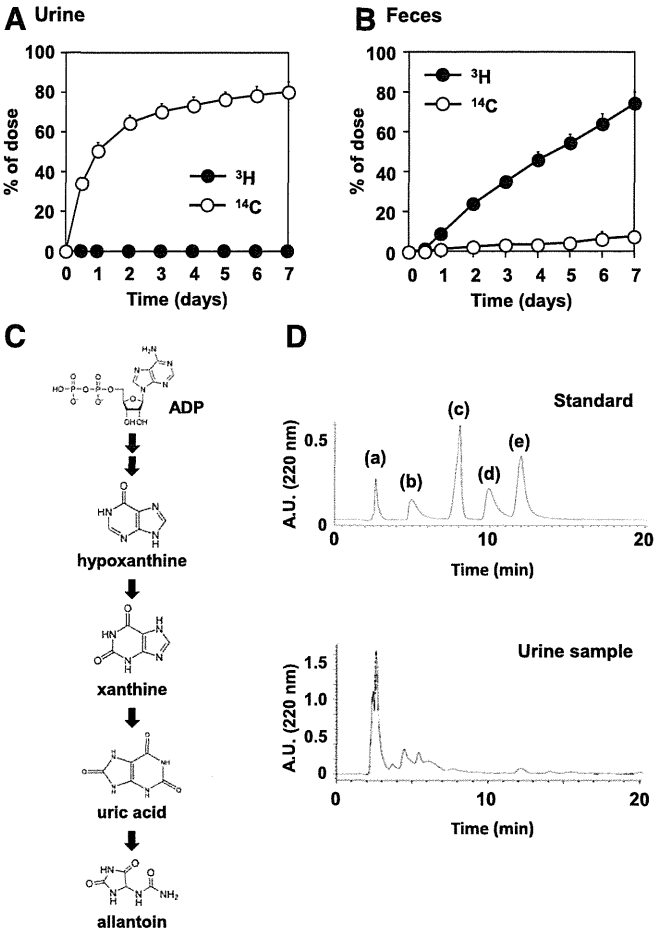

Fig. 3. Time course for radioactivity in urine (A) and feces (B) after the administration of ^{3}H- and ^{14}C-radiolabeled H12-(ADP)-liposome to rats. Each point represents the mean ± S.D. (n = 4). (C) Scheme of metabolism pathway from ADP to allantoin in rodents. (D) Chromatogram of standard mixture and urine sample analyzed by HPLC. The standard peaks are (a) allantoin, (b) ADP, (c) uric acid, (d) hypoxanthine, and (e) xanthine. The urine sample was collected 6 hours after intravenous injection of ^{3}H- and ^{14}C-radiolabeled H12-(ADP)-liposome at a dose of 10 mg lipids/kg to rats.

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 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 ^{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. 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 hematostatic effects.

Retention in the blood is also an important factor in the evaluation of the hematostatic 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. This

TABLE 2

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

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

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

N.D., not determined.

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 ^{3}H and ^{14}C 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 ^{14}C, ^{3}H-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

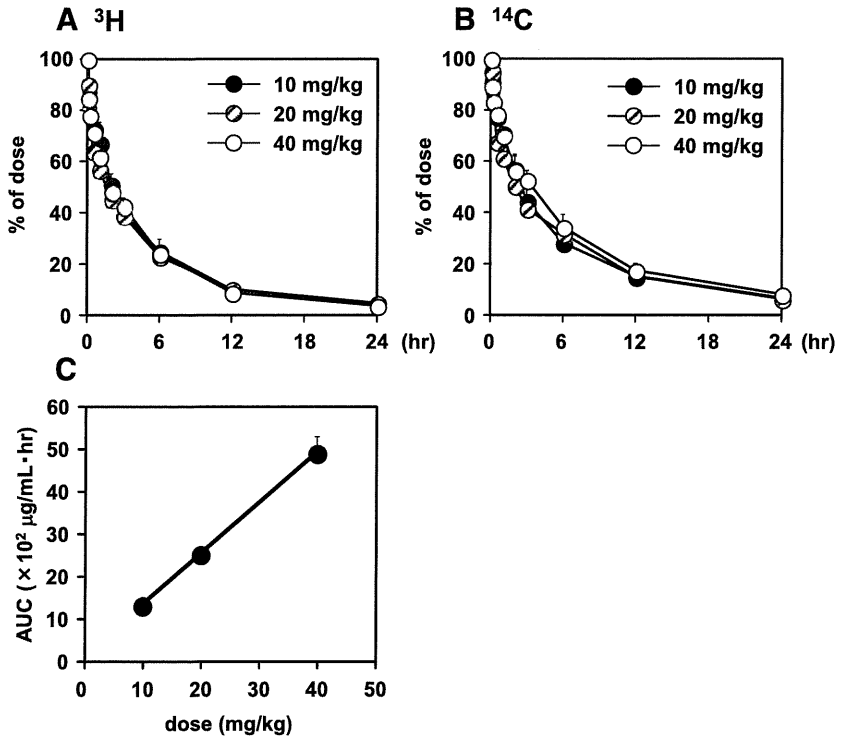

Fig. 4. Dose-dependent plasma concentration curve of (A) ^{3}H- and (B) ^{14}C-radiolabeled H12-(ADP)-liposome after intravenous injection at doses of 10, 20, and 40 mg lipids/kg to rats. Each point represents the mean ± S.D. (n = 4). (C) Relationship between the dose of H12-(ADP)-liposome and the area under the blood concentration-time curve. The linear regression of logarithmic values was calculated using the least-squares method (y = 98.33x+124.98, r^2 = 1)

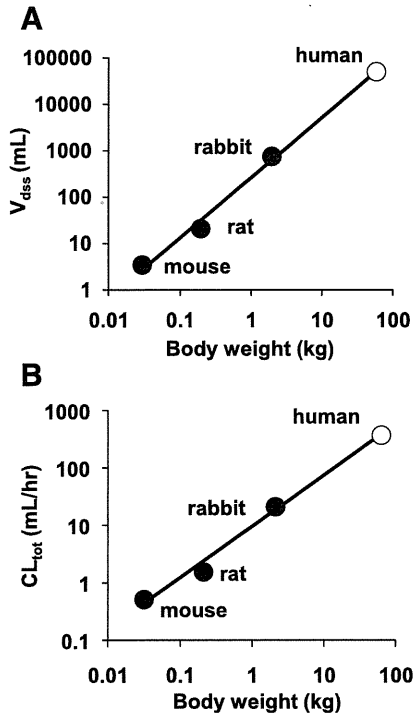

Fig. 5. Allometric relationships between body weight and distribution volume (V_{dss}) (A) and body weight and clearance (CL) (B). The linear regression of the logarithmic values was calculated using the least-squares method (A, $y = 257.71x^{1.2947}$, $r^2 = 0.965$; B, $y = 10.246x^{0.8928}$, $r^2 = 0.97$). The extrapolated human values based on a body weight of 70 kg (open circle) and the values from individual animals (gray circle) are also shown.

converts most of uric acid into allantoin, a form that allows it to be excreted in the urine more readily (Friedman and Byers, 1947). Taken together, these findings indicate that the ADP encapsulated by H12-(ADP)-liposome was completely metabolized and excreted into the urine even though ADP was encapsulated within liposome. However, ^{14}C radioactivity was not completely recovered until 7 days after ^{14}C-labeled H12-(ADP)-liposome administration. Although we could not explain the reason why the recovery of ^{14}C radioactivity was less than 100% at higher doses, it was suggested that a part of encapsulated ADP was used in the body as endogenous ADP.

The [^{3}H]cholesterol in H12-(ADP)-liposomes was mainly excreted into feces within 7 days after the injection of ^{3}H-labeled H12-(ADP)-liposomes. This result is in good agreement with the disposition of HbV, using HbV labeled with [^{3}H]cholesterol after an injection of HbV, which revealed that the majority of outer lipids component (cholesterol) was excreted via feces within 7 days (Taguchi et al., 2009). Kuipers et al. (1986) previously reported that cholesterol in vesicles reappear in the blood mainly as lipoprotein-cholesterol complexes after entrapment in Kupffer cells and should then be excreted in the bile after entrapment of the lipoprotein-cholesterol complex by hepatocytes. Therefore, a knowledge of whether the behavior of cholesterol as the lipid components of H12-(ADP)-liposome is the same as that of endogenous cholesterol after the metabolization of H12-(ADP)-liposome in the MPS would be highly desirable. On the other hand, we did not directly examine the disposition of the DPPC, DHSG, PEG-DSPE, and H12-PEG-Glu2C18 in H12-(ADP)-liposomes. Previous reports have shown that the phospholipids in liposomes are metabolized in the MPS and reused as cell membranes or are excreted into the bile (Dijkstra et al., 1985; Verkade et al., 1991). Therefore, 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 administrated, 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.

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Address correspondence to: Dr. Toru Maruyama, Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: tomaru@gpo.kumamoto-u.ac.jp

シンポジウム

3. 診療ガイドラインの社会的意義と問題点
3) ガイドライン作成の社会的意義と評価および法的側面：信頼されるガイドラインへ

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Key words：診療ガイドライン，コミュニケーション，評価，医療訴訟，医療の質

1. 社会的意義

診療ガイドラインは，臨床での意思決定を支援するために系統的な方法で作成されたものである．ガイドラインの機能として，コミュニケーションの基盤としての役割が大きいと考えられる（図1）．臨床医と患者を結ぶものであるが，同僚や他科専門医，あるいは研究者や医師以外の医療従事者を結ぶものでもある．また診療現場を離れて公衆衛生，医療政策とも関わってくる．さらに広く社会の中で医療の実情を伝える役割を担い，医療訴訟でガイドラインが引用されるのはその例と言える．

ガイドラインの有用性を検討した多くの研究で，診療プロセスや患者アウトカムの改善が認められている．Grimshawらの系統的レビューによれば，診療プロセスに関連した59件の報告中55件で改善が報告され，アウトカムに関連した11件の研究中9件で改善が報告されていたという[1]．国内でも同様の検討がなされている．ガイドライン公表後の診療プロセスの改善，ガイドラインに関する知識と望ましい診療プロセスとの関連，ガイドラインに一致した診療と患者アウトカムとの関連などが検討されてきた．検索した範囲で，国内から診療プロセスに関して5件の報告がありそのうち4件で改善，アウトカムに関しても他に5件の報告があり4件で改善することが記載されていた．

診療ガイドラインが診療現場で，どの程度定着しているか，また医師特性により利用状況に違いがあるかを検討するために，インターネットを利用した断面的調査を行った．調査対象は調査会社にモニター登録された臨床医1,342名であり，ガイドライン利用状況やガイドラインに対する態度について質問をした．「診療に関して

国立国際医療研究センター臨床研究センター医療情報解析研究部

110th Scientific Meeting of the Japanese Society of Internal Medicine：Symposium：3. Social Impacts and Controversial Points of Clinical Practice Guidelines；3) Impact, evaluation and legal issues of clinical practice guidelines.

Takuro Shimbo：Department of Clinical Study and Informatics, Center for Clinical Sciences, National Center for Global Health and Medicine, Japan.

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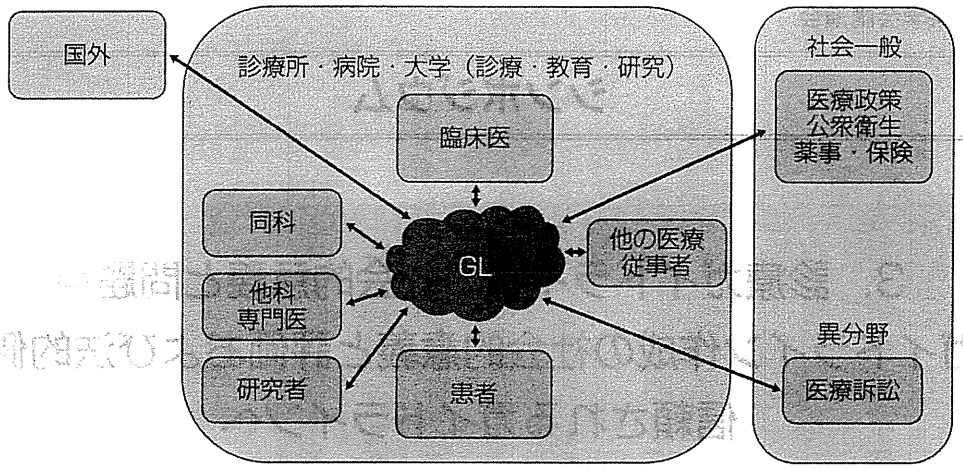

図 1. コミュニケーションの基盤としてのガイドライン
*GL（ガイドライン）

疑問があるとき，ガイドラインを検索しますか？」などの診療，教育，研究での利用状況に関する6項目の質問では，「いつもする，しばしばする」など利用度の高い回答は28～55% と高かった．普段利用しているガイドラインの件数は，1～4件が61%，5～9件が28% であった．

「患者さんに説明するとき，ガイドラインの内容を意識しますか？」という質問項目に対して，利用度の高い回答の多変量調整後の平均的な予測率を計算した（図2）．これは諸要因を調整したあとの結果を示し，他の要因が同一の場合でのその要因の影響を示す．年齢による影響が大きく，40歳未満と60歳以上では25% 程度の差が認められた．また診療科や勤務する医療機関による違いも認められた．他の質問項目でも同様の結果が示された．

従来からガイドラインの普及を妨げる障壁として，信頼感の欠如が報告されている[2]．信頼感に対する態度と使用状況の関連をみると，信頼感の高い群ではそうでない群に比べて利用度の高い回答が約25% 高かった．ガイドライン作成において信頼されるガイドラインを目指すことが重要と考えられた．

2. 評　　価

信頼されるガイドラインであるためには，評価を活かした作成や改訂が必要である．ガイドラインの診療プロセスやアウトカムに及ぼす影響を評価することも重要だが，妥当性のある評価ツールを用いて作成方法や記載内容を確認することも有用である．このような評価ツールにAGREE II[3,4]などがあり，一例として日本癌治療学会での活動について紹介したい．

日本癌治療学会では「がん診療ガイドライン」を専門分科会の協力のもとで収集し公開する活動を行っている[5]．そして癌診療ガイドライン委員会，ガイドライン評価委員会を設置し，ガイドラインの評価を実施してきた．2005年以降38件のガイドラインを評価している．

AGREE IIでは6つの領域，1）対象と目的，2）利害関係者の参加，3）作成の厳密さ，4）明確さと提示のしかた，5）適用可能性，6）編集の独立性，を23項目の質問によって評価しスコア化する．2005年～2007年当初は各領域のスコアは低値であったが，2007年以降各領域のスコアは改善してきた[6]．しかし6領域の中でも6）編集の独立性はスコアが低値であった．23項目の質問の個別スコアを見てみると，患者の視点へ

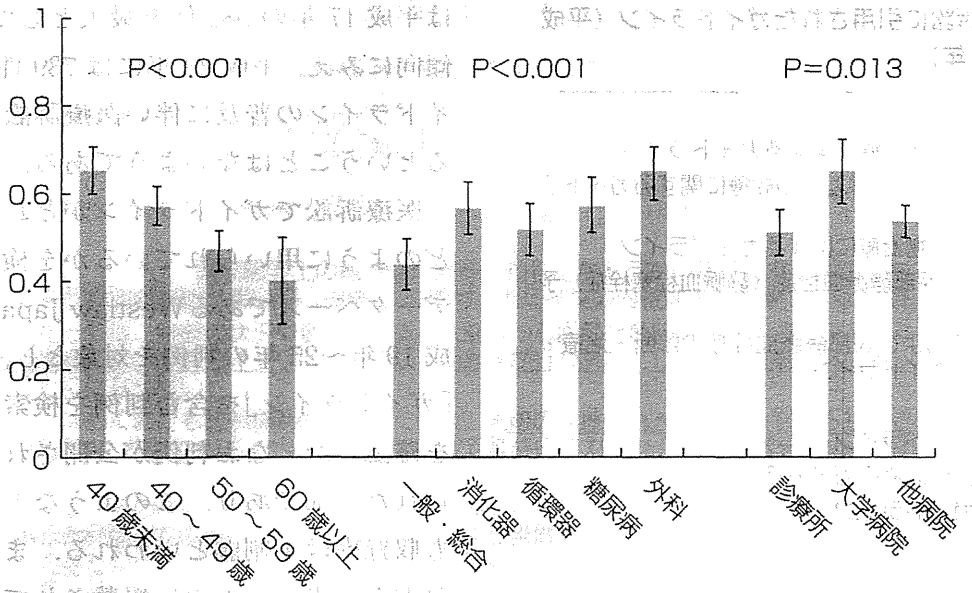

図2. 医師特性によるガイドライン利用状況

＊「患者さんに説明するとき，ガイドラインの内容を意識しますか？」という質問に対して，「いつもする，しばしばする」の回答割合．年齢，性別，診療科，医療機関，週の診療日数を調整したうえで，それぞれの水準での平均予測確率をロジスティック回帰で計算．そのため，他の要因が同じ場合の各水準での割合を示している．

＊調査対象は調査会社にモニター登録された臨床医1,342名．平均年齢（SD）は46.5（9.6）歳であり，男性1,222（91%），女性120（9%）．内科系の診療科として，一般・総合内科301名（22.4%），消化器内科261名（19.5%），循環器内科262名（19.5%），糖尿病・内分泌・代謝内科257名（19.2%），また外科系のうち高いガイドライン利用が予想された一般・消化器・乳腺外科261名（19.5%）を調査対象とした．勤務する医療機関は，医院・診療所が398名（29.7%），大学病院が187名（13.9%），大学病院以外のその他病院757名（56.4%）であった．

＊エラーバーは95%信頼区間．

の配慮，費用に関する記載，conflicts of interest（COI）に対する記載，が特に不十分であった．2009年以降の16件のガイドラインにつき，COIの記載があるガイドラインとそうでないガイドラインで6領域のスコアを比較すると，COIの記載のないガイドラインでは押し並べて各領域のスコアが低値であった．

米国のInstitute of Medicineは，診療ガイドライン作成におけるCOIに関する推奨を発表している[7]．基本的にはCOIのない個人の参加を求めているが，COIのある個人が参加する場合の方法として例えば，COIのある人を委員長にはしない，COIのある人が多数派にはならないようにする，COIのある人が推奨決定時の討論，投票，原稿執筆などには加わらないようにする，などの管理方法が記載されている．

3. 法的側面

従来からガイドラインの普及に伴い，医療訴訟が増えるのではないか，ガイドラインと異なる診療をすると不法行為や債務不履行とみなされるのではないか，との懸念があった．既に1995年にはこのような問題に関する報告もあり，医療訴訟においてガイドラインが原告患者側に用いられる場合と，被告病院側に活用される場合

表1. 医療訴訟に引用されたガイドライン（平成19年～25年）

循環器疾患
循環器病の診断と治療に関するガイドライン
急性心筋梗塞（ST上昇型）の診療に関するガイドライン
弁膜疾患の非薬物治療に関するガイドライン
肺血栓塞栓症/深部静脈血栓症（静脈血栓塞栓症）予防ガイドライン
肺血栓塞栓症および深部静脈血栓症の診断・治療・予防に関するガイドライン
脳卒中
脳卒中治療ガイドライン
脳血管障害画像診断のガイドライン
脳ドックのガイドライン
がん
肺癌診療ガイドライン
ゲフィチニブ使用に関するガイドライン
胃癌治療ガイドライン
大腸癌治療ガイドライン
肝癌診療ガイドライン
膵癌診療ガイドライン
造血幹細胞移植の適応ガイドライン
同種末梢血幹細胞移植のための健常人ドナーからの末梢血幹細胞の動員・採取に関するガイドライン
卵巣がん治療ガイドライン
乳癌診療ガイドライン4. 検診・診断
乳房超音波診断ガイドライン
プライマリ・ケア，救急
成人市中肺炎診療ガイドライン
急性胆管炎・胆嚢炎の診療ガイドライン
急性膵炎診療ガイドライン
動脈硬化性疾患予防ガイドライン
高血圧治療ガイドライン
喘息予防・管理ガイドライン
褥瘡の予防・治療ガイドライン
わが国の新しい救急蘇生ガイドライン（骨子）
外傷初期診療ガイドライン
医療安全・手技
病院感染対策ガイドライン
消化器内視鏡ガイドライン
内視鏡の洗浄・消毒に関するガイドライン
その他
薬剤性肺障害の評価，治療についてのガイドライン
深在性真菌症の診断・治療ガイドライン

の両者があることが記載されている[8]．また福井らも詳細に検討し平成24年の内科学会で報告している[9]．

国内の医療訴訟の第一審通常訴訟新受事件数は平成17年の982件を最大として，その後低下傾向にみえ，平成23年には739件であった．ガイドラインの普及に伴い医療訴訟が増加しているということはないようである．

医療訴訟でガイドラインがどれぐらい，またどのように用いられているかを検討した．判例データベースであるWestlaw Japanを用いた．平成19年～25年の判例を対象とし，「医療」「医師」「ガイドライン」を含む判例を検索し，記述内容を確認した．なお判例が公開されているのは限られた一部であり，このようなデータベースでも収録率は1割弱といわれる．また和解に至り双方が譲歩したものは掲載されていない．

診療行為に関わる判例でのガイドライン引用割合は，平成19年から24年にかけ約20～30%で推移しており，一定の割合で利用されていることが伺えた．

内科領域で，医療訴訟で引用されたガイドラインを表1に記載した．循環器，脳卒中，がんなどの重篤になりがちな疾患に関連して医療訴訟となり，ガイドラインが引用されていた．またプライマリ・ケア領域でも市中肺炎，胆嚢炎・胆管炎，急性膵炎のガイドラインが引用されていた．また慢性疾患ではあるが，薬物療法の副作用と関連して動脈硬化性疾患予防ガイドラインが引用されていた．医療安全と関連して，病院感染対策ガイドラインも用いられていた．ガイドラインの利用方法として，一般的な医学知識の情報源や医療水準を知る手懸りとしての利用が多かった．

判例中の裁判所判断において，ガイドライン自体に関して言及されていた．その内容を大別すると，「ガイドラインは標準的な診療を判断する手懸り」とする記載と，「実際の診療では個別性も考慮されるべきである」とする記載がされていた（表2）．ガイドラインは医療水準そのものではないが医療水準の判断に寄与するものとしての見方がある一方で，診療での個別性も十分考慮されていた．合理的な理由があれば，裁

表2. 判例におけるガイドライン自体に関する言及

- 一般に診療ガイドラインは，作成時点で最も妥当と考えられる手順をモデルとして示したものであることが認められ，具体的な医療行為を行うにあたって，ガイドラインに従わなかったとしても，直ちに診療契約上の債務不履行又は不法行為に該当すると評価することができるものではないが，当該ガイドラインの内容を踏まえた上で医療行為を行うことが必要であり，医師はその義務を負っていると解される．（平成19年9月19日大阪地裁）
- ガイドラインは，その緒言において，ガイドライン自体，乏しい資料に基づいた未だ発展途上のものであり，今後研究を重ねて，より完成度の高いガイドラインに改訂されていくべきことがうたわれている．（平成21年9月29日大阪地裁）
- 本件ガイドラインは（中略），臨床医学上の標準的治療が行われたかどうかの基準となるものである．（前略），少なくとも被告病院のような総合病院において（中略），本件ガイドラインの内容は医療水準であったというべきである．

  実際の治療にあたっては，施設の状況や個々の患者の状態等から医師が最終的な対処法を決定するものであるから，合理的理由がある場合には，本件ガイドラインと異なる治療等が行われたとしても，医療水準に従った治療等がなされなかったということにはならない…．

  本件ガイドラインはあくまでも最も標準的な指針であり，実際の診療行為を決して強制するものではなく，施設の状況（人員，経験，機器等）や個々の患者の個別性を加味して最終的に対処法を決定すべきものとされる．（平成21年1月27日仙台地裁）
- 診療ガイドラインは，その時点における標準的な知見を集約したものであるから，それに沿うことによって当該治療方法が合理的であると評価される場合が多くなるのはもとより当然である．もっとも，診療ガイドラインはあらゆる症例に適応する絶対的なものとまではいえないから，個々の患者の具体的症状が診療ガイドラインにおいて前提とされる症状と必ずしも一致しないような場合や，患者固有の特殊事情がある場合において，相応の医学的根拠に基づいて個々の患者の状態に応じた治療方法を選択した場合には，それが診療ガイドラインと異なる治療方法であったとしても，直ちに医療機関に期待される合理的行動を逸脱したとは評価できない．（平成22年6月30日仙台地裁）
- ガイドラインをそのまま適用できる患者は約60%，斟酌しおおむね利用できるのが30%程度と考えられており全く適用できない患者も存在する．（平成23年3月23日東京地裁・イレッサ第1審）
- 個々の患者に対していかなる医療行為を行うかは，患者と十分に協議した上，最終的には担当医の責任において決定すべき…，医療ガイドラインは，その決定を支援するための指針にすぎず，担当医の医療行為を制限するものでも，当該ガイドラインの推奨する医療行為を実施することを医療従事者に義務付けるものでもない．

  少なくとも本件において，予防ガイドライン等に従った医療行為が実施されなかった場合には，このことにつき特段の合理的理由があると認められない限り，これは医師としての合理的裁量の範囲を逸脱するものというべきである．（平成23年12月9日東京地裁）

*網掛けの箇所は，「ガイドラインは標準的な診療を判断する手懸り」とする記載．下線の箇所は，「実際の診療では個別性も考慮されるべきである」とする記載．

量も認められる．

また表3に医療の不確実性や推奨レベルに関してどのように記載されているかを要約した．推奨レベルCでは，必ずしも実施されるべきではないという記載がみられた．医療の不確実性や限界にも配慮がされているようにみえた．

結局，ガイドラインは医療訴訟の中で一定の割合で利用されており，医療水準を考える手掛かりとされていた．しかし合理的な理由があれば，必ずしもその通りに診療すべきものではないことは理解されており，推奨レベルにみられるような医学の不確実性についても記載されていた．このようなことは，診療ガイドラインが社会一般の医学に関する理解を深めるのに寄与していることを伺わせた．

ガイドラインは医療訴訟において標準的診療を知る手懸りとされているが，一方，診療場面での個別性も配慮されている．このため，合理的な理由があればガイドライン通りに診療しなくても注意義務違反になるわけではない．しかし標準的なものであるだけにその通りの診療を行わないのであれば，患者に説明する必要があるし，説明がなければ説明義務違反に問われる可能性はある．ガイドライン作成者は，信頼に足る質の高いガイドラインを作成する必要がある．この中で適応範囲を明確にし，限界も明らかにするべきである．また診療にあたる医師にとって，ガイドラインを知っていることは標準となる．合理的な診療を心がけ独り善がりではない裁量を発揮し，患者への説明を的確に行ってこれを記録するべきである．

表3. 判例における推奨度に関する言及

- 持続動注療法は，急性膵炎ガイドラインにおいてオプション扱いで推奨度Cとされていること，（中略）ことからすると，担当医師において，同日ころに持続動注療法を実施すべき義務があったとはいえない．
  CHDF及びSDDは，急性膵炎ガイドラインにおいてオプション扱いで推奨度Cとされていることからすれば，担当医師において，同日ころCHDF，SDDを実施すべきであったとはいえない．（平19年7月26日東京地裁）
- 卵巣がんの（中略），初回治療後のフォローアップについて，診察・検査項目のエビデンスレベル及び推奨度は，2004年版においてはそれぞれⅣ，Eであり，2007年版においても Ⅲ，Cとされていることからすれば，上記ガイドラインの記載を前提にしても，少なくとも1年に1度は胸部X線検査を実施すべき義務があることを導き出すことはできない．（平22年6月3日東京地裁）
- 肝癌診療ガイドラインにおいてサーベイランスの至適間隔に関する明確なエビデンスはないとされており，推奨の強さはグレードC1（中略）と位置づけられていることからすれば，サーベイランスの間隔については一義的に標準化されているとまでは認めがたいのであるから，上記間隔については医師の裁量が認められる余地は相対的に大きくなるものと解される．（平成22年6月30日仙台地裁）

＊エビデンスに基づいた急性膵炎の診療ガイドライン（第1版）（2007）では，推奨度分類Cは，「その推奨の効果を支持する（あるいは否定する）根拠が不十分であるか，その効果が有害作用・不都合（毒性や薬剤の相互作用，コスト）を上回らない可能性がある」と記載されていた．なお第2版，第3版では推奨度分類Cは，C1，C2と分けられている．
卵巣がん治療ガイドライン（2004年版）では，推奨グレードCは「タイプⅡ，Ⅲ，Ⅳのエビデンスがあり，調査結果が一貫していない」とされていた．タイプⅡ，Ⅲ，Ⅳはそれぞれ，少なくとも一つのランダム化比較試験，観察研究，専門家の意見に該当するものと思われる．その後推奨グレードCはC1とC2に分けられている．
肝癌診療ガイドライン（2005年版）ではグレードC1は「行うことを考慮してもよいが，十分な科学的根拠がない」とされていた．

4. 結　論

ガイドラインは国内の診療現場で定着しており，診療，教育，研究に大きな影響を及ぼす．医療機関の中やまた広く社会一般に対して，コミュニケーションの基盤として重要である．このため評価などを活かしつつ，質の高い信頼されるガイドラインが作成される必要がある．そのようなガイドラインによってはじめて医療の質の向上に資することができ，また医療における多くの問題で社会の理解と協力を得ることができるようになる．

著者のCOI（conflicts of interest）開示：本論文発表内容に関連して特に申告なし

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Inhibitory Effects of Caffeic Acid Phenethyl Ester Derivatives on Replication of Hepatitis C Virus

Hui Shen[1], Atsuya Yamashita[1], Masamichi Nakakoshi[2], Hiromasa Yokoe[3], Masashi Sudo[3], Hirotake Kasai[1], Tomohisa Tanaka[1], Yuusuke Fujimoto[1], Masanori Ikeda[4], Nobuyuki Kato[4], Naoya Sakamoto[5], Hiroko Shindo[6], Shinya Maekawa[6], Nobuyuki Enomoto[6], Masayoshi Tsubuki[3]*, Kohji Moriishi[1]*

1 Department of Microbiology, Division of Medicine, Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan, **2** Faculty of Pharmaceutical Sciences, Toho University, Chiba, Japan, **3** Institute of Medical Chemistry, Hoshi University, Tokyo, Japan, **4** Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan, **5** Department of Gastroenterology and Hepatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan, **6** First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan

Abstract

Caffeic acid phenethyl ester (CAPE) has been reported as a multifunctional compound. In this report, we tested the effect of CAPE and its derivatives on hepatitis C virus (HCV) replication in order to develop an effective anti-HCV compound. CAPE and CAPE derivatives exhibited anti-HCV activity against an HCV replicon cell line of genotype 1b with EC_{50} values in a range from 1.0 to 109.6 μM. Analyses of chemical structure and antiviral activity suggested that the length of the n-alkyl side chain and catechol moiety are responsible for the anti-HCV activity of these compounds. Caffeic acid n-octyl ester exhibited the highest anti-HCV activity among the tested derivatives with an EC_{50} value of 1.0 μM and an SI value of 63.1 by using the replicon cell line derived from genotype 1b strain Con1. Treatment with caffeic acid n-octyl ester inhibited HCV replication of genotype 2a at a similar level to that of genotype 1b irrespectively of interferon signaling. Caffeic acid n-octyl ester could synergistically enhance the anti-HCV activities of interferon-alpha 2b, daclatasvir, and VX-222, but neither telaprevir nor danoprevir. These results suggest that caffeic acid n-octyl ester is a potential candidate for novel anti-HCV chemotherapy drugs.

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* E-mail: tsubuki@hoshi.ac.jp (MT); kmoriishi@yamanashi.ac.jp (KM)

Introduction

Hepatitis C virus (HCV) is well known as a major causative agent of chronic liver disease including cirrhosis and hepatocellular carcinoma and is thought to persistently infect 170 million patients worldwide [1]. HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae* and possesses a viral genome that is characterized by a single positive strand RNA with a nucleotide length of 9.6 kb [2]. The single polypeptide coded by the genome is composed of 3,000 amino acids and is cleaved by host and viral proteases, resulting in 10 proteins, which are classified into structural and nonstructural proteins [3]. The viral genome is transcribed by a replication complex consisting of NS3 to NS5B and host factors [4]. NS3 forms a complex with NS4A and becomes a fully active form to cleave the C-terminal parts of the nonstructural proteins. The advanced NS3/4A protease inhibitors, telaprevir and boceprevir, have been employed in the treatment of chronic hepatitis C patients infected with genotype 1 [5]. Sustained virologic response (SVR) was reportedly 80% in patients infected with genotype 1 following triple combination therapy with pegylated interferon, ribavirin, and telaprevir [6], although the therapy exhibits side effects including rash, severe cutaneous eruption, influenza-like symptoms, cytopenias, depression, and anemia [7]. In addition, there is the possibility of the emergence of drug-resistant viruses following treatment with those anti-HCV drugs [8] Thus, further study is required for development of safer and more effective anti-HCV compounds.

Several recent reports indicate that silbinin [9], epigallocatechin-3-gallate [10], curcumin [11], quercetin [12] and proanthocyanidins [13], which all originate from natural sources, have exhibited inhibitory activity against HCV replication in cultured cells. Caffeic acid phenethyl ester (CAPE) is an active component included in propolis prepared from honeybee hives, and has a similar structure to flavonoids (Fig. 1A). CAPE has multifunctional properties containing anti-inflammatory [14], antiviral [15], anticarcinogenic [16], and immunomodulatory activities [15]. CAPE also inhibits enzymatic activities of endogenous and viral proteins [17–19] and transcriptional activity of NF-kappaB [14,20]. In addition, CAPE could suppress HCV replication enhanced by using the NF-kappaB activation activity of morphine [21], although it has been unknown which of moieties including CAPE is responsible for anti-HCV activity. Furthermore, it is not clear whether chemical modification of CAPE could enhance anti-HCV activity or not. In this report, we examined the effect of

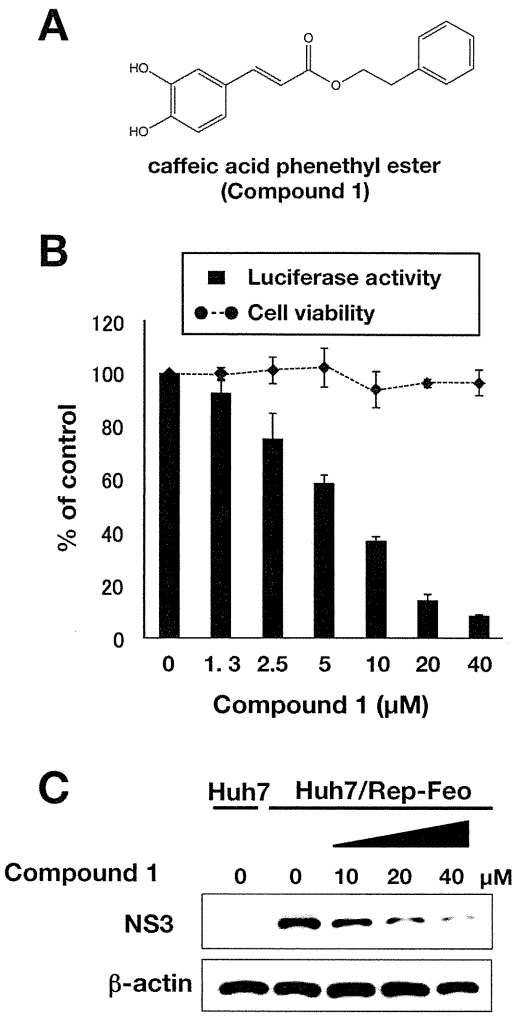

Figure 1. Effect of CAPE on viral replication in the replicon cell line of genotype 1b. (A) Molecular structure of CAPE. (B) Huh7/Rep-Feo cells were incubated for 72 h in a medium containing various concentrations of CAPE. Luciferase and cytotoxicity assays were carried out by the method described in Materials and Methods. Error bars indicate standard deviation. The data represent results from three independent experiments. (C) Protein extract was prepared from Huh7/Rep-Feo cells treated for 72 h with the indicated concentration of CAPE and it was then was subjected to Western blotting using antibodies to NS3 and beta-actin.
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CAPE derivatives on HCV proliferation to develop more effective and safer anti-HCV compounds.

Results

Effect of CAPE on HCV RNA replication in HCV subgenomic replicon cells

CAPE is composed of ester of caffeic acid and phenethyl alcohol (Fig. 1A). We examined the effect of CAPE (compound **1**) on both viral replication and cell growth in the HCV subgenomic replicon cell line Huh7/Rep-Feo. The replicon cell line was treated with various concentrations of compound **1**. The replication level of the HCV RNA was measured as an enzymatic activity of luciferase, which is bicistronically, encoded on the replicon RNA. Compound **1** suppressed HCV RNA replication at concentrations from 1.3 to 40 μM in a dose-dependent manner, but did not affect cell viability (Fig. 1B). HCV NS3, which is a viral protease, was decreased at the protein level by treatment with CAPE in a dose-dependent manner, corresponding to the viral replication, whereas beta-actin was not changed in the replicon cell line (Fig. 1C). Based on the calculation using a dose dependency of CAPE, compound **1** exhibited an EC_{50} value of 9.0 μM and a CC_{50} value of 136.1 μM, giving a selectivity index estimate (SI) of 17.9 (Table 1). These results suggest that treatment with CAPE inhibits HCV replication in HCV subgenomic replicon cells.

Structure-activity relationship of CAPE analogues

To clarify the structure-activity relationship of CAPE analogues, we examined the effect of hydroxyl groups on the aromatic ring (catechol moiety), the alkenyl moieties on alpha, beta-unsaturated esters, and the ester parts as follows (Figure S1).

We tested whether commercially available CAPE-related compounds **2** to **6** (Fig. S1) affected HCV replication (Table 1). All these compounds showed weaker inhibitory activity than CAPE (**1**), but are not toxic. Compound **2**, which is the acid component of CAPE, showed a slightly lower value of EC_{50} than compound **3**, which is the compound **2** derivative replaced a hydroxyl group with a methoxyl group of catechol moiety, while compound **4**, which is the derivative lacking two hydroxyl groups within catechol moiety, exhibits a higher value of EC_{50} than compounds **1** and **2**. These data suggest that the catechol moiety of CAPE is required for anti-HCV activity. Interestingly, compounds **5** and **6**, which are natural products including polyhydroxylated acid moieties in the ester parts, showed much weaker inhibitions than compound **1** and exhibits low *Clog P* values. The position of hydroxyl group or/and the structure of the ester part may affect the inhibitory activity and/or hydrophobicity.

We next examined the effects of caffeic acid ester compounds **7** to **11**, which include various lengths of alkyl side chains, on HCV replication (Table 2 and Figure S2). The EC_{50} values decreased in the order methyl ester (compound **7**), n-butyl ester (compound **8**), n-hexyl ester (compound **9**), and n-octyl ester (compound **10**), suggesting that elongation of the n-alkyl side chain increased the inhibitory activity. However, the EC_{50} value of n-dodecyl ester (compound **11**) was higher than that of compound **10**. Thus, n-octyl ester (compound **10**) showed the lowest EC_{50} value and the highest SI among the tested compounds shown in Tables 1 and 2. Compounds **7** to **11** gradually increased own *Clog P* values,

Table 1. Effect of CAPE (**1**) and related compounds **2–6** on HCV replication.

Compound (Number)	EC_{50} [a] (μM)	CC_{50} [b] (μM)	SI [c]	*Clog P* [d]
CAPE (**1**)	9.0±0.7	136.1±1.9	17.9	3.30
caffeic acid (**2**)	36.6±6.7	>320	>8.7	0.98
ferulic acid (**3**)	71.9±5.8	>320	>4.5	1.42
cinnamic acid henethyl ester (**4**)	86.1±6.3	>320	>3.7	4.56
chlorogenic acid (**5**)	103.0±3.4	>320	>3.1	−0.96
rosmarinic acid (**6**)	109.6±1.1	>320	>2.9	1.10

a: Fifty percent effective concentration based on the inhibition of HCV replication.
b: Fifty percent cytotoxicity concentration based on the reduction in cell viability.
c: Selectivity index (CC_{50}/EC_{50}).
d: Determined with ChemDraw software (Chem Bio Office Ultra, 2008).
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