Effect of Repeated Injections of Adenosine Diphosphate-Encapsulated Liposomes Coated with a Fibrinogen γ-Chain Dodecapeptide Developed as a Synthetic Platelet Substitute on Accelerated Blood Clearance in a Healthy and an Anticancer Drug-Induced Thrombocytopenia Rat Model

KAZUAKI TAGUCHI,[1] MAI HASHIMOTO,[2] SHIGERU OGAKI,[2] HIROSHI WATANABE,[2,3] SHINJI TAKEOKA,[4] YASUO IKEDA,[4] MAKOTO HANDA,[5] MASAKI OTAGIRI,[1,2,6] TORU MARUYAMA[2,3]

[1]Faculty of Pharmaceutical Sciences, Sojo University, Nishi-ku, Kumamoto 862-0082, Japan
[2]Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, Chuo-ku, Kumamoto 862-0973, Japan
[3]Center for Clinical Pharmaceutical Sciences, Kumamoto University, Chuo-ku, Kumamoto 862-0973, Japan
[4]Department of Life Science and Medical Bioscience, Graduate School of Advanced Science and Engineering, Waseda University, Shinjuku-ku, Tokyo 162-8480, Japan
[5]Center for Transfusion Medicine and Cell Therapy, Keio University, Shinjuku-ku, Tokyo 160-8582, Japan
[6]DDS Research Institute, Sojo University, Nishi-ku, Kumamoto 862-0082, Japan

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ABSTRACT: Adenosine diphosphate (ADP)-encapsulated liposomes coated with a fibrinogen γ-chain dodecapeptide [H12 (dodecapeptide (^{400}HHLGGAKQAGDV^{411}))-(ADP)-liposome] is a synthetic platelet substitute, in which the surface is covered with polyethylene glycol (PEG). It has been reported that repeated injections of PEGylated liposomes induce an accelerated blood clearance (ABC) phenomenon, which involves a loss in the long-circulation half-life of the material when administered repeatedly to the same animals. The objective of this study was to determine whether the ABC phenomenon was induced by repeated injections of H12-(ADP)-liposome in healthy and anticancer drug-induced thrombocytopenia model rats. The findings show that the ABC phenomenon was induced by healthy rats that were repeatedly injected with H12-(ADP)-liposomes at the interval of 5 days at a dose of 10 mg lipids/kg. The ABC phenomenon involves the production of anti-H12-(ADP)-liposome immunoglobulin M (IgM) and complement activation. On the other hand, when thrombocytopenia model rats were repeatedly injected with H12-(ADP)-liposomes under the same conditions, no ABC phenomenon, nor was any suppression of anti-H12-(ADP)-liposome IgM-mediated complement activation observed. We thus conclude that the repeated injection of H12-(ADP)-liposome treatment in rat model with anticancer drug-induced thrombocytopenia did not induce the ABC phenomenon. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci
Keywords: liposome; adenosine-diphosphate; dodecapeptide; accelerated blood clearance phenomenon; thrombocytopenia; platelet substitute; pegylation; disposition; clearance; pharmacokinetics

INTRODUCTION

To further enhance the quality and efficiency of drug delivery substances, they are frequently modified with polyethylene glycol (PEG).[1] There is now little doubt that PEGylation is useful and is in widespread use because it results in a prolonged half-life, a higher stability and a lower immunogenicity. In fact, some of PEGylated products, such as PEGylated epoetin-β[2] and a PEGylated liposomal formulation of doxorubicin,[3] take advantage of these characteristics, and have been approved for use in clinical settings.

However, it is widely known that the intravenous injection of PEGylated products, including liposomes and micelles, causes a second dose of PEGylated products to lose their long-circulating characteristics and accumulate in the liver, when administrated twice in the same animal [referred to as the "accelerated blood clearance (ABC) phenomenon"].[4] This phenomenon has been reported for a number of animal species, including rhesus monkeys, dogs, rabbits, guinea pigs, rats, and mice.[4–7] Moreover, Ishida and coworkers[8,9] reported that the abundant secretion of anti-PEG immunoglobulin M (IgM), produced by the spleen in response to the first dose of PEGylated liposomes, plays a crucial role in the induction of the ABC phenomenon. Interestingly, the probable existence of naturally occurring anti-PEG antibodies in human beings has also been reported,[10,11] which indicate that repeated injections of PEGylated products might induce the ABC phenomenon, even in a clinical situation. Therefore, it would be advisable to examine

Abbreviations used: ADP, adenosine diphosphate; H12, dodecapeptide (^{400}HHLGGAKQAGDV^{411}); ABC, accelerated blood clearance; PEG, polyethylene glycol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DHSG, 1,5-dihexadecyl-*N*-succinyl-L-glutamate; DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-(monomethoxyPEG); HbV, hemoglobin-vesicles.

Correspondence to: Toru Maruyama (Telephone: +81-96-361-4150; Fax: +81-96-362-7690; E-mail: tomaru@gpo.kumamoto-u.ac.jp)

Kazuaki Taguchi and Mai Hashimoto contributed equally to this work.

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the pharmacokinetic properties of PEGylated products with respect to the effects of repeated injections before the beginning of a clinical trial, because the ABC phenomenon may cause adverse effects or decrease the pharmacological effects of a formulation, because of the development of altered pharmacokinetics of PEGylated products.

The dodecapeptide (^{400}HHLGGAKQAGDV^{411}) (H12)-(ADP)-liposome was developed as a synthetic platelet substitute, of which the surface of phospholipid vesicles is modified with PEG. In order to enhance the hemostatic effect, this PEGylated liposome-based artificial platelet substitute bears a synthetic H12 on its surface, corresponding to the carboxy-terminus of the fibrinogen γ-chain, and the physiologic platelet agonist adenosine diphosphate (ADP) in the interior. In fact, it was reported that these modifications enable the liposomes to accumulate at the site of an injury *in vivo* by specifically binding to glycoprotein IIb/IIIa on activated platelet membranes,[12,13] thus decreasing bleeding time in a dose-dependent manner in both thrombocytopenic rat and rabbit models.[14,15] Furthermore, we recently reported that H12-(ADP)-liposomes have an adequate circulation time in the blood to permit them to function as a platelet substitute in healthy animals and a thrombocytopenic model rat.[16,17] Because of these characteristics, H12-(ADP)-liposomes show hemostatic effects comparable to platelet-rich plasma and also result in an improved survival in rabbits with acute thrombocytopenia.[18,19]

As it is likely that repeated injections of H12-(ADP)-liposome would be required, as a platelet substitute, in clinical settings, the possibility remains that repeated injections of the H12-(ADP)-liposome might induce the ABC phenomenon. If the ABC phenomenon were induced by repeated injections, then the hemostatic effects of H12-(ADP)-liposomes could be compromised. In addition, changes in the pharmacokinetic properties of H12-(ADP)-liposomes may cause unexpected adverse effects. Therefore, clarifying the pharmacokinetics of H12-(ADP)-liposomes after repeated injections at a dose is effective for producing therapeutic effects in animal studies, and thereby for possible use in clinical settings as a platelet substitute should provide useful information for future clinical applications.

In the present study, we investigated the issue of whether the first injection of H12-(ADP)-liposome at a dose of 10 mg lipids/kg that is proposed for use in clinical practice as a platelet substitute affects the pharmacokinetic behavior of H12-(ADP)-liposome after the second injection in healthy rats and rats models of an adaptation disease (thrombocytopenia). In addition, we also investigated the issue of whether anti-H12-(ADP)-liposome IgM-mediated complement activation occurs after the first injection, and which lipid component of the H12-(ADP)-liposome is recognized by IgM.

MATERIALS AND METHODS

Reagents

Cholesterol and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) were purchased from Nippon Fine Chemical (Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-(monomethoxyPEG) (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 reported previously.[20]

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

^{14}C, ^{3}H double-labeled H12-(ADP)-liposomes were prepared as previously reported.[16] The diameter and zeta-potential of the ^{14}C-labeled H12-(ADP)-liposomes used in this study were in the range of 250 ± 50 nm and −10 ± 0.9 mV, respectively. Before being used in pharmacokinetic experiments, all of the samples were mixed with unlabeled H12-(ADP)-liposomes. The 5%–10% of added 1 mM ADP was encapsulated in the inner space of the vesicle.

The Pharmacokinetic Experimental Protocol in Healthy Rats

All animal experiments were undertaken in accordance with the guidelines principles and procedures of Kumamoto University for the care and use of laboratory animals. All animals were maintained under conventional housing conditions, with food and water *ad libitum* in a temperature-controlled room with a 12-h dark/light cycle. All male Sprague–Dawley (SD) rats (270–300 g) were purchased from Kyudou Company (Kumamoto, Japan).

Eight male SD rats were anesthetized using ether and received a single injection of a nonlabeled H12-(ADP)-liposomes (10 mg lipids/kg) via the tail vein. This dose is lowest recommended dosage to exert a sufficient hematostatic effect in thrombocytopenic rats.[14] Five days after the first injection of the nonlabeled H12-(ADP)-liposomes, the same SD rats received a ^{14}C, ^{3}H-labeled H12-(ADP)-liposomes (10 mg lipids/kg) via the tail vein under ether anesthesia. Four rats were randomly selected to undergo a plasma concentration test. Under ether anesthesia, approximately 200 μL of a blood sample from all administered groups were collected from the tail vein 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, and 24 h) and the plasma was separated by centrifugation (3000*g*, 5 min). An additional four rats were sacrificed and their livers were collected at 2 h after an injection of ^{14}C, ^{3}H-labeled H12-(ADP)-liposomes.

Pharmacokinetic Studies in Thrombocytopenic Rats

Thrombocytopenic rats induced by busulphan (Sigma–Aldrich, St Louis, Missouri) treatment were created as in our previous report.[17] Ten days after the final administration of busulphan, eight thrombocytopenic rats were anesthetized with ether and given a single injection of nonlabeled H12-(ADP)-liposomes (10 mg lipids/kg) via the tail vein. Five days after the first injection of the nonlabeled H12-(ADP)-liposomes, the same thrombocytopenic rats received ^{14}C, ^{3}H-labeled H12-(ADP)-liposomes (10 mg lipids/kg) via the tail vein under ether anesthesia. Four rats were randomly selected to undergo a plasma concentration test. Under ether anesthesia, approximately 200 μL blood sample from all administered groups were collected from the tail vein 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, and 24 h) and the plasma was separated by centrifugation (3000*g*, 5 min). An additional four thrombocytopenic rats were sacrificed and their livers were collected 2 h after an injection of ^{14}C, ^{3}H-labeled H12-(ADP)-liposomes.

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

The ^{14}C, ^{3}H radioactivity in plasma and livers were determined by liquid scintillation counting (LSC-5121; Aloka, Tokyo, Japan) with Hionic Fluor (Perkin Elmer, Yokohama, Japan) as previously reported.[16]

Quantitative Determination of Anti-H12-(ADP)-Liposome IgM

Normal ($n = 4$) or thrombocytopenic rats ($n = 4$) were injected with H12-(ADP)-liposome (10 mg lipids/kg) via the tail vein under ether anesthesia. At each time point (1–7, 10, and 14 days) after the injection, blood was collected from the tail vein. Plasma was collected after centrifugation (3000g, 5 min), and was stored at −80°C until used.

ELISA was employed to detect IgM against H12-(ADP)-liposome using a previously described method, with minor modifications.[6] A 100-μL of 475 ng lipids/mL H12-(ADP)-liposomes (comprising DPPC, cholesterol, DHSG, PEG-DSPE, and H12-PEG-Glu2C18) were added to 96-well plates (NUNC, New York). The plates were incubated for 2 h at 25°C. After incubation, the wells were washed three times with a wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). A blocking solution [50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin (BSA), pH 8.0] was then added to each well and the plate incubated for 2 h at 25°C. After incubation, the wells were washed three times with wash solution and 100 μL of plasma sample, diluted 1:100 with sample solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, 1% BSA, pH 8.0), and were added to the wells. After incubation for 90 min, the wells were washed three times with wash solution and 100 μL of peroxidase-labeled affinity purified antibody to mouse IgM (μ), diluted 1:1000 with sample solution, and were added to each well. After incubation for 60 min, the wells were washed three times with wash solution. Coloration was initiated by adding 100 μL of *o*-phenylene diamine (1 mg/mL). After incubation, the reaction was terminated by adding 100 μL of 1 N H_2SO_4 and the absorbance was measured at 490 nm using a Microplate reader (Model 680; BIO-RAD, Tokyo, Japan).

Quantitative Determination of Anti-Lipid IgM

A 10-nmol aliquot of each lipid (DPPC, cholesterol, DHSG, PEG-DSPE, or H12-PEG-Glu2C18) in 50 μL of 100% ethanol was added to 96-well plates (NUNC). The plates were incubated for 4 h at 37°C to dry completely. Following processes were identical to those described in the *Quantitative Determination of Anti-H12-(ADP)-Liposome IgM* section.

Measurement of Complement Activity (CH50)

Normal rats ($n = 4$) or thrombocytopenic rats ($n = 4$) were injected with H12-(ADP)-liposomes (10 mg lipids/kg) via the tail vein under ether anesthesia. At stipulated time point, blood was collected from the tail vein. The blood was centrifuged (3000g, 5 min) to obtain plasma samples for analysis. All plasma samples were stored at −80°C prior to analysis by a commercial clinical testing laboratory (SRL, Tokyo, Japan). The CH 50 was detected by the method of Mayer.[21]

Data Analysis

A noncompartment model was used for the pharmacokinetic analysis. Each parameter was calculated using the moment analysis program available on Microsoft Excel. Data are shown as the mean ± SD for the indicated number of animals. Statistical evaluation of pharmacokinetic data was compared using unpaired Student's t-test. Statistical significance of the results for CH50 was tested with paired Student's t-test, and any other statistical evaluation among each group was compared using one-way analysis of variance, followed by Bonferron post hoc test. A probability value of $p < 0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Pharmacokinetic Properties of H12-(ADP)-Liposome After Repeated Injection in Healthy Rats

In a previous study, we found that repeated injection of a low dose of hemoglobin-vesicles (HbV), the liposomal characteristics of which were similar in terms of liposomal structure to H12-(ADP)-liposomes, resulted in the induction of the ABC phenomenon accompanied by the production of anti-HbV antibodies.[6] However, it is well known that a variety of factors, including the lipid dose used, physicochemical properties (degree of PEGylation, PEG chain length, surface charge, and size) and the encapsulated drug of the initially injected liposome, dosing intervals, all can strongly affect the pharmacokinetic response to a subsequent injection.[22] It is noteworthy that the end of the PEG lipids on the surface of H12-(ADP)-liposomes is partly modified with H12. Therefore, we first investigated the pharmacokinetic properties of the second injected H12-(ADP)-liposomes in the healthy rats using ^{14}C, ^{3}H-labeled H12-(ADP)-liposomes, in which the encapsulated ADP and membrane component (cholesterol) were labeled with ^{14}C and ^{3}H, respectively. The time interval for the injection was selected for 5 days based on a previous report, in which the ABC phenomenon in rats was observed the most strongly, when the time interval for the injection was 4–5 days.[23]

Figure 1a shows the time course for the plasma concentration curve for the ^{14}C, ^{3}H-labeled H12-(ADP)-liposomes administrated once or twice to healthy rats at a dose of 10 mg lipids/kg. The plasma concentration curves for ^{14}C radioactivity and ^{3}H radioactivity indicate that, in the second injection, the radioactivity was rapidly cleared compared with the first injection (Fig. 1a). The plasma clearance for both ^{14}C radioactivity and ^{3}H radioactivity in the second injection was increased significantly compared with the corresponding values for the first injection (Table 1).

As the second injected PEG-liposomes accumulate at relatively high levels in the liver when the ABC phenomenon occurs,[23] the effect of repeated injections on the hepatic distribution of ^{14}C, ^{3}H-labeled H12-(ADP)-liposomes was examined. Figure 1b shows the hepatic distribution for ^{14}C and ^{3}H radioactivity (% of dose/g tissue) at 2 h after the administration of ^{14}C, ^{3}H-labeled H12-(ADP)-liposomes once or twice to healthy rats at a dose of 10 mg lipids/kg. As expected, the hepatic distribution for both the ^{14}C and ^{3}H radioactivity in the second injection was much higher than the values for the first injection at 2 h after the administration of ^{14}C, ^{3}H-labeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg (Fig. 1b). These results indicate that the ABC phenomenon was clearly induced at 5 days after the injection when healthy rats were injected with H12-(ADP)-liposomes at a dose of 10 mg lipids/kg.

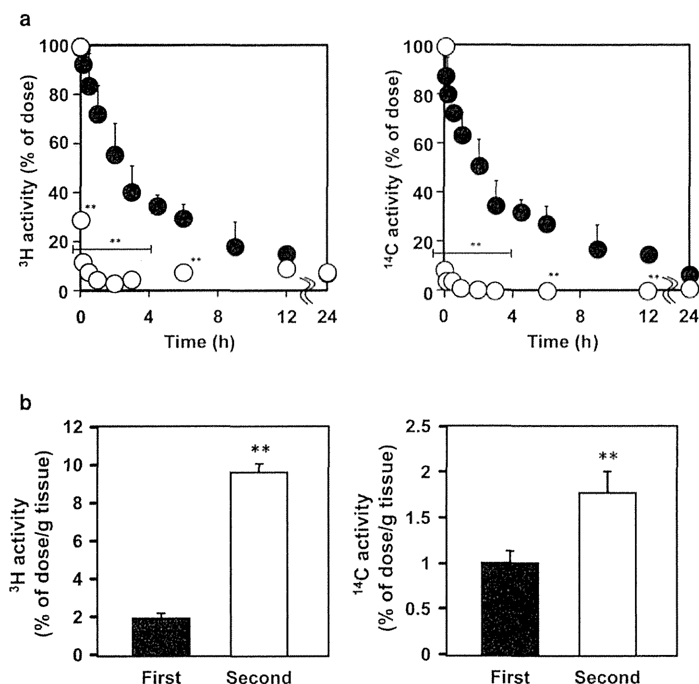

Figure 1. (a) Plasma concentration curve for ^{14}C and ^{3}H radiolabeled H12-(ADP)-liposomes after the first injection (closed symbol) or the second injection (open symbol) of ^{3}H, ^{14}C radiolabeled H12-(ADP)-liposome at a dose of 10 mg lipid/kg to healthy rats. (b) The hepatic distribution of ^{14}C and ^{3}H radioactivity at 2 h after the first injection (closed bar) or the second injection (open bar) of ^{3}H, ^{14}C radiolabeled H12-(ADP)-liposome at a dose of 10 mg lipid/kg to healthy rats. The data for the first injection were cited from our previously reported paper.[16] Each point represents the mean ± SD (n = 4). $^{**}p < 0.01$ versus first injection.

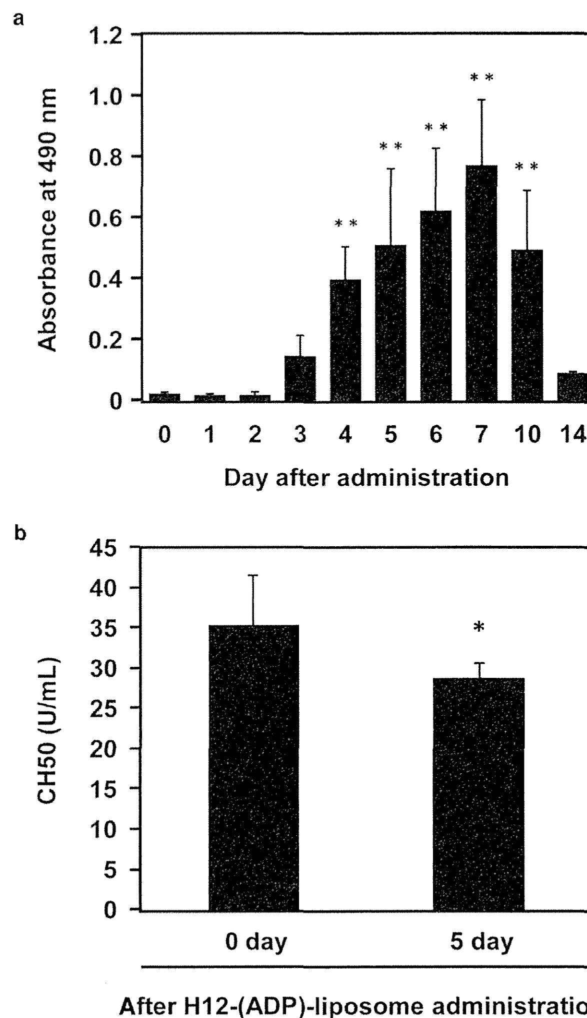

Figure 2. (a) Determination of IgM against H12-(ADP)-liposomes after a single intravenous injection of H12-(ADP)-liposomes at a dose of 10 mg lipid/kg in healthy rats. (b) CH50 values in healthy rats before treatment with H12-(ADP)-liposomes (0 day) or 5 days after a single intravenous injection of H12-(ADP)-liposomes at a dose of 10 mg lipid/kg. Each bar represents the mean ± SD (n = 4). $^{**}p < 0.01$ versus 0 day, $^{*}p < 0.05$ versus 0 day.

Production of IgM Against H12-(ADP)-Liposome and Complement Activity After H12-(ADP)-Liposome Injection in Healthy Rats

It is known that the ABC phenomenon is induced by the selective binding of IgM to the second injected PEGylated liposome, resulting in the activation of complement and consequently an enhanced uptake of the second injected PEGylated liposome, by Kupffer cells via complement receptor-mediated endocytosis.[23] Therefore, we examined the issue of whether IgM against H12-(ADP)-liposomes is elicited by an initial injection of H12-(ADP)-liposome at a dose of 10 mg lipids/kg in healthy rats. Figure 2a shows the values for the quantitative determination of plasma IgM against H12-(ADP)-liposomes after the injection of H12-(ADP)-liposomes at a dose of 10 mg lipids/kg in healthy rats. In line with a previous report in which rats were used,[24] IgM against H12-(ADP)-liposome was elicited starting from 3 days after the injection of H12-(ADP)-liposomes (Fig. 2a).

Table 1. The Pharmacokinetic Parameters of Inner ADP [(8–^{14}C)ADP] and Outer Lipids Membranes [(1,2–^{3}H(N))-Cholesterol] Derived from ^{3}H, ^{14}C-Radiolabeled H12-(ADP)-Liposomes After One or Two Intravenous Injections at a Dose of 10 mg Lipids/kg to Normal Rats

	First Injection		Second Injection	
	^{3}H	^{14}C	^{3}H	^{14}C
$t_{1/2}$ (h)	8.18 ± 0.77	8.21 ± 1.01	1.32 ± 0.62^a	0.64 ± 0.22^a
AUC (h·% of dose/mL)	58.4 ± 6.5	54.2 ± 10.1	2.65 ± 0.42^a	1.36 ± 0.10^a
CL (mL/h)	1.73 ± 0.18	1.89 ± 0.32	38.3 ± 6.6^a	73.8 ± 5.4^a
V_{dss} (mL)	17.7 ± 3.5	19.5 ± 3.6	34.9 ± 13.2^a	18.8 ± 8.0

$^a p < 0.01$ versus first injection. Each value represents the mean ± SD (n = 4).
The data for the first injection are cited from our previously reported paper.[16]
$t_{1/2}$, half-life; AUC, area under the concentration–time curve; CL, clearance; V_{dss}, distribution volume.

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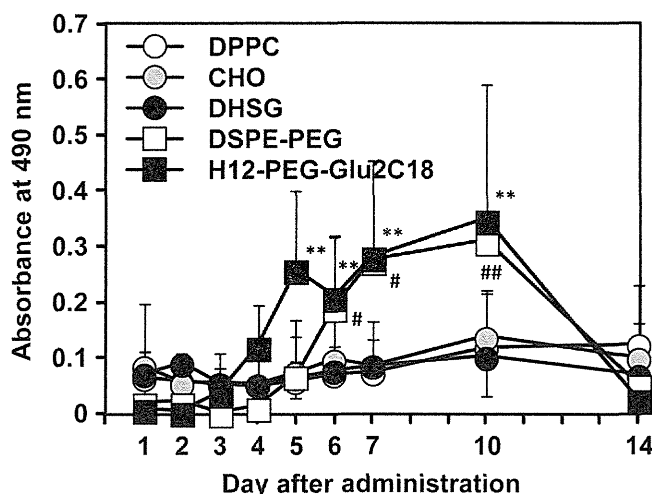

Figure 3. Determination of the specific recognition site of IgM against H12-(ADP)-liposome after a single intravenous injection of H12-(ADP)-liposome at a dose of 10 mg lipid/kg in healthy rats. Each point represents the mean ± SD ($n = 4$). $^{**}p < 0.01$ versus 1 day of H12-PEG-Glu2C18, $^{\#\#}p < 0.01$ versus 1 day of DSPE-PEG, $^{\#}p < 0.05$ versus 1 day of DSPE-PEG.

Furthermore, we also measured the CH50 levels in healthy rats before and at 5 days after the injection of H12-(ADP)-liposomes. The findings show that at 5 days after the H12-(ADP)-liposome injection, the CH50 values decreased compared with that before H12-(ADP)-liposome injection (Fig. 2b). In previous, Hashimoto et al.[25] reported that subsequent complement activation following IgM binding is the most important step in dictating the *in vivo* fate of PEGylated products. Therefore, the production of IgM against H12-(ADP)-liposomes and complement activation would relate to the induction of the ABC phenomenon of H12-(ADP)-liposomes in the healthy rats.

Determination of the Specific Recognition Site of IgM Against H12-(ADP)-Liposome in Healthy Rats

To evaluate the specific recognition site of IgM against H12-(ADP)-liposomes, a modified ELISA was employed using each lipid component (DPPC, cholesterol, DHSG, DSPE-PEG, and H12-PEG-Glu2C18) of the H12-(ADP)-liposome. Figure 3 shows data for the quantitative determination of the specific recognition site of IgM against H12-(ADP)-liposomes during 14 days after the H12-(ADP)-liposome at doses of 10 mg of lipid/kg. IgM was observed to bind strongly to DSPE-PEG and H12-PEG-Glu2C18. On the contrary, IgM against other lipid components (DPPC, cholesterol, and DHSG) were negligible during all times examined after the injection of H12-(ADP)-liposomes. Previous reports have emphasized that the antigenic epitope capable of generating anti-PEG IgM is the repeating -(O-CH_2-CH_2)- subunit in the PEG moiety.[26,27] The fact is that modification of the liposome surface with a polyglycerol-derived lipid, in which the repeating -(O-CH_2-CH_2)$_n$- subunit in the PEG is changed to a -(O-CH_2-CH(CH_2OH))$_n$- subunit, enables both the production of anti-polyglycerol IgM and the induction of the ABC phenomenon to be avoided.[28,29] It therefore appears that anti-H12-(ADP)-liposome IgM would react with both DSPE-PEG and H12-PEG-Glu2C18, even though the end of PEG is modified with H12.

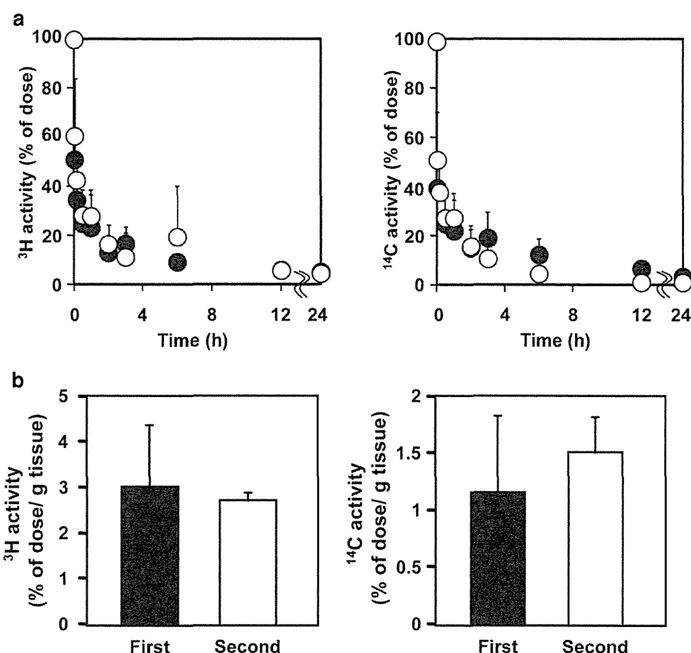

Figure 4. (a) Plasma concentration curve of ^{14}C and ^{3}H-radiolabeled H12-(ADP)-liposome after the first injection (closed symbol) or the second injection (open symbol) of ^{3}H, ^{14}C-radiolabeled H12-(ADP)-liposome at a dose of 10 mg lipid/kg to busulphan-induced thrombocytopenic model rats. (b) The hepatic distribution of ^{14}C and ^{3}H radioactivity at 2 h after the first injection (closed bar) or the second injection (open bar) of ^{3}H,^{14} C-radiolabeled H12-(ADP)-liposome at a dose of 10 mg lipid/kg to busulphan-induced thrombocytopenic model rats. The data for the first injection were cited from a previous report.[17] Each point represents the mean ± SD. ($n = 4$).

Pharmacokinetic Properties of H12-(ADP)-Liposome After Repeated Injection in Anticancer Drug-Induced Thrombocytopenic Rats

We next investigated whether repeated injections of H12-(ADP)-liposomes resulted in the development of the ABC phenomenon in busulphan-induced thrombocytopenic rats, as was observed in the case of healthy rats. Figure 4a shows the time course for the plasma concentration of ^{14}C, ^{3}H-labeled H12-(ADP)-liposomes that were injected into thrombocytopenic rats at a dose of 10 mg lipid/kg, which was lowest recommended dosage to exert a sufficient hematostatic effect in thrombocytopenic rats.[14] Contrary to healthy rats, the plasma concentration curves for ^{14}C radioactivity and ^{3}H radioactivity in the second injection were not significantly different from those for the first injection. The pharmacokinetic parameters, plasma clearance, for both ^{14}C radioactivity and ^{3}H radioactivity were also not different between the first and a significant reduction was observed compared with the values for the first injection (Table 2). In addition, the hepatic distributions of ^{14}C and ^{3}H radioactivity (% of dose/g tissue) at 2 h after the administration of ^{14}C, ^{3}H-labeled H12-(ADP)-liposomes were similar between the first and the second injections (Fig. 4b). Furthermore, as shown in Figure 5a, anti-H12-(ADP)-liposome IgM was elicited at negligible levels during the 14 days after the first injection. Accompanying the minor changes in anti-H12-(ADP)-liposome IgM production, the CH50 values were not changed at 5 days after the H12-(ADP)-liposome injection as compared with

Table 2. The Pharmacokinetic Parameters of Inner ADP [(8–^{14}C)ADP] and Outer Lipids Membranes [(1,2–^{3}H(N))-Cholesterol] Derived from ^{3}H, ^{14}C-Radiolabeled H12-(ADP)-Liposomes After One or Two Intravenous Injections at a Dose of 10 mg Lipids/kg to Thrombocytopenic Rats

	First Injection		Second Injection	
	^{3}H	^{14}C	^{3}H	^{14}C
$t_{1/2}$ (h)	1.81 ± 0.39	1.68 ± 0.78	1.96 ± 1.35	1.53 ± 0.60
AUC (h·% of dose/mL)	10.7 ± 4.1	10.2 ± 4.3	7.34 ± 2.69	7.40 ± 3.93
CL (mL/h)	10.4 ± 4.2	11.4 ± 5.4	16.0 ± 8.78	17.0 ± 10.4
V_{dss} (mL)	20.7 ± 2.9	20.2 ± 1.9	28.3 ± 13.5	24.1 ± 6.54

Each value represents the mean ± SD (n = 4).
The data for the first injection are cited from our previously reported paper.[17]
$t_{1/2}$, half-life; AUC, area under the concentration–time curve; CL, clearance; V_{dss}, distribution volume.

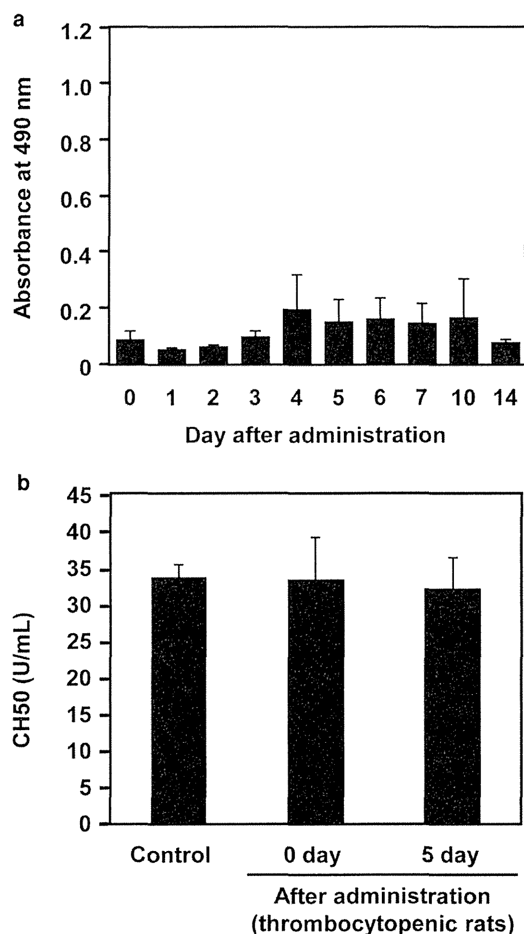

Figure 5. (a) Determination of IgM against H12-(ADP)-liposomes after a single intravenous injection of H12-(ADP)-liposomes at a dose of 10 mg lipid/kg in busulphan-induced thrombocytopenic model rats. (b) CH50 values in busulphan-induced thrombocytopenic model rats before the administration of busulphan (control) and H12-(ADP)-liposomes (0 day) or 5 days after a single intravenous injection of H12-(ADP)-liposomes at a dose of 10 mg lipid/kg. Each bar represents the mean ± SD (n = 4).

before the administration of busulphan and H12-(ADP)-liposomes (Fig. 5b).

The induction of the ABC phenomenon appears to be time-dependent, and to involve two phases; an induction phase, following the first injection, during which the immune system is primed (reflected in the production of antiliposome IgM), and an effectuation phase, following the second injection, during which PEG-liposomes are rapidly cleared from the bloodstream (reflected by the enhanced uptake by Kupffer cells).[30] It was previously reported that the intravenous injection of doxorubicin-encapsulated liposomes did not induce the ABC phenomenon, when they are administrated twice in the same murine model.[30,31] However, Laverman et al.[30] reported that the effects of a second injectection of doxorubicin-encapsulated liposomes were altered dramatically when empty PEGylated liposome were injected 1 week prior to the first injection. On the basis of these fact, it is thought that a cytotoxic drug (e.g., doxorubicin) delivered via PEGylated liposomes inhibits the secretion of anti-PEG IgM from MZ-B cells, and consequently attenuates the ABC phenomenon, which means that a cytotoxic drug inhibits the induction phase. To induce a thrombocytopenic condition, rats were intraperitoneally injected with a total busulphan at a dose of 20 mg/kg before the injection of H12-(ADP)-liposomes. Busulphan is a bifunctional alkylating agent and is mainly cytotoxic for proliferating tissues and depletes noncycling primitive stem cells, including spleen.[32] Therefore, the busulphan pretreatment may have impaired splenic MZ-B cells and anti-H12-(ADP)-liposome molecules might not be produced by the first injection of H12-(ADP)-liposomes, resulting in abrogating the induction of the ABC phenomenon in thrombocytopenic rats induced by busulphan. In fact, the suppression of anti-H12-(ADP)-liposome IgM-mediated complement activation was observed (Fig. 5).

Our present study clearly shows that H12-(ADP)-liposomes themselves did not induce the ABC phenomenon under the conditions of these experiments, that is, thrombocytopenia induced by an anticancer drug. However, predosing with topotecan, a cell-cycle phase-specific drug-containing PEGylated liposomes did induce the ABC phenomenon as the result of a second dose in mice, rats, and dogs.[33,34] In addition, it is well known that not only anticancer drugs, but also other noncytotoxic drugs are capable of inducing thrombocytopenia.[35] Furthermore, Suzuki et al.[5,36] reported that doxorubicin-encapsulated liposomes induce the ABC phenomenon in mice, rats, dogs, minipigs, and monkeys when injected repeatedly at very lower doses. Therefore, the possibility of the ABC phenomenon being induced by the repeated injection of H12-(ADP)-liposomes with the production of IgM against H12-(ADP)-liposome in patients with thrombocytopenia who take noncytotoxic drugs (including noncytotoxic anticancer drug) or low doses of a cytotoxic anticancer drug, cannot be completely excluded. It will be necessary to accumulate further evidence in these points.

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CONCLUSION

Repeated injections of H12-(ADP)-liposomes to rat models of an adaptation disease (thrombocytopenia) at the putative dose for a clinical situation (10 mg lipids/kg) did not appear to induce the ABC phenomenon accompanied with the suppression of anti-H12-(ADP)-liposome IgM-mediated complement activation. As the thrombocytopenic rats used in this study reflect the clinical features of patients with thrombocytopenia induced by anticancer drugs, a similar phenomenon would be expected in clinical situations. Therefore, the results obtained in this study suggest that, in a clinical situation, the repeated use of H12-(ADP)-liposomes would not be expected to induce the ABC phenomenon. The above findings clearly show that H12-(ADP)-liposomes have potential for the treatment in the patients with thrombocytopenia from the view point of pharmacokinetics.

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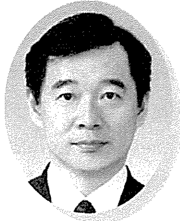
人工赤血球、人工血小板の臨床応用

防衛医科大学校免疫微生物学講座
木下　学



PROFILE
木下　学　防衛医科大学校免疫微生物学講座 准教授
Manabu Kinoshita

1988年：防衛医科大学校卒業
1994年：防衛医科大学校医学研究科(大学院、消化器外科)
1998年：自衛隊岐阜病院外科 医長
2000年：航空自衛隊第一航空団 衛生隊長(浜松)
2003年：防衛医科大学校防衛医学研究センター外傷研究部門 指定講師
2007年：現職

趣味：阪神タイガース、水泳、ジョギング

はじめに

ナノテクノロジーの進歩は社会の様々な分野に恩恵を及ぼしているが、医学領域も例外ではない。本特集で紹介されている、粒子径わずか200nm前後の脂質分子二層膜にヒトヘモグロビンを内包させた人工赤血球や、同様に脂質分子二層膜に血小板活性化物質であるアデノシン二リン酸(adenosine diphosphate：ADP)を内包し膜表面に活性化血小板と結合するフィブリノゲン活性化部位を担持させた人工血小板は、日本のナノテク科学技術の粋を集め、世界に先駆け開発に成功した血液代替物である。人工赤血球の酸素運搬体としての基本性能や、人工血小板の止血血栓形成能は、酒井、半田両氏の解説にある如く、既に動物実験では十分に満足出来るレベルに達していると思われる。しかし、残念ながら人工赤血球、人工血小板共に未だヒトへの投与は始まっていない。人工血液の今後、すなわち臨床応用について、危機管理の視点からも考えてみたい。

来るべき大震災と血液備蓄の必要性(東日本大震災の教訓から)

1923年の関東大震災、1995年には阪神・淡路大震災が、そして記憶にも新しい2011年には東日本大震災が我が国では発生している。周知のように私たちの住む日本列島は環太平洋火山帯の上にあり、かつフィリピン海プレート(西日本)や太平洋プレート(東日本)といった海側プレートが今も日本列島の太平洋岸にもぐり続け、ひずみが溜まり続けている。太平洋プレートの大きく溜まったひずみがとれ、陸側プレートが一気に跳ね上がり起こったのが東日本大震災である。未だひずみが溜まり続けているフィリピン海プレートもいずれ跳ね上がるであろうことは地球史の視点でみれば必然である。これが南海トラフ大地震であるが、その被害想定は正に想像を絶するものである。警察や消防、自衛隊などは「国民の安心安全」のためにこれらの巨大自然災害に備えなければならないが、中でも人命の救助は最優先されるべきである。大災害では多種多様な重症外傷患者が大量に発生することは容易に想像出来るが、このうち治療により救命出来る可能性が最も高い症例、いわゆるpre-ventable deathの代表例は出血性ショックである。大量失血した血液の補充(輸血)と止血制御を迅速適切に行えれば、死に至ったであろう患者を救命出来るのである。

これには災害時の輸血用血液供給体制の確立が急務であるが、東日本大震災では救助が最も必要な被災地に、救援物資が最も届きにくくなるという厳しい現実を再認識させられた。これは自衛隊の有する圧倒的な輸送能力を持ってしても例外では

なく、被災周辺地域では輸血用血液が十二分に供給されるが、真に切迫した輸血需要のある激甚な被災地域にはほとんど供給されないといった事態を招きかねない。日本人の誇るべき特性であるが、今回の東日本大震災でも震災直後、献血量は大幅に増加した。しかし、これを被災地に届ける輸送ルートが寸断されていた。巨大津波が全てを流し去った東日本大震災では輸血需要量は見掛け上、決して高くはなかったが、大震災時には外傷患者の大量発生を必ず念頭に置くべきである。被災地における医療必需品は極端な供給低下と大幅な需要増加により、深刻な欠乏状態に陥ると考えられる。被災想定地域での予めの備蓄が唯一の解決策となるが、輸血用血液に関しては保存期間が赤血球で3週間、血小板では3日間と短く、これが大きな制約となってくる。しかも、これらは災害時のpreventable deathを防ぐ最も有効な治療手段であるにもかかわらずである。そこで本特集でも紹介されている保存期間の長い人工赤血球や人工血小板に期待が寄せられる。

人工赤血球の臨床応用に向けての課題

人工赤血球には、欧米で主に開発されているヘモグロビンを裸のまま重合させただけの重合ヘモグロビン、すなわち「非細胞型人工赤血球」と、本邦で酒井氏らが開発しているヘモグロビンを脂質分子二層膜で包んだ「細胞型人工赤血球(Hemoglobin-vesicles)」の大きく分けて2つのタイプがある。ウシ由来のヘモグロビンを重合させたタイプは、既に南アフリカで認可されているが(Hemopure®、Biopure社)、急激な血圧上昇と半減期の短さによる重度の貧血が報告されていた[1]。このように既に欧米では幾つかの種類の非細胞型人工赤血球がヒトに投与されている。しかし、米国食品医薬品局(FDA)は2008年にHemopure®を含めた5種類の非細胞型人工赤血球の臨床検討結果を統合的に解析し、心筋梗塞合併率と死亡率が増加すると結論するに至った[2]。これらの副作用は、おそらくはヘモグロビンが一酸化炭素(NO)を消去することで起こる血管収縮によると考えられる。本来、ヘモグロビンは強力な酸素運搬能と共にNO消去作用も持つため、裸のままでは存在し得ず、進化の過程で脂質分子二層膜で包まれた赤血球として存在するようになったと思われる。ミミズなどの下等生物では赤血球ではなく重合した巨大ヘモグロビンを持っている。この点、ヘモグロビンを脂質分子二層膜で包み込むことで本来の赤血球の構造に近づけた細胞型人工赤血球は理想的とも言えるが、その粒子径は200nmと赤血球より遥かに小さい。粒子径を大きくし、かつ毛細血管を通過させるには赤血球のようにドーナツ型にする必要があるが、これは現在の科学技術では克服できないレベルにある。更なる半減期の延長とNO消去作用の減弱化が、今後の臨床応用に向けての鍵となるが、研究開発グループではこれらの諸問題を解決すべく取り組んでいる最中である。

骨髄輸血と人工赤血球

人工赤血球輸血が一般病棟で日常的に行われている濃厚赤血球輸血にとって代わるには、そのメリットが赤血球輸血をはるかに上回り、かつ副作用が下回らなければ難しいと推察される。しかし、前述のように災害時における使用を前提とした備蓄には、血液型に関係なく投与出来、かつ約2年間も保存可能な人工赤血球は極めて魅力的であり、危機管理の視点からもニーズは高い。

筆者らは、この細胞型人工赤血球の小さい粒子径を逆に利用して、出血性ショック時の有効な救命蘇生を考えている。医療設備の整った病院での輸血は従来の赤血球輸血で十分との意見もあり、人工赤血球はむしろ輸血サービスが望めないプレホスピタルでの、しかも致死的な出血性ショック症例患者の救命にその力を発揮すると考えている。しかし、このような状況下では末梢血管は虚脱し血管確保は至難で、これを前提とした人工赤血球の治療戦略は現実的でないと考える。そこで骨髄輸液

のように出血性ショック時の骨髄ルートからの人工赤血球の輸血を考案した。通常、赤血球はその大きさから骨髄投与は困難であるが、人工赤血球は200nmと小さく、輸液と同様にすみやかに循環系へ移行した[3]。その結果、赤血球輸血よりはるかに優れた救命効果が得られ(Fig.1)、NOの顕著な消去も認められず、実践的な使用法の1つと考えられた。

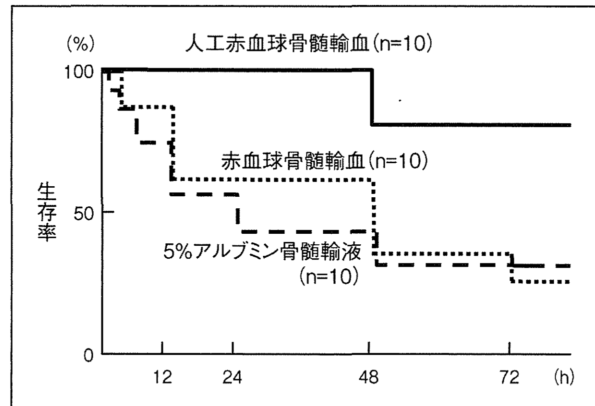


Fig.1. 人工赤血球の骨髄輸血による出血性ショックマウスの救命蘇生効果 (文献3より引用)

人工血小板による止血制御

出血性ショック症例では出血源の止血制御が救命に重要であるが、赤血球輸血には止血効果はなく、凝固因子の補充のみでも血小板減少時の止血制御には不十分で、このような病態では血小板輸血こそが有効な止血救命効果を発揮すると考える。しかし、輸血用血小板の保存は22℃という厳密な温度管理下で水平振盪させなければならない上に、保存期間もわずか4日程度と極めて短い。従って、その顕著な止血救命効果が期待されるにもかかわらず、被災想定地域での血小板の備蓄は不可能である。一方、半田氏らが開発した人工血小板は常温静置で半年間は保存可能で、備蓄に適している。しかも完全合成系で血液汚染の危険がない。出血部位に付着した活性化血小板にフィブリノゲン活性化部位を介して接着し、これを起点に血中にわずかに残った血小板を集めて血小板同士を架橋することで血栓形成を促進する。これにより最も懸念される血栓症の合併が、動物実験の段階ではあるが全く認められていない[4,5]。筆者らは、急性血小板減少病態の家兎に致死性の肝臓出血を作製し、人工血小板を輸血したが、血小板輸血と同様に100％救命出来、その顕著な止血効果を確認した(Fig.2)。人工赤血球では半減期が長い方が酸素運搬能の維持には有利であるが、人工血小板では止血完了後はすみやかに消退した方が血栓症の回避には都合がよいため、半減期は短くてよい。しかし、血中にわずかに残る血小板を集めて血栓形成を誘導することから、残存血小板の機能低下時や、血小板が全くない状態では有効な止血機能を発揮出来ない危険性がある。人工血小板の保存の簡便性と優れた止血能は特筆に値するが、出血性ショック病態で救命に直結するのはやはり赤血球の補充、すなわち輸血であることに変わりはない。今後は人工赤血球と人工血小板をその特長を巧みに活かしながら輸血する、実際の臨床に則した治療戦略を研究開発することが重要となってくるであろう。

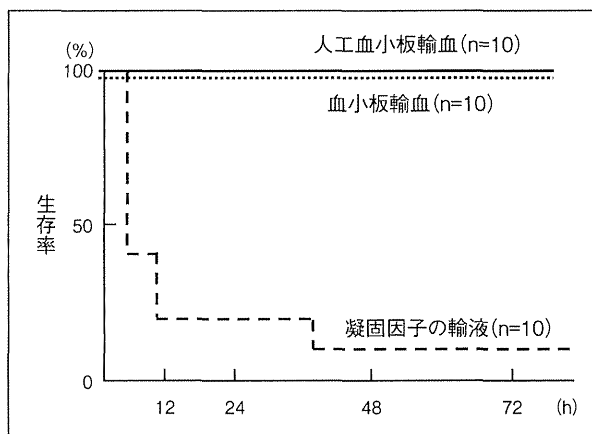


Fig.2. 人工血小板の急性血小板減少家兎での肝臓出血に対する止血救命効果（文献5より引用改変）

おわりに

人工赤血球や人工血小板は、世界に誇るジャパンオリジナルの素材であり、臨床応用が可能になると、医学が飛躍的に進歩することは間違いないであろう。また、災害時での有用性を考えると人工血液の開発は「国民の安心安全」に直結するもので、その臨床応用が社会に与える恩恵は計り知れないものがある。さらに、少子高齢化が進む日本では輸血ドナーの確保自体も困難になってくるのは想像に難くない。一日も早く、人工赤血球や人工血小板が臨床応用されることを願って止まない。

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衝撃波による致死的肺出血マウスに対する人工血小板（H12-ADP-liposome）の救命効果

防衛医科大学校生理学講座[1]、同　免疫微生物学講座[2]、同　救急部[3]、同　防衛医学研究センター情報システム研究部門[4]、日本医科大学形態解析共同研究施設[5]、早稲田大学先進理工学部[6]、防衛医科大学校・防衛医学研究センター外傷研究部門[7]
萩沢　康介[1]、木下　　学[2]、宮脇　博基[3]、佐藤　俊一[4]、鈴木　英紀[5]
土井　麻美[6]、武岡　真司[6]、小野　　聡[7]、斎藤　大蔵[7]、西田　育弘[1]

要旨

【はじめに】爆発などの衝撃波による外傷では、中空臓器である肺損傷から、致死性の肺出血を合併することが多い。われわれはレーザー誘起衝撃波を用いて、ヒトの Blast Lung Injury の病像に近似した致死性肺出血マウスモデルの作製に成功しており、今回、H12-ADP-liposome 投与が救命効果を発揮するか検討した。【方法】H12-ADP-liposome をマウスの尾静脈から投与した後に、レーザー誘起衝撃波（8 J）を右側背から照射した（n＝13）。生理食塩水投与（n＝12）、分子標的性のない ADP-liposome 投与（n＝10）、PBS-liposome 投与（n＝10）にも同様にレーザー誘起衝撃波を照射して、5日後の生存率を比較した。【結果】H12-ADP-liposome 群が他群に比して有意に生存した。病理学的検討では H12-ADP-liposome 群では生食投与群に比べて非照射側（左側）肺での出血面積が縮小していた。また気管支肺胞洗浄液中の TNF-α などの炎症性サイトカイン濃度の上昇が抑制されていた。【結論】H12-ADP-liposome の投与は、衝撃波による致死的肺出血の救命に有用である可能性が示唆された。

1.背景

Blast Lung Injury は戦傷者のとくに多発外傷で頻発する。1944 年のモンテ・カッシーノの戦闘で外見からは胸部外傷の所見なく死亡した兵士 87 名の剖検例の 34.5%で Blast Lung Injury の合併を認めた[1]。また 1969 年から 1974 年の北アイルランド紛争の犠牲者の 47%にびまん性肺挫傷を認めている[2]。そもそも肺は鼓膜に次いで爆発・衝撃波による損傷を蒙りやすい臓器である。それは衝撃圧が肺胞と毛細血管の境界を通過する際に、肺胞壁の破断を生じ、出血、肺挫傷さらには気胸・血胸・皮下気腫などを引き起こすためである。戦闘用の防弾服は、弾丸そのものや爆発物の破片による 2 次的な被害から体幹を防護する効果はあるものの、1 次的な Blast Injury すなわち衝撃圧損傷に対しては無力である[3]。

現在に至るまで Blast Lung Injury に対しての特異的な治療法は確立していない。治療ガイドラインは頸椎保護、気道確保、酸素供給、呼吸管理、出血の制御と循環管理を推奨しているに過ぎない[4]。

Blast Lung Injury の病態生理は、いくつかの急性肺傷害の病態が複合している。肺胞壁の機械的過進展という点では、人工呼吸による肺損傷（VILI）に類似している。また低酸素症/低血圧の遷延に関しては、虚血再灌流傷害モデルに類似している。これらに共通するのは肺胞への出血と滲出液・炎症細胞の浸潤である。その病態制御に、内因性アデノシン産生とアデノシン受容体活性が重要であることがわかってきた。

われわれは人工血小板として H12-ADP-liposome（平均径 210 nm）を開発してきた。これは血小板が凝集するために不可欠な血小板膜糖タンパクの IIb/IIIa 複合体をターゲットに、フィブリノゲン γ 鎖カルボキシル末端にある 12 個のペプチド（HHLGGAKQAGDV 配列：H12）をリポゾーム膜の表面に組み込むとともに、リポソーム内部にアデノシン 5′-二リン酸（ADP）を含有させている[5]。H12-

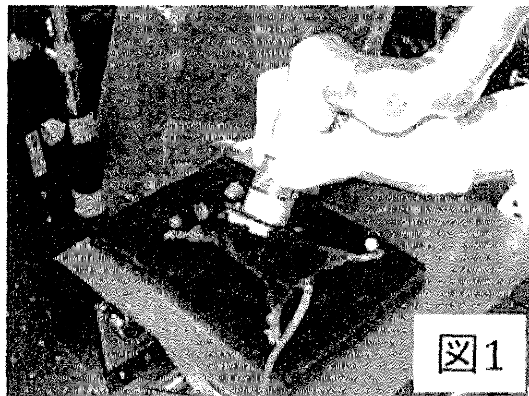


図１　マウスへのレーザー誘起衝撃波（LISW）照射

ADP-liposome は出血局所において血小板同士の凝集を補強するとともに内封していた ADP を放出する。H12-ADP-liposome（20 mg/kg）の投与で致死的な低血小板性凝固障害の止血凝固能を回復させ救命が可能になった[6]。この ADP は、P2Y 受容体を介した血小板凝集促進作用だけではなく、アデノシンに代謝され臓器保護に作用すると考えられることから、H12-ADP-liposome 投与が Blast Lung Injury の新しい治療法につながるものと考えた。本研究はヒトの Blast Lung Injury の病像に近似した致死性のマウス肺出血モデルを用いて、H12-ADP-liposome による Blast Lung Injury の低減・救命効果を検証するとともに、そのメカニズム解明を目的とした。

2.方法

防衛医科大学校動物実験倫理委員会による承認を受け、計 144 匹の C57BL/6 マウス（25＋2 g）を使用した。

1）レーザー誘起衝撃波（LISW）装置

波長 694 nm の Q スイッチ・ルビー・レーザーをターゲット（天然ゴム＋アクリル板：直径 12.0 mm・厚さ 0.5 mm）に単発照射し、プラズマ現象から衝撃波を形成した[7]。

2）LISW 強度による Blast Lung Injury の死亡閾値

マウスを剃毛後、腹臥位に固定した（図 1）。レーザー装置をマウス右側背より密着させたうえで LISW を右肺全体に照射し、Blast Lung Injury を作成した。6 J、6.5 J、7 J、7.5 J、8 J、8.5 J（n＝3 ずつ）、9 J（n＝2）の LISW 照射後、気道閉塞による窒息死を防止する体位をとりながら、60 分間呼吸状態などを観察し、回復後は飼育ケージに戻した。

3）H12-ADP-liposome 投与による救命効果

H12-ADP-liposome（n＝13）、ADP-liposome（n＝10）、PBS-liposome（n＝10）、生理食塩水（n＝12）を 120 μL 尾静脈から投与した。その後、LISW 8 J を右肺に照射し、気道閉塞による窒息死を防止する体位をとりながら、60 分間呼吸状態などを観察し、回復後は飼育ケージに戻した。

4）H12-ADP-liposome 投与による臓器保護のメカニズム解明

LISW 実験の 1 時間前に、A2A 受容体拮抗薬（ZM241385）10 mg/kg をマウスに皮下注射した（n＝6）。同様に A2B 受容体拮抗薬（PSB 1115）10 mg/kg をマウスの腹腔内に注入した（n＝6）。その後、H12-ADP-liposome 120 μL を尾静脈から投与し、LISW 8 J を右肺に照射した。気道閉塞による窒息死を防止する体位をとりながら、60 分間呼吸状態などを観察し、回復後は飼育ケージに戻した。

5) Sonoclot®による血液凝固評価と血算測定

①H12-ADP-liposome、ADP-liposome、PBS-liposome、生理食塩水 120 μL を尾静脈から投与した（n＝2 ずつ）。5 分後に腹部大動脈から採血した。

②H12-ADP-liposome（n＝6）、ADP-liposome（n＝5）、PBS-liposome（n＝5）、生理食塩水（n＝6）を 120 μL、尾静脈から投与した。5 分後に LISW 8 J を照射してただちに腹部大動脈から採血した。

いずれのサンプルも血算測定とともに、Sonoclot®（Sienco、CO）を使用し、凝集から凝固開始までの時間を反映する clotting time（CT）と凝固の完成を示す clotting rate（CR）を測定し、止血凝固能を総合的に評価した[6]。

6) 病理組織学的検討

H12-ADP-liposome、生理食塩水を 120 μL 尾静脈から投与した（n＝5 ずつ）。5 分後に LISW 8 J を照射。3 時間後にマウスの肺、心臓、肝臓、脾臓および腎臓を病理学検査用に採取した。但し生理食塩水投与群は観察中死亡した場合、ただちに検体を採取。HE 染色を行って、定量評価として Yelvertons らの Pathological injury スコア[8]を修正して、使用した。

Pathological injury スコア＝(E＋G＋ST)×(SD)　64 点満点

- Extent：肺の受傷面積として、いくつの肺葉に損傷が及んでいるか（1～7 点）
- Grade：病変の重症度として、各肺葉の何割が受傷しているか（1～4 点）
- Severity Type：出血や損傷の重症度として、損傷は散在性か融合性であるか（1～5 点）
- Severity Depth/Disruption：深度として、損傷は胸膜・実質まで及んでいるか（1～4 点）

7) 電子顕微鏡による観察

H12-ADP-liposome、生理食塩水を 120 μL 尾静脈から投与した（n＝2 ずつ）。LISW の 24 時間後に採取した。但し生理食塩水投与群は観察中死亡した場合、その時点で検体を採取した。

8) 気管支肺胞洗浄液（BAL）分析

H12-ADP-liposome（n＝7）、ADP-liposome（n＝3）、PBS-liposome（n＝3）、生理食塩水（n＝3）、H12-ADP-liposome＋ZM241385（n＝4）、H12-ADP-liposome＋PSB 1115（n＝3）、前述のとおりそれぞれの薬物を投与してから LISW 8 J を右肺に照射し、3 時間後にマウスから BAL 液を採取した。BAL 液中アルブミン濃度は Bradford 法（Bio Rad）で測定した。BAL 液中の TNF-α および MIP-2 濃度は ELISA 法（R & D Systems）で定量した。

9) 統計解析

生存率は Wilcoxon signed rank test によって比較した。2 群間の統計は Student's *t* test を使用した。他は ANOVA を使用し、Bonferroni post-hoc test を行った。いずれも $p<0.05$ を統計的有意とした。

3. 結果

胸部 X 線は Blast Lung Injury 出血により右肺に浸潤影を示した。びまん性の出血が右葉および左肺門部に生じた（図 2 破線円内）。融合性の出血が、右肺下葉に生じた。LISW 強度による死亡閾値の検討では、8 J 以上で鼻出血（喀血）ありのものはすべて 1 時間以内に死亡した。よって以後の生存率の検討では鼻出血（喀血）のない場合を除外した。

1) 生存率比較

図 3 に示すように、H12-ADP-liposome 群が他群に比して有意に生存率が高かった。H12-ADP-liposome（9/13）、ADP-liposome（2/10）、PBS-liposome（0/10）、生理食塩水（1/12）であった。アデノシン受容体拮抗薬は、H12-ADP-liposome による救命効果を阻害した。（ZM241385：0/6、

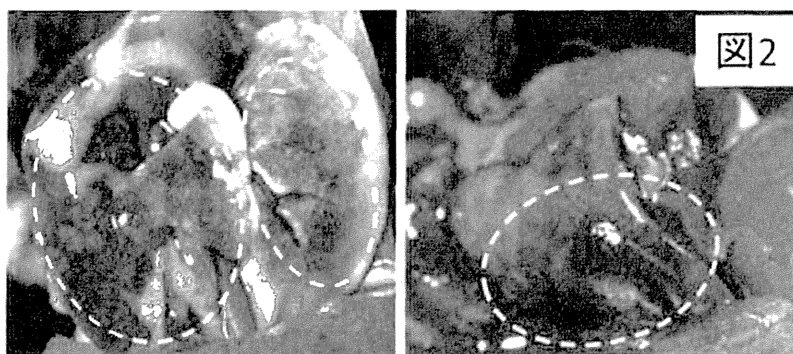
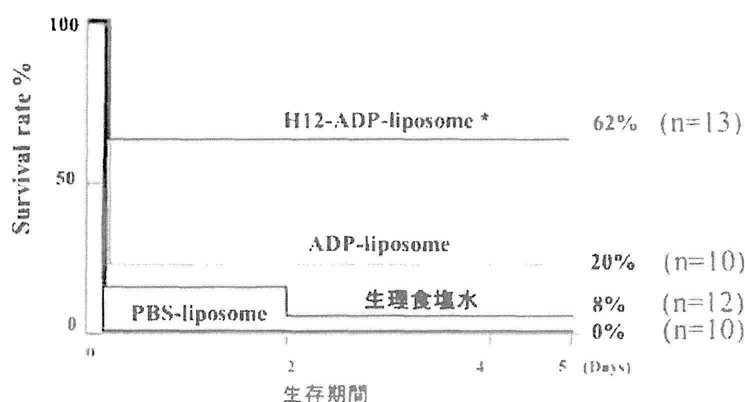


図２　Blast Lung Injury のマクロ像



* p=0.014, mxn Chi square and Yates test

図３　予後の比較

表１　血算ならびに血液凝固機能データ

	LISW (−) (n=6)	Normal Saline (n=6)	H12-(ADP) -liposomes (n=6)	(ADP) -liposomes (n=5)	(PBS) -liposomes (n=5)
WBC count (×10³/μL)	5.6±2.1	2.1±2.4*	4.0±2.2	4.8±1.8	5.3±1.7
RBC count (×10⁶/μL)	8.0±0.7	5.5±2.2**	8.3±1.0	8.0±0.5	7.4±1.1
Hemoglobin concentrations (g/dL)	12.8±1.4	8.6±3.7*	13.6±3.1	13.3±1.2	13.1±1.8
Platelet counts (×10³/μL)	238±71†	99±54	111±29	109±19	99±23
Clotting time (CT) (sec)	65±14†	131±79	134±58	119±33	129±19
Clotting rate (CR)	32.5±5.5†	15.0±6.7	14.0±6.1	19.2±8.1	14.70±1.4

Hematological parameters and coagulation factors/activities in mice after LISW exposure (followed by administration of Normal Saline, H12-(ADP)-liposomes, (ADP)-liposomes or (PBS)-liposomes.) Data are mean ±SD, *p<0.05, **p<0.01 vs. LISW (−), H12-(ADP)-liposomes, (ADP)-liposomes or (PBS)-liposomes. †p <0.01 vs. Normal Saline, H12-(ADP)-liposomes, (ADP)-liposomes or (PBS)-liposomes

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2) Sonoclot®による血液凝固評価と血算

LISW（－）すなわち Blast Lung Injury なしでは、H12-ADP-liposome、ADP-liposome、PBS-liposome、生理食塩水のいずれを投与した場合でも Sonoclot®波形に相違はなく、CT や CR は対照

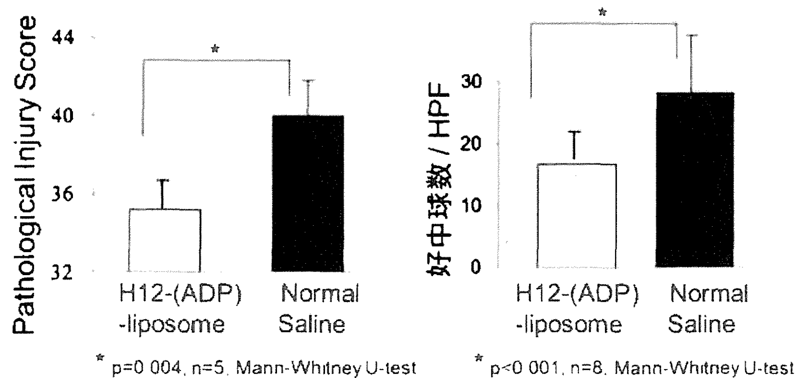

図４　肺病理の定量評価

群と変化なかった。LISW による Blast Lung Injury ありでは、H12-ADP-liposome、ADP-liposome、PBS-liposome、生理食塩水のいずれを投与した場合でも同様に CT が２倍に延長し CR は半減した。血小板数も半減した（表１）。

3）病理組織所見

肺以外の心臓、肝臓、脾臓および腎臓には血栓症などの異常所見はなかった。肉眼所見としては、どちらの群でも右肺には融合性の出血が生じた。左肺では、生理食塩水投与群で融合性出血が生じた一方で、H12-ADP-liposome 投与群では、軽度の散在性出血がみられるに過ぎなかった。Pathological injury スコアは H12-ADP-liposome 投与群（40.0＋2.0）が生理食塩水投与群（35.2＋2.3）に対して有意に軽減していた（図４左）。さらに、肺胞における１視野あたりの好中球数についても、H12-ADP-liposome 投与群（16.7＋6.2）が生理食塩水投与群（28.4＋8.5）に対して有意に軽減していた（図４右）。

4）電子顕微鏡所見

肺胞壁の毛細血管の変形・破断とともに肺胞内に赤血球が漏出していた（図 5A）。好中球浸潤も認められた（図 5B）。H12-ADP-liposome 投与群では毛細血管内で血小板と結合する H12-ADP-liposome 像が認められた（図 5C）。

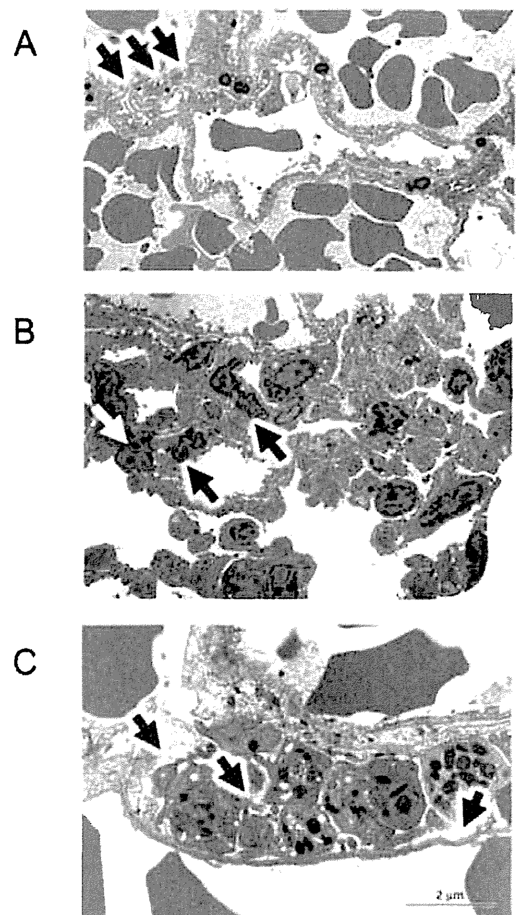

図５　肺透過型電子顕微鏡像

5）BAL 分析

LISW 照射３時間後の BAL 液中のアルブミン漏出は H12-ADP-liposome 投与群においてのみ有意に減少していた（図 6A）。さらに H12-ADP-liposome 投与は、BAL 液中の TNF-α および MIP-2 の過剰発現を有意に軽減した。アデノシン A2A 受容体拮抗薬は、H12-ADP-liposome 投与による BAL 液中の TNF-α 減少効果を阻害した。アデノシン A2B 受容体拮抗薬は H12-ADP-liposome 投

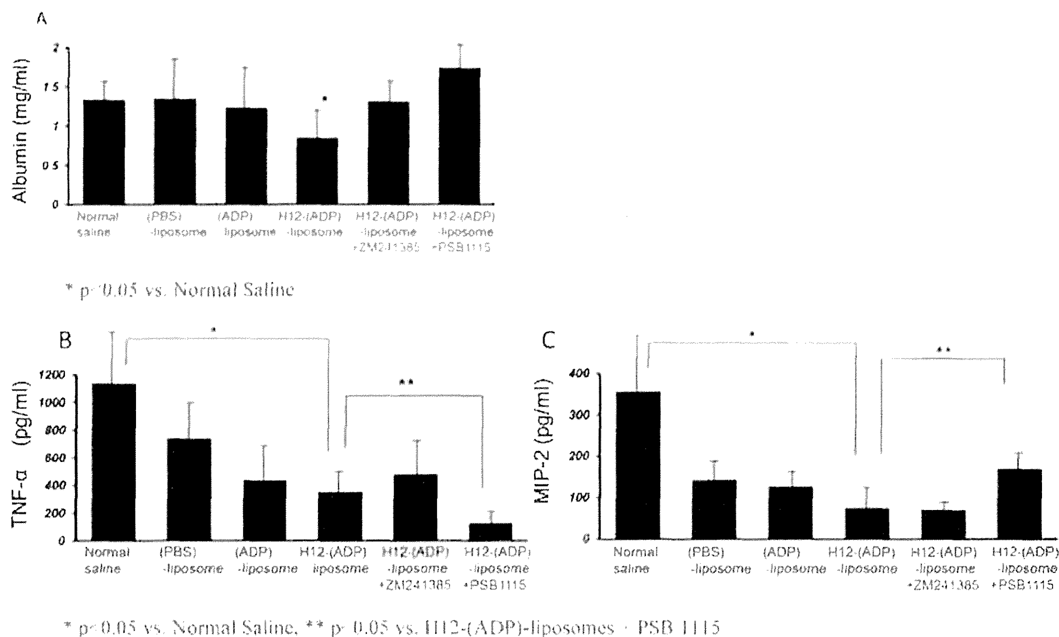

図６　気管支肺胞洗浄液解析結果

与によるBAL液中のMIP-2の減少効果を阻害した（図6B、C)。

4.考察

LISW衝撃波による致死性肺出血マウスにおいて、H12-ADP-liposomeは肺出血を軽減し、生存率を増加させた。病理学的には肺胞壁の伸展と毛細血管の破壊に伴う肺胞出血の所見は、Tsokosらによって報告されたヒトのBlast Lung Injuryの病理所見[9]と合致した。

Yelvertonsらは Pathological injury スコア36点以上を重症肺傷害と定義している[8]が、今回H12-ADP-liposome投与により、36点以下に軽減できており、生命予後を改善したことと合致している。LISW照射側の右肺はH12-ADP-liposome群でも生理食塩水投与群と同様に、融合性の出血が臓器の深部に亘り、所見に差異はない。むしろ非照射側である左肺の出血が限局されたことが予後の改善につながっていると推察される。これはLISWの衝撃波は経気道的に反対側の肺胞を傷害すると同時に、照射側肺の肺胞マクロファージから放出された炎症性サイトカインが反対側の肺胞上皮、毛細血管に作用することをH12-ADP-liposomeが抑制しているものと考えられた。すなわち、H12-ADP-liposomeは本来の止血作用とともに、局所で放出されるADPが病変部で代謝されアデノシン作用を示すのではないかと考えられる。一般に、生理学的濃度でアデノシンはA1、A2AおよびA3受容体を活性化する。対照的に、アデノシンA2B受容体は高濃度のアデノシンで作用すると考えられている[10]。出血性ショックではアデノシンは病変局所で細胞外に放出される。とくに低酸素病態を伴う場合にアデノシンA2B受容体作用が活性化することが報告されている[11]。

今回、アデノシン受容体拮抗薬を使った薬理学的検討では、Blast Lung Injuryによる好中球誘導性ケモカインであるMIP-2放出亢進と好中球の遊走浸潤をH12-ADP-liposome投与がA2B受容体を介して抑制し、急性期の予後の改善につながっていたことが示唆された。しかしながら、炎症性サイトカインのTNF-αについては確定的なデータは得られなかった。

このBlast Lung Injuryモデルでは急性肺傷害の病態が複合している。肺胞壁の機械的過進展に関

しては、人工呼吸による肺外傷（VILI）に類似している。また低酸素症/低血圧の遷延に関しては、虚血再灌流傷害モデルに類似している。VILI に関しては、Eckle らが肺胞・毛細血管バリアの欠損が肺の A2B 受容体を介して減じられると報告した[12]。さらに、A2B 受容体を介して VILI による毛細血管から肺胞への vascular leak を軽減することに寄与し、A2B 受容体作動薬がある種の「肺利尿」効果を示し、肺胞クリアランスが改善して臓器保護につながっていると述べている[12]。さらに Eckle らは低酸素換気モデルで生じる肺胞への好中球の遊走浸潤が A2B 受容体を介して抑制されることも報告している[11]。

Haskó らは、A2A 受容体を介するアデノシンの外傷/出血性ショック軽減効果を報告している[13]。一般的にも A2A 受容体を介したアデノシンの抗炎症効果の報告が多い。しかしながら、衝撃波により誘起された臓器傷害病態におけるアデノシンの役割は不明な点が多い。血漿中のアデノシンの半減期が非常に短い（1～2 秒）ことが、外因性に投与したアデノシンの実験結果の解釈を難しくしている。本研究では H12-ADP-liposome の止血作用が病態にどの程度貢献していたかは不明であり、今後さらなる検討が必要である。

結論

H12-ADP-liposome 投与は衝撃波による致死的肺出血の救命に有用である可能性が示唆された。

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