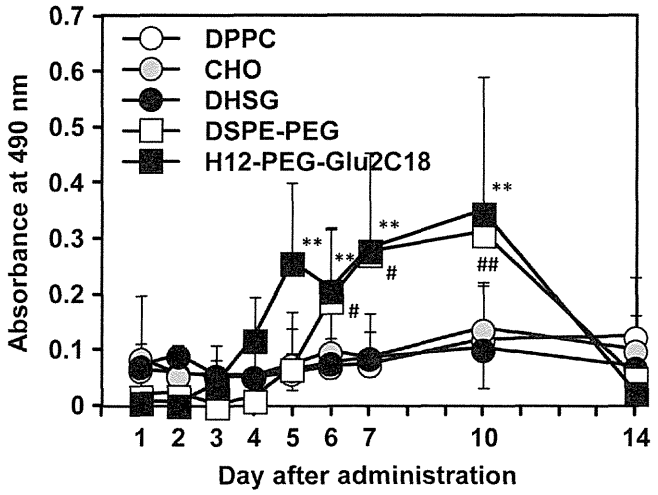

Figure 3. Determination of the specific recognition site of IgM against H12-(ADP)-liposome after a single intravenous injection of H12-(ADP)-liposome at a dose of 10 mg lipid/kg in healthy rats. Each point represents the mean ± SD ($n = 4$). $^{**}p < 0.01$ versus 1 day of H12-PEG-Glu2C18, $^{\#\#}p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

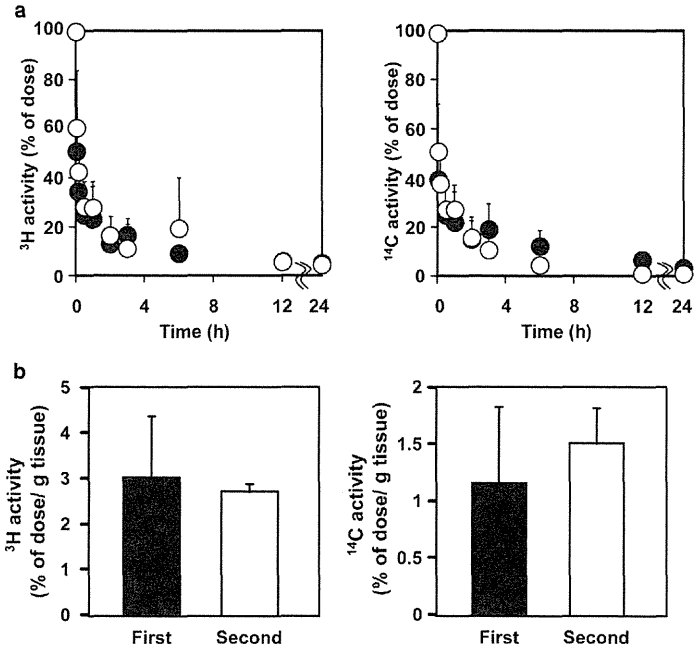

Figure 4. (a) Plasma concentration curve of ^{14}C and ^{3}H-radiolabeled H12-(ADP)-liposome after the first injection (closed symbol) or the second injection (open symbol) of ^{3}H, ^{14}C-radiolabeled H12-(ADP)-liposome at a dose of 10 mg lipid/kg to busulphan-induced thrombocytopenic model rats. (b) 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. ($n = 4$).

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.

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.

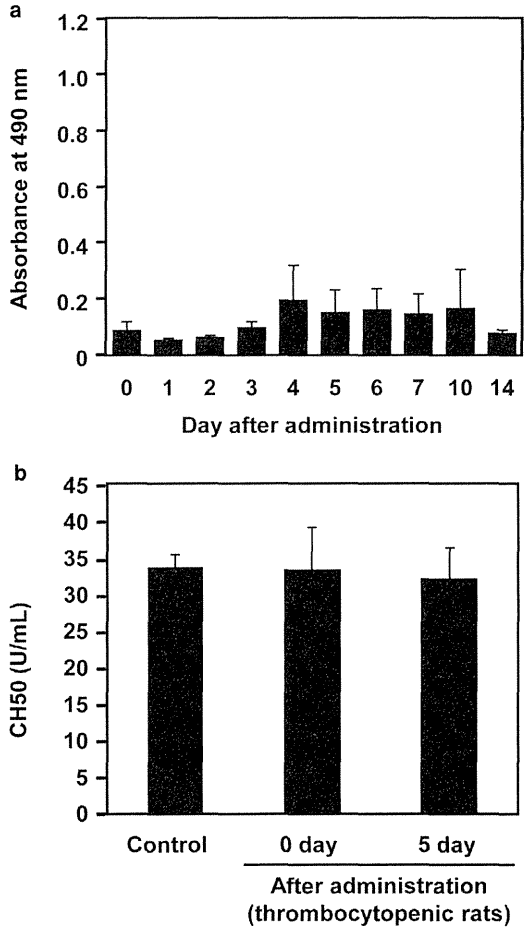

Figure 5. (a) Determination of IgM against H12-(ADP)-liposomes after a single intravenous injection of H12-(ADP)-liposomes at a dose of 10 mg lipid/kg in busulphan-induced thrombocytopenic model rats. (b) CH50 values in busulphan-induced thrombocytopenic model rats before the administration of busulphan (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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REFERENCES

1. Verkade HJ, Derksen JT, Gerding A, Scherphof GL, Vonk RJ, Kuipers F. 1991. Differential hepatic processing and biliary secretion of head-group and acyl chains of liposomal phosphatidylcholines. Biochem J 275 (Pt 1):139–144.

2. McGahan L. 2008. Continuous erythropoietin receptor activator (Mircera) for renal anemia. Issues Emerg Health Technol (113):1–6.

3. James ND, Coker RJ, Tomlinson D, Harris JR, Gompels M, Pinching AJ, Stewart JS. 1994. Liposomal doxorubicin (Doxil): An effective new treatment for Kaposi's sarcoma in AIDS. Clin Oncol (R Coll Radiol) 6(5):294–296.

4. Dams ET, Laverman P, Oyen WJ, Storm G, Scherphof GL, van Der Meer JW, Corstens FH, Boerman OC. 2000. Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. J Pharmacol Exp Ther 292(3):1071–1079.

5. Suzuki T, Ichihara M, Hyodo K, Yamamoto E, Ishida T, Kiwada H, Ishihara H, Kikuchi H. 2012. Accelerated blood clearance of PEGylated liposomes containing doxorubicin upon repeated administration to dogs. Int J Pharm 436(1–2):636–643.

6. Taguchi K, Urata Y, Anraku M, Watanabe H, Kadowaki D, Sakai H, Horinouchi H, Kobayashi K, Tsuchida E, Maruyama T, Otagiri M. 2009. Hemoglobin vesicles, polyethylene glycol (PEG)ylated liposomes developed as a red blood cell substitute, do not induce the accelerated blood clearance phenomenon in mice. Drug Metab Dispos 37(11):2197–2203.

7. Xu H, Ye F, Hu M, Yin P, Zhang W, Li Y, Yu X, Deng Y. 2014. Influence of phospholipid types and animal models on the accelerated blood clearance phenomenon of PEGylated liposomes upon repeated injection. Drug Deliv. [doi:10.3109/10717544.2014.885998]

8. Abu Lila AS, Ichihara M, Shimizu T, Ishida T, Kiwada H. 2013. Ex-vivo/in-vitro anti-polyethylene glycol (PEG) immunoglobulin M production from murine splenic B cells stimulated by PEGylated liposome. Biol Pharm Bull 36(11):1842–1848.

9. Shimizu T, Ishida T, Kiwada H. 2013. Transport of PEGylated liposomes from the splenic marginal zone to the follicle in the induction phase of the accelerated blood clearance phenomenon. Immunobiology 218(5):725–732.

10. Armstrong JK, Hempel G, Koling S, Chan LS, Fisher T, Meiselman HJ, Garratty G. 2007. Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. Cancer 110(1):103–111.

11. Liu Y, Reidler H, Pan J, Milunic D, Qin D, Chen D, Vallejo YR, Yin R. 2011. A double antigen bridging immunogenicity ELISA for the detection of antibodies to polyethylene glycol polymers. J Pharmacol Toxicol Methods 64(3):238–245.

12. Okamura Y, Eto K, Maruyama H, Handa M, Ikeda Y, Takeoka S. 2010. Visualization of liposomes carrying fibrinogen gamma-chain dodecapeptide accumulated to sites of vascular injury using computed tomography. Nanomedicine 6(2):391–396.

13. Tokutomi K, Tagawa T, Korenaga M, Chiba M, Asai T, Watanabe N, Takeoka S, Handa M, Ikeda Y, Oku N. 2011. Decoration of fibrinogen gamma-chain peptide on adenosine diphosphate-encapsulated liposomes enhances binding of the liposomes to activated platelets. Int J Pharm 407(1–2):151–157.

14. Okamura Y, Katsuno S, Suzuki H, Maruyama H, Handa M, Ikeda Y, Takeoka S. 2010. Release abilities of adenosine diphosphate from phospholipid vesicles with different membrane properties and their hemostatic effects as a platelet substitute. J Control Release 148(3):373–379.

15. Okamura Y, Takeoka S, Eto K, Maekawa I, Fujie T, Maruyama H, Ikeda Y, Handa M. 2009. Development of fibrinogen gamma-chain peptide-coated, adenosine diphosphate-encapsulated liposomes as a synthetic platelet substitute. J Thromb Haemost 7(3):470–477.

16. Taguchi K, Ujihira H, Ogaki S, Watanabe H, Fujiyama A, Doi M, Okamura Y, Takeoka S, Ikeda Y, Handa M, Otagiri M, Maruyama T. 2013. Pharmacokinetic study of the structural components of adenosine diphosphate-encapsulated liposomes coated with fibrinogen gamma-chain dodecapeptide as a synthetic platelet substitute. Drug Metab Dispos 41(8):1584–1591.

17. Taguchi K, Ujihira H, Watanabe H, Fujiyama A, Doi M, Takeoka S, Ikeda Y, Handa M, Otagiri M, Maruyama T. 2013. Pharmacokinetic study of adenosine diphosphate-encapsulated liposomes coated with fibrinogen gamma-chain dodecapeptide as a synthetic platelet substitute in an anticancer drug-induced thrombocytopenia rat model. J Pharm Sci 102(10):3852–3859.

18. Hagisawa K, Nishikawa K, Yanagawa R, Kinoshita M, Doi M, Suzuki H, Iwaya K, Saitoh D, Seki S, Takeoka S, Handa M, Nishida Y. 2014. Treatment with fibrinogen gamma-chain peptide-coated, adenosine 5'-diphosphate-encapsulated liposomes as an infusible hemostatic agent against active liver bleeding in rabbits with acute thrombocytopenia. Transfusion55(2):314-325.

19. Nishikawa K, Hagisawa K, Kinoshita M, Shono S, Katsuno S, Doi M, Yanagawa R, Suzuki H, Iwaya K, Saitoh D, Sakamoto T, Seki S, Takeoka S, Handa M. 2012. Fibrinogen gamma-chain peptide-coated, ADP-encapsulated liposomes rescue thrombocytopenic rabbits from non-compressible liver hemorrhage. J Thromb Haemost 10(10):2137–2148.

20. Okamura Y, Maekawa I, Teramura Y, Maruyama H, Handa M, Ikeda Y, Takeoka S. 2005. Hemostatic effects of phospholipid vesicles carrying fibrinogen gamma chain dodecapeptide in vitro and in vivo. Bioconjug Chem 16(6):1589–1596.

21. Mayer M. 1961. Complement and complement fixation. 2nd ed. Springfield, Massachusetts: Thomas, pp 133–240.

22. Abu Lila AS, Kiwada H, Ishida T. 2013. The accelerated blood clearance (ABC) phenomenon: Clinical challenge and approaches to manage. J Control Release 172(1):38–47.

23. Ishida T, Ichihara M, Wang X, Yamamoto K, Kimura J, Majima E, Kiwada H. 2006. Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes. J Control Release 112(1):15–25.

24. Ishida T, Wang X, Shimizu T, Nawata K, Kiwada H. 2007. PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner. J Control Release 122(3):349–355.

25. Hashimoto Y, Shimizu T, Mima Y, Abu Lila AS, Ishida T, Kiwada H. 2014. Generation, characterization and in vivo biological activity

of two distinct monoclonal anti-PEG IgMs. Toxicol Appl Pharmacol 277(1):30–38.

26. Cheng TL, Chen BM, Chern JW, Wu MF, Roffler SR. 2000. Efficient clearance of poly(ethylene glycol)-modified immunoenzyme with anti-PEG monoclonal antibody for prodrug cancer therapy. Bioconjug Chem 11(2):258–266.

27. Cheng TL, Wu PY, Wu MF, Chern JW, Roffler SR. 1999. Accelerated clearance of polyethylene glycol-modified proteins by anti-polyethylene glycol IgM. Bioconjug Chem 10(3):520–528.

28. Abu Lila AS, Nawata K, Shimizu T, Ishida T, Kiwada H. 2013. Use of polyglycerol (PG), instead of polyethylene glycol (PEG), prevents induction of the accelerated blood clearance phenomenon against long-circulating liposomes upon repeated administration. Int J Pharm 456(1):235–242.

29. Abu Lila AS, Uehara Y, Ishida T, Kiwada H. 2014. Application of polyglycerol coating to plasmid DNA lipoplex for the evasion of the accelerated blood clearance phenomenon in nucleic acid delivery. J Pharm Sci 103(2):557–566.

30. Laverman P, Carstens MG, Boerman OC, Dams ET, Oyen WJ, van Rooijen N, Corstens FH, Storm G. 2001. Factors affecting the accelerated blood clearance of polyethylene glycol-liposomes upon repeated injection. J Pharmacol Exp Ther 298(2):607–612.

31. Ishida T, Atobe K, Wang X, Kiwada H. 2006. Accelerated blood clearance of PEGylated liposomes upon repeated injections: Effect of doxorubicin-encapsulation and high-dose first injection. J Control Release 115(3):251–258.

32. Ten Brink MH, Zwaveling J, Swen JJ, Bredius RG, Lankester AC, Guchelaar HJ. 2014. Personalized busulfan and treosulfan conditioning for pediatric stem cell transplantation: The role of pharmacogenetics and pharmacokinetics. Drug Discov Today 19(10):1572–1586.

33. Li C, Cao J, Wang Y, Zhao X, Deng C, Wei N, Yang J, Cui J. 2012. Accelerated blood clearance of pegylated liposomal topotecan: Influence of polyethylene glycol grafting density and animal species. J Pharm Sci 101(10):3864–3876.

34. Ma Y, Yang Q, Wang L, Zhou X, Zhao Y, Deng Y. 2012. Repeated injections of PEGylated liposomal topotecan induces accelerated blood clearance phenomenon in rats. Eur J Pharm Sci 45(5):539–545.

35. Thushara RM, Hemshekhar M, Kemparaju K, Rangappa KS, Devaraja S, Girish KS. 2014. Therapeutic drug-induced platelet apoptosis: An overlooked issue in pharmacotoxicology. Arch Toxicol 88(2):185–198.

36. Suzuki T, Ichihara M, Hyodo K, Yamamoto E, Ishida T, Kiwada H, Kikuchi H, Ishihara H. 2014. Influence of dose and animal species on accelerated blood clearance of PEGylated liposomal doxorubicin. Int J Pharm 476(1–2):205–212.

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Treatment with fibrinogen γ-chain peptide-coated, adenosine 5′-diphosphate–encapsulated liposomes as an infusible hemostatic agent against active liver bleeding in rabbits with acute thrombocytopenia

Kohsuke Hagisawa,[1] Kahoko Nishikawa,[2] Rempei Yanagawa,[3] Manabu Kinoshita,[4] Mami Doi,[5] Hidenori Suzuki,[6] Keiichi Iwaya,[7] Daizoh Saitoh,[8] Shuhji Seki,[4] Shinji Takeoka,[5] Makoto Handa,[9] and Yasuhiro Nishida[1]

BACKGROUND: We evaluated the hemostatic efficacy of H12-(adenosine 5′-diphosphate [ADP])-liposomes in the setting of active liver bleeding in rabbits with dilutional thrombocytopenia after massive transfusion.
STUDY DESIGN AND METHODS: Acute thrombocytopenia (platelet [PLT] count < 50 × 10^9/L) was induced in rabbits by repeated blood withdrawal and isovolemic transfusion of autologous washed red blood cells. Liver hemorrhage was initiated by a penetrating liver injury. Subsequently, the animals received tamponade treatment for the liver hemorrhage for 5 minutes and were intravenously administered H12-(ADP)-liposomes with PLT-poor plasma (PPP), PLT-rich plasma (PRP), PPP alone, H12-(phosphate-buffered saline [PBS])-liposome/PPP, or H12-(ADP)-liposomes/PPP plus fibrinogen concentrate during the tamponade.
RESULTS: Administration of H12-(ADP)-liposomes/PPP rescued 60% of the rabbits from the liver hemorrhage; PRP administration rescued 50%. In contrast, rabbits receiving PPP or H12-(PBS)-liposome/PPP achieved only 10 or 17% survival, respectively, for the first 24 hours. H12-(ADP)-liposomes/PPP as well as PRP consistently reduced bleeding volumes and shortened clotting times (CTs) in comparison to PPP administration. Specifically, bleeding volumes in the initial 5 minutes averaged 11 mL (H12-(ADP)-liposomes/PPP) and 17 mL (PRP) versus 30 mL (PPP; $p < 0.05$); CTs averaged 270 and 306 seconds versus 401 seconds ($p < 0.05$). H12-(ADP)-liposomes were observed at the bleeding site with thrombus formation, suggesting an induction of thrombi. Neither macro- nor microthrombi were detected in the lung, kidney, spleen, or liver in rabbits treated with H12-(ADP)-liposomes. Supplementation of fibrinogen to H12-(ADP)-liposomes/PPP did not significantly improve rabbit survival.
CONCLUSIONS: H12-(ADP)-liposomes might be a safe and effective therapeutic tool during damage control surgery for trauma patients with acute thrombocytopenia and massive bleeding.

Fluid resuscitation after massive hemorrhage in severe trauma might result in extensive hemodilution and coagulopathy.[1] Coagulopathy, hypothermia, and acidosis are identified as a lethal triad for patients presenting with exsanguinating

ABBREVIATIONS: APTT = activated partial thromboplastin time; AT = antithrombin; CR(s) = clotting rate(s); CT(s) = clotting time(s); MAP = mean arterial pressure; PPP = platelet-poor plasma; PRP = platelet-rich plasma; PT(s) = prothrombin time(s).

From the [1]Department of Physiology, the [2]Department of Traumatology and Critical Care Medicine, the [3]Department of Defense Medicine, the [4]Department of Immunology and Microbiology, and the [7]Department of Basic Pathology, National Defense Medical College, Tokorozawa, Saitama, Japan; the [5]Department of Life Science and Medical Bioscience, Graduate School of Advanced Science and Engineering, Waseda University, TWIns, and the [6]Division of Morphological and Biomolecular Research, Graduate School of Medicine, Nippon Medical School, Tokyo, Japan; the [8]Division of Traumatology, National Defense Medical College Research Institute, Tokorozawa, Japan; and the [9]Department of Transfusion Medicine and Cell Therapy, School of Medicine, Keio University, Tokyo, Japan.

Address reprint requests to: Manabu Kinoshita, Department of Immunology and Microbiology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan; e-mail: manabu@ndmc.ac.jp.

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hemorrhage.[2] Platelet (PLT) transfusion is more effective for management of actual coagulopathy involved in trauma injury or surgery than the transfusion with fresh-frozen plasma (FFP), cryoprecipitate, desmopressin, or recombinant activated Factor VII.[3-5] The current US military resuscitation practice is to use a balanced approach, using FFP, PLTs, and red blood cells (RBCs; 1:1:1) as the primary resuscitation fluid for the most seriously injured casualties.[5,6] Nevertheless, FFP is usually preferred over PLTs for the treatment of coagulopathy after massive transfusion because, from a logistic point of view, the availability of PLT concentrates is restricted due to their short shelf life.[7] PLT concentrates expire within several days in spite of shaking preservation at 22°C.

We have developed liposome-based artificial PLTs bearing synthetic HHLGGAKQAGDV (H12) peptides corresponding to the carboxyl terminal of the fibrinogen γ-chain on the surface. The liposomes also contain the physiologic PLT agonist adenosine 5′-diphosphate (ADP) inside.[8] Preliminary observations indicate that this compound can be stored for at least 6 months at 4°C without shaking (S. Takeoka et al., unpublished observation, 2014). We previously demonstrated that H12-(ADP)-liposomes were a synthetic PLT substitute preventing uncontrollable traumatic hemorrhage confounded by acute thrombocytopenia after massive RBC transfusion.[9] However, the administration of H12-(ADP)-liposomes preceded liver injury by 30 minutes in that study. Thus it did not precisely simulate a clinical situation in which PLT transfusion starts after active bleeding. In this study, we examined the hemostatic effects of H12-(ADP)-liposomes after the occurrence of acute bleeding from liver injury.

MATERIALS AND METHODS

This study was conducted according to the guidelines of the institutional review board for the Care of Animal Subjects of the National Defense Medical College. The institutional review board approved this study and the ethical approval number was Number 13042.

Rabbits and reagents

A total of 68 New Zealand white rabbits (2.0 ± 0.2 kg, male; Japan SLC, Hamamatsu, Japan) were used in this study as follows: 52 rabbits for monitoring survival, 12 for pathologic examination, and four for electron microscopic observation. Cholesterol and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine were purchased from Nippon Fine Chemical (Osaka, Japan), 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[monomethoxypoly(ethylenglycol)] (PEG-DSPE, 5.1 kDa) was obtained from NOF (Tokyo, Japan), and adenosine 5′-diphosphate (ADP) was from Sigma-Aldrich (St Louis, MO). We synthesized 1,5-dihexadecyl-*N*-succinyl-L-glutamine (DHSG) and H12-PEG-Glu2C18. The fibrinogen γ-chain dodecapeptide (C-HHLGGAKQAGDV, Cys-H12) was conjugated to the end of the PEG-lipids, as described elsewhere.[10]

Preparation of H12-(ADP)-liposomes

H12-(ADP)-liposomes were prepared as described elsewhere.[10] Briefly, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (1 g, 1.36 mmol), cholesterol (527 mg, 1.36 mmol), DHSG (189 mg, 272 μmol), PEG-DSPE (52 mg, 9 μmol), and H12-PEG-Glu2C18 (47 mg, 9 μmol) were dissolved in benzene and freeze-dried. The resulting mixed lipids were hydrated with phosphate-buffered saline (PBS) containing 1 mmol/L ADP with filter membranes (pore size, 0.45, 0.22 μm; Millipore, Tokyo, Japan) to prepare H12-(ADP)-liposomes. After liposomes were washed with PBS followed by centrifugation (100,000 × *g*, 30 min, 4°C), the remaining ADP was removed using gel filtration medium (Sephadex G25, GE Healthcare, Tokyo, Japan). H12 liposomes without ADP (H12-(PBS)-liposome) were also prepared by skipping ADP encapsulation.

Acute thrombocytopenic rabbit model

Acute thrombocytopenia was induced in rabbits, as described elsewhere.[9] Briefly, rabbits were anesthetized using intramuscular injections of ketamine (25 mg/kg) and xylazine (10 mg/kg), followed by maintaining anesthesia with intravenous (IV) injections of pentobarbital (15 mg/kg) every 30 minutes during the experiment. The adequacy of anesthesia was monitored by the loss of the ear pinch reflex. Anaesthetized rabbits were placed on a warming plate to maintain the body temperature at 37°C. Aseptic techniques were adopted for all surgical procedures. Surgical catheters (polyethylene indwelling 20-gauge needle; Terumo Co., Tokyo, Japan) were inserted into the femoral artery and vein in each rabbit (Fig. 1). Thereafter, 12.5 mL/kg blood (Sample 1) was drawn from the femoral artery, and the same volume of dextran 40 (308 mOsm/L, Otsuka, Tokushima, Japan) was simultaneously transfused via the femoral vein (Fig. 2). Forty minutes later, the next blood sample (12.5 mL/kg, Sample 2) was withdrawn and the same volume of washed RBCs prepared using Sample 1 was transfused. This isovolemic blood exchange was repeated eight times, and the PLT counts were approximately fewer than 50×10^9/L. The last transfusion of washed RBCs was performed without simultaneous blood withdrawal (Fig. 2). Arterial pH was spontaneously maintained at 7.35 to 7.45 in rabbits.

Preparation of washed RBCs, PLT-rich plasma, and PLT-poor plasma

Blood samples drawn with a 10% volume of 3.8% (wt/vol) sodium citrate were centrifuged at 100 × *g* for 15 minutes,

and the supernatant was used as PLT-rich plasma (PRP; Fig. 1). The remaining sample was further centrifuged at 500 × *g* for 10 minutes and the supernatant was used as PLT-poor plasma (PPP). Thereafter, remaining cells were washed with saline, diluted in 12.5 mL/kg lactated Ringer's solution containing 5% human serum albumin, and transfused into the rabbit as washed RBCs (Fig. 1).

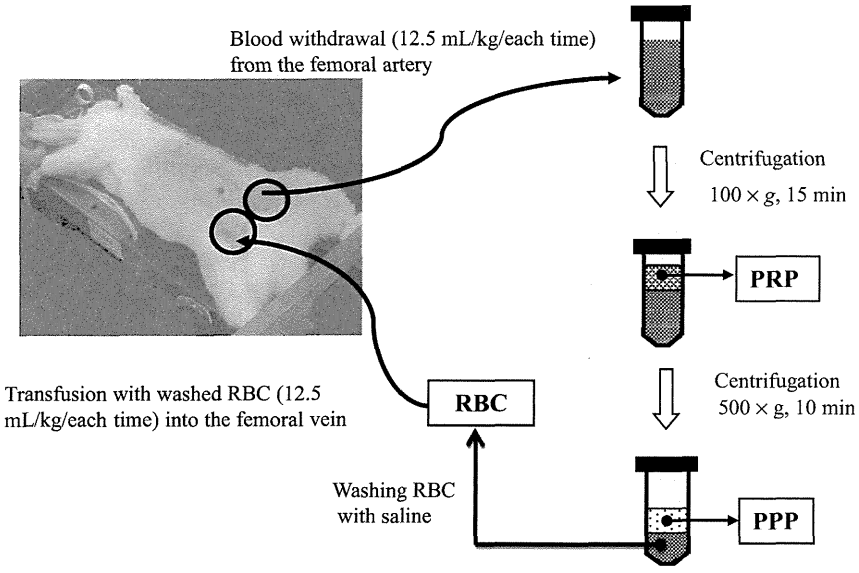
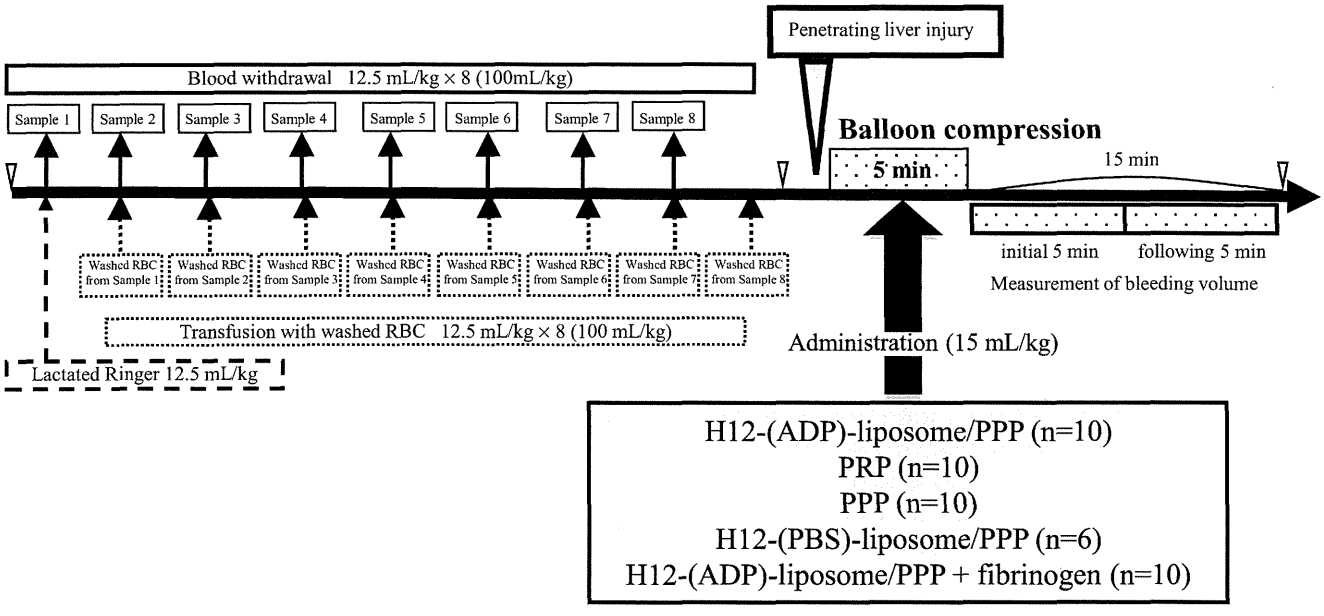


Fig. 1. Scheme of blood withdrawal from rabbits: preparation of PRP, PPP, and washed RBCs and transfusion of washed RBCs into rabbits.

Penetrating liver injury followed by balloon compression in the bleeding site

After it was confirmed that the PLT count was fewer than 50×10^9/L, rabbits underwent laparotomy to expose a liver lobe. We cut a hole in a surgical glove and passed the lobe through the hole to collect exsanguinating blood in the glove and precisely evaluate bleeding volume. Thereafter, the liver was penetrated using a disposable punch biopsy apparatus (5 mm in diameter; DermaPunch, Nipro Medical Industries Ltd., Tokyo, Japan; Figs. 3A and 3B). Immediately after the injury was induced, a 4-Fr urologic catheter (Bardex Biocath, 0165PL 8 ch/Fr 2.7 mm, C. R. Bard, Inc., Murray Hill, NJ) was inserted into the lesion and the balloon was inflated to compress the lesion for 5 minutes, simulating manual compression as damage control surgery[11] (Figs. 3C and 3D). During the balloon compression, H12-(ADP)-liposomes, PRP, PPP, H12-(PBS)-liposomes, or fibrinogen concentrates were administered to the rabbits. After deflating and removing the balloon catheter, we then measured the bleeding volume from the site of the liver injury for the initial 5-minute period (0 to 5 min) and the following 5 minutes (5 to 10 min). Bleeding time from the



▽ Collection of blood samples for the measurements of coagulation factors and Sonoclot analyses

Fig. 2. Experimental design for acute thrombocytopenia and liver hemorrhage in rabbits, followed by balloon compression and infusion of hemostatic agents.

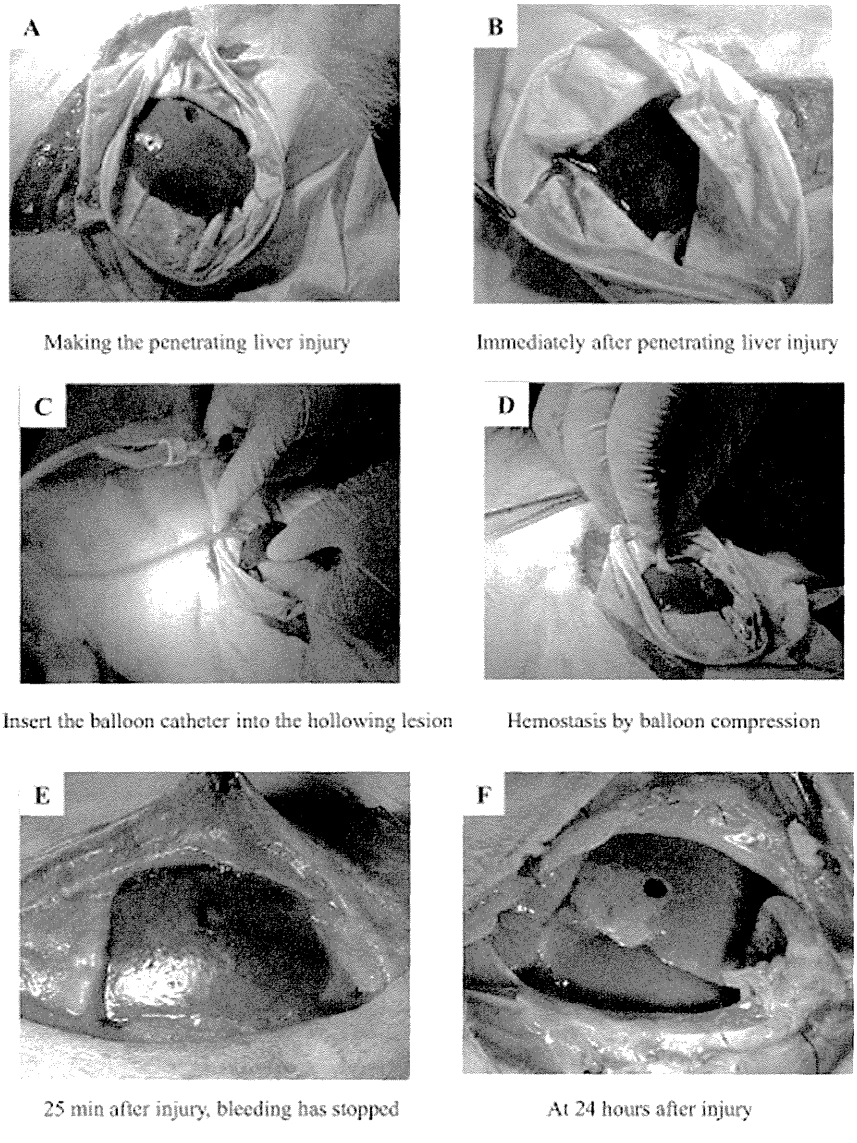


Fig. 3. Hemostasis after balloon compression. (A, B) Making a penetrating liver injury. (C, D) Balloon compression against penetrating liver injury by indwelling urethral catheter. (E) Hemostasis was achieved after administration of H12-(ADP)-liposomes/PPP following balloon compression. (F) Complete hemostasis was observed with ischemic change in the lesion 24 hours later.

penetrating liver injury was monitored for 60 minutes. Thereafter, the rabbit's abdomen was closed to monitor survival for 72 hours under ad libitum feeding with laboratory diet and water. Postoperative analgesia was performed with two intramuscular injections of buprenorphine (0.02 mg/kg), immediately after wound closure and 12 hours later.

Administration of H12-(ADP)-liposomes, PRP, PPP, and H12-(PBS)-liposomes

PRP or PPP was prepared from the blood taken at the first and second phlebotomy. These PRP and PPP samples showed similar coagulation activities (fibrinogen, approximately 150 mg/dL; antithrombin [AT] III activity, 99%; prothrombin time [PT], 12 sec; activated partial thromboplastin time [APTT], 32 sec).[10] During balloon compression against liver bleeding, H12-(ADP)-liposomes (20 mg/4 mL/kg) were administered IV into the rabbits, followed by administration of 11 mL/kg PPP (n = 10, Fig. 2). In our previous studies of rabbits with busulfan-induced thrombocytopenia, H12-(ADP)-liposomes administered at a dose of 20 mg/kg gave optimal bleeding time-shortening effects, similar to those of PRP.[9,10] Therefore, this dose was selected for all of the present experiments. Similarly, 15 mL/kg PRP or PPP was administered to the rabbits during the balloon compression (n = 10 in each group, Fig. 2). Also, H12-(PBS)-liposomes that contain no ADP were administered IV to the rabbits, followed by administration of 11 mL/kg PPP (n = 6). To supplement an adequate amount of fibrinogen, 70 mg/kg fibrinogen concentrate (Haemocompletan, CSL Behring, Marburg, Germany) was injected into the rabbits, after the administration of H12-(ADP)-liposomes (20 mg/4 mL/kg) and PPP (11 mL/kg, n = 10; Fig. 2). For negative controls, two rabbits with thrombocytopenia treated with balloon compression alone (without PLT and/or plasma replacement) were evaluated. Also, the rabbits with thrombocytopenia treated with H12-(ADP)-liposome or PRP alone (without balloon compression) were evaluated (n = 2 in each group).

Analyses of whole blood coagulation activity

Whole blood samples were analyzed for hemostatic function three times: before and after blood exchange and 20 minutes after liver injury. The coagulation activity of whole blood was examined using the a coagulation and PLT function analyzer (Sonoclot, Sienco, Morrison, CO).[9] Briefly, a tubular probe mounted on an ultrasonic transducer and vibrating vertically with a distance of 1 μm and a frequency of 200 Hz is immersed to a fixed depth in a cuvette containing 400 μL of whole blood obtained from the femoral artery without anticoagulant. As the sample clots, the increasing impedance to the probe vibration is

detected by the sensor and converted to an output signal that reflects the viscoelastic properties of the developing clot. The signal typically describes coagulation variables including "clotting time (CT)," which indicates the period up to the beginning of fibrin formation, and "clotting rate (CR)," which indicates the slope of fibrin gel formation that is affected by both the rate of the fibrinogen to fibrin conversion and the amount of fibrinogen (Fig. 4A).

Measurements of mean arterial pressure, hematologic variables, and coagulation factors

Mean arterial pressure (MAP) was measured from the cannulated femoral artery with a polygraph recording system (RM-6000, Nihon Kohden, Tokyo, Japan). Blood samples were also collected from the femoral artery. PLT count, hemoglobin (Hb) concentration, and white blood cell count were measured using a hematology analyzer (PEC 170, Erma, Inc., Tokyo, Japan). Plasma concentration of fibrinogen, AT III activity, PT, and APTT were measured at the BML Laboratory (Tokyo, Japan).

Electron microscopic examinations

For electron microscopy, four rabbits treated with H12-(ADP)-liposomes were prepared. Liver specimens were obtained at 1 hour after liver hemorrhage. These were fixed with a fixative containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 3 hours at 4°C, followed by postfixing in 1% osmium tetroxide in 0.1 mol/L phosphate buffer (pH 7.4) for 2 hours at 4°C, dehydration, and embedding in epoxy resin. To select the bleeding site lesion, semithin sections were stained with toluidine blue. Ultrathin sections stained with uranyl acetate and lead citrate were then examined under an electron microscope (JEM 1010, JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.[12]

Histopathologic examinations

For histopathologic examinations, the rabbits treated with H12-(ADP)-liposomes, PRP, or H12-(ADP)-liposomes/PPP plus fibrinogen were euthanized 24 hours after liver hemorrhage (n = 3 in each group). Three rabbits with PPP that died after several minutes were also examined. The liver (uninjured lobe), lung, spleen, and kidney were removed from the subject rabbits. Excised organs were fixed by 20% formalin for 2 days and processed to paraffin embedding blocks to stain with hematoxylin and eosin.

Statistical analyses

Statistical analyses were performed with a software package (Stat View 4.02J, Abacus Concepts, Berkeley, CA). Survival rates were compared by Wilcoxon signed rank test. Statistical evaluations between two groups were compared using the t test, and any other statistical evaluations were compared using the one-way analysis of variance, followed by Bonferroni post hoc test. Data are presented as means ± standard deviation (SD), with p values of less than 0.05 considered to be significant.

RESULTS

Acute thrombocytopenia in rabbits

After isovolemic blood exchange and plateletpheresis, PLT counts in rabbits were decreased to $45 \times 10^9 \pm 8 \times 10^9$/L, indicating acute thrombocytopenia (Table 1). Although the subject rabbits maintained MAP as a result of isovolemic exchanges, their Hb concentrations were decreased to approximately 6 g/dL due to inevitable loss of RBCs during plateletpheresis, which we attribute to mechanical destruction of RBCs in the processes of centrifugation and washing with saline. However, apparent hemolysis was not observed in the blood samples obtained from rabbits with thrombocytopenia (Table 1). Their coagulation factors were also decreased to very low levels (Table 1), because they had not yet received PPP or PRP administration. CT and CR were also markedly worsened by the blood exchange (Table 1).

Survival from liver hemorrhage in the rabbits with acute thrombocytopenia

Balloon compression alone did not rescue any of the rabbits with thrombocytopenia from hemorrhage induced by the penetrating liver injury, as the treatment did not stop the bleeding and death occurred within 1 hour. However, administration of H12-(ADP)-liposomes/PPP as well as PRP after balloon compression significantly increased the survivals from liver hemorrhage in the rabbits with thrombocytopenia in comparison to administration of PPP (Fig. 5). Unlike H12-(ADP)-liposomes/PPP, administration of H12-(PBS)-liposomes/PPP after balloon compression was not effective (Fig. 5). Supplementation with fibrinogen concentrate did not increase the survival of the rabbits with thrombocytopenia treated with H12-(ADP)-liposomes/PPP (Fig. 5). We also treated the rabbits with thrombocytopenia with H12-(ADP)-liposome/PPP or PRP alone (without balloon compression, n = 2 in each group). However, neither treatment with H12-(ADP)-liposomes nor treatment with PRP rescued any rabbits from hemorrhage. Specifically, after treatment with H12-(ADP)-liposomes, two animals failed to stop bleeding and died within 12 hours; after treatment with PRP, bleeding stopped in one of two animals but both died within 6 hours. These outcomes suggested the importance of damage control intervention.

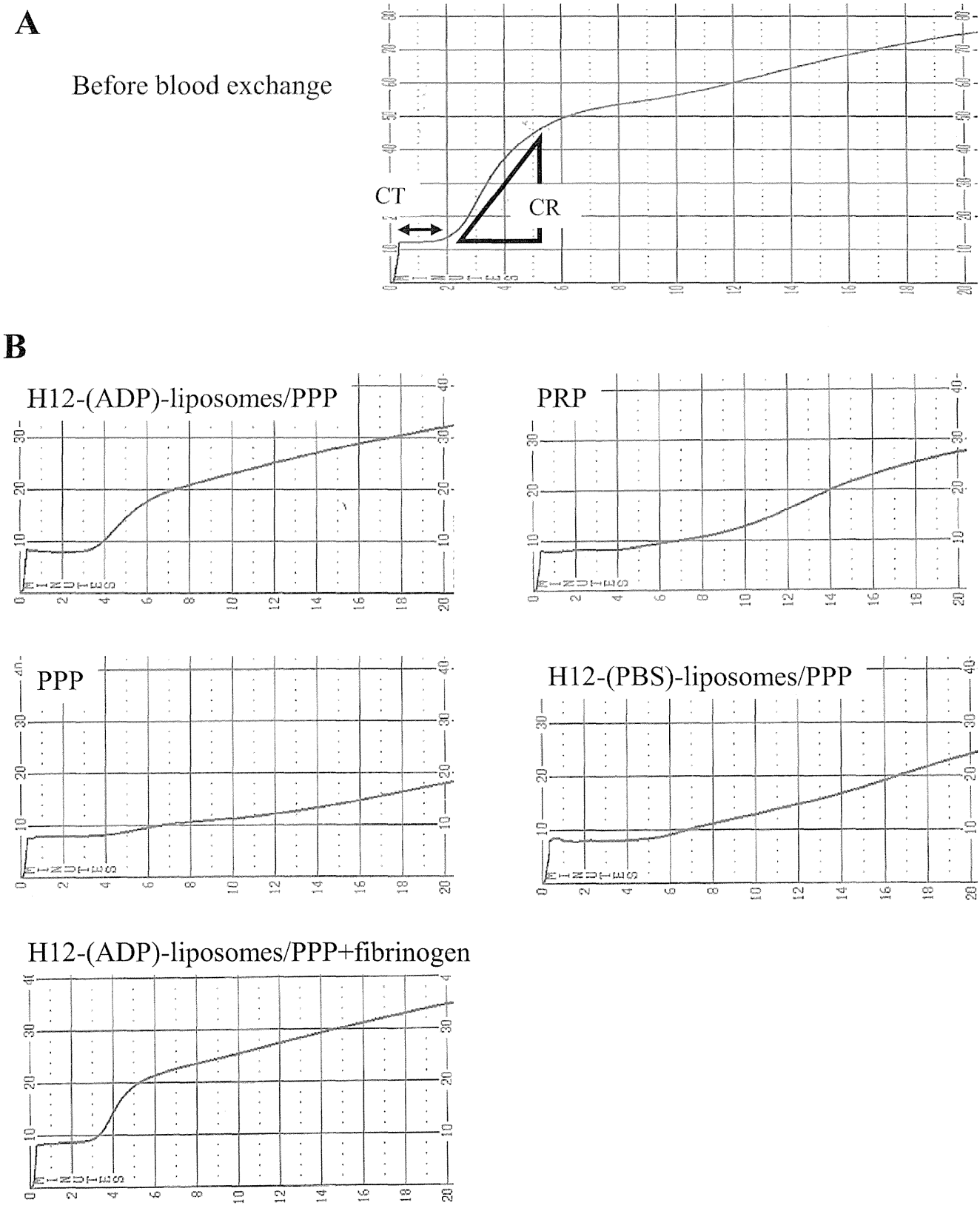


Fig. 4. Analyses of whole blood coagulation activities. (A) Whole blood coagulation activities in rabbits before blood exchanges. CT and CR are indicated by arrow and triangle, respectively. (B) Blood coagulation activities in each group 20 minutes after liver injury. Representative data with similar results are shown.

Bleeding time of the liver injury site

Administration of H12-(ADP)-liposomes/PPP as well as PRP achieved significant hemostasis in nine of 10 rabbits in both groups, while administration of PPP stopped bleeding in only two of 10 rabbits (90% vs. 20%, $p < 0.01$; Fig. 6A). Administration of H12-(PBS)-liposomes/PPP stopped bleeding in four of six rabbits; however, this treatment only rescued one rabbit, suggesting that H12-(PBS)-liposomes induced a weak (ineffective) hemostasis (Fig. 6A). Interestingly, although supplementation of fibrinogen to the H12-(ADP)-liposomes/PPP achieved hemostasis in all rabbits, six of 10 rabbits eventually died (Fig. 6A). We further analyzed the bleeding time in rabbits showing hemostasis. All treated groups showed significantly shorter bleeding times than those receiving the PPP group (Fig. 6A).

TABLE 1. Changes in the hematologic variables and coagulation factors in rabbits before and after blood exchange*

Variable	Before blood exchange (n = 68)	After blood exchange (n = 68)
MAP (mmHg)	71 ± 12	67 ± 14
Hb concentrations (g/dL)	11.3 ± 2.1	6.3 ± 2.3†
PLT counts ($\times 10^9$/L)	217 ± 66	45 ± 8†
Fibrinogen concentration (mg/dL)	186 ± 38	<55
AT III activity (%)	115 ± 18	35 ± 11†
PT (sec)	10 ± 1	>30
APTT (sec)	24 ± 8	>75
CT (sec)	114 ± 16	387 ± 185†
CR (sec)	17 ± 5	2.1 ± 1.3†

* Hematologic variables and coagulation factors were measured in rabbits before and after blood exchange. Data are reported as mean ± SD.
† $p < 0.01$ versus before blood exchange.

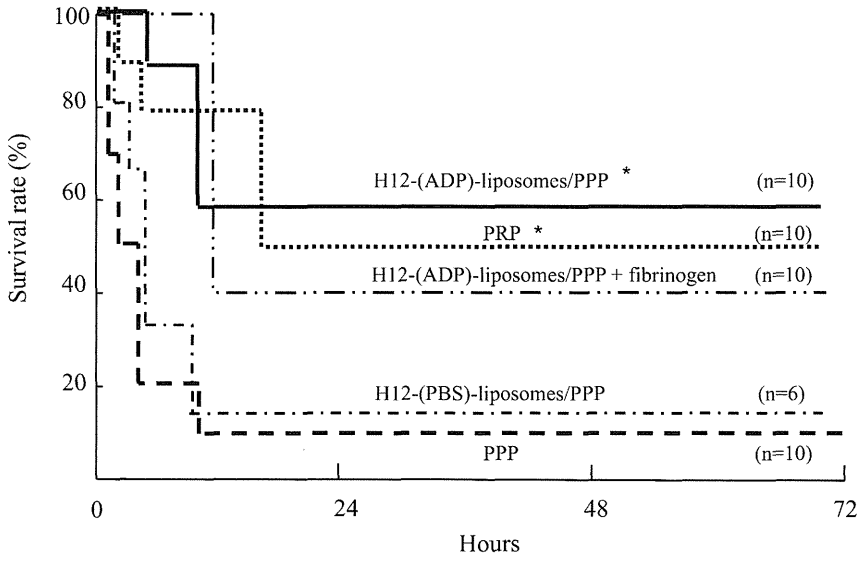


Fig. 5. Survival rates of rabbits with acute thrombocytopenia after liver hemorrhage. Balloon compression and administration of H12-(ADP)-liposomes/PPP, PRP, PPP, H12-(PBS)-liposome/PPP, or H12-(ADP)-liposomes/PPP with fibrinogen were used to treat liver hemorrhage in rabbits with acute thrombocytopenia. *$p < 0.01$ versus PPP, $p < 0.05$ versus H12-(PBS)-liposome.

Bleeding volume from the liver injury site

Administration of H12-(ADP)-liposomes/PPP as well as PRP significantly reduced the bleeding volume from the site of the liver injury in the initial 5 minutes compared to that of PPP (Fig. 6B). However, administration of H12-(PBS)-liposomes/PPP resulted in a substantial amount of bleeding in the rabbits during the initial 5 minutes compared to treatment with H12-(ADP)-liposomes/PPP or PRP alone (Fig. 6B), suggesting that H12-(PBS)-liposome did not promptly exert a hemostatic effect. Supplementation of fibrinogen to H12-(ADP)-liposomes/PPP resulted in a significant reduction of bleeding volume as did administration of H12-(ADP)-liposomes/PPP alone. H12-(ADP)-liposomes/PPP and PRP markedly reduced the bleeding volume in the subsequent 5 minutes (5 to 10 min) in comparison to those in the initial 5 minutes, suggesting achievement of effective hemostasis (Fig. 6B). PPP and H12-(PBS)-liposomes/PPP groups also showed a marked reduction of bleeding volume in the subsequent 5 minutes in comparison to those in the initial 5 minutes. However, both groups remarkably decreased their blood pressure 20 minutes after liver injury (Table 2), presumably owing to their substantial hemorrhage in the initial 5 minutes. In turn, this severe hypotension (shock) might have affected reducing the hemorrhagic volume in the next 5 minutes and caused subsequent death.

Immediately after making the penetrating liver injury, rabbits showed exsanguinating hemorrhage at the injured site (Figs. 3A and 3B). The rabbits received balloon compression for 5 minutes and were administered H12-(ADP)-liposomes/PPP (in this case) during balloon compression (Figs. 3C and 3D). Twenty-five minutes after penetrating liver injury, blood coagulation or hemostasis was observed in the rabbits, and 24 hours later, complete hemostasis in the liver's site of injury was confirmed (Figs. 3E and 3F).

Changes in hemodynamics, hematologic variables, and coagulation factors

Twenty minutes after liver injury, rabbits in the PPP group and the H12-(PBS)-liposomes/PPP group showed

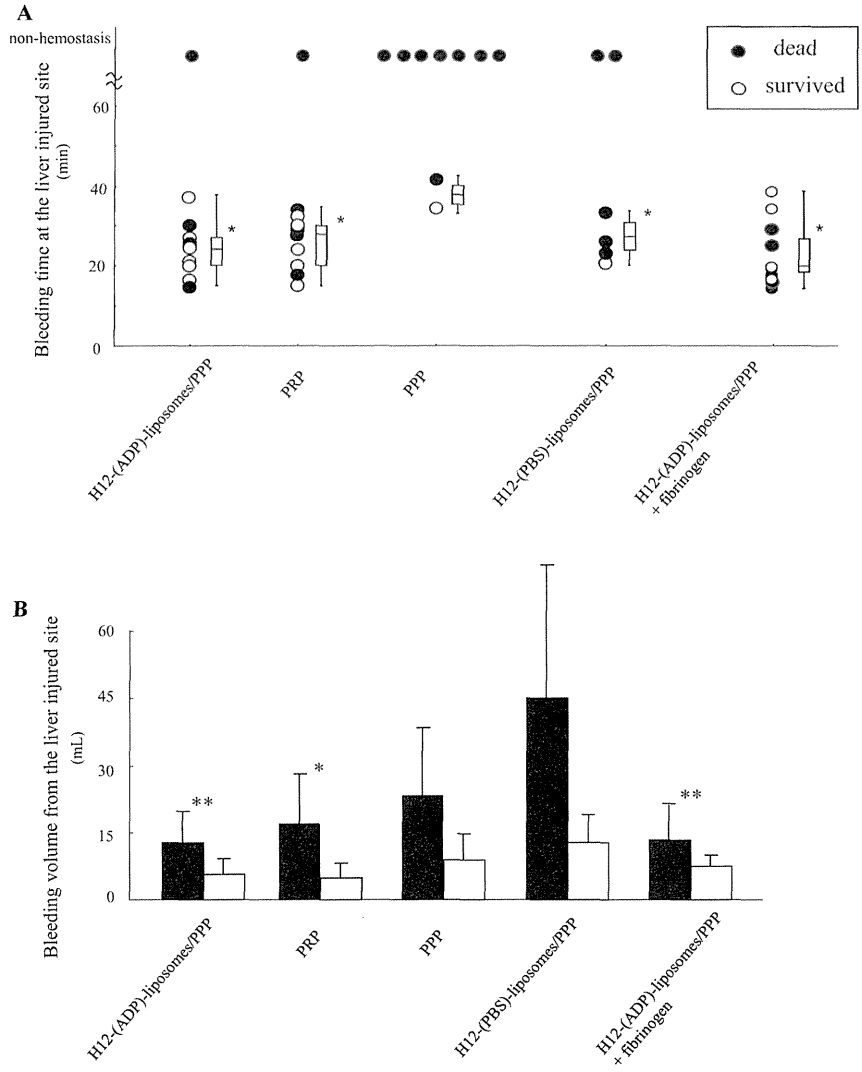


Fig. 6. Liver hemorrhage in rabbits with thrombocytopenia after administration of H12-(ADP)-liposomes/PPP, PRP, PPP, H12-(PBS)-liposome/PPP, or H12-(ADP)-liposomes/PPP with fibrinogen. (A) Bleeding time from liver injury. *p < 0.05 versus PPP. (B) Bleeding volumes from the penetrating liver injury. (■) 0 to 5 minutes; (□) 5 to 10 minutes. *p < 0.05 versus PPP, p < 0.01 versus H12-(PBS)-liposomes/PPP; **p < 0.01 versus PPP, H12-(PBS)-liposomes/PPP.

significantly lower MAP (<40 mmHg) than the other groups (approx. 60 mmHg; Table 2). These two groups also showed severe anemia (Hb < 4 g/dL) due to massive hemorrhage (Table 2). As expected, only rabbits receiving PRP showed a significantly higher PLT count, which might have contributed to the cessation of bleeding (Table 2). Plasma fibrinogen was below the lower limit of detection except for the H12-(ADP)-liposomes/PPP plus fibrinogen group (Table 2), suggesting that supplementation of coagulation factors such as fibrinogen by PPP or PRP administration appeared to be insufficient. However, there were no significant differences in the AT III activities or PTs among the H12-(ADP)-liposomes/PPP, PRP, and H12-(ADP)-liposomes/PPP plus fibrinogen groups, although PPP and H12-(PBS)-liposomes/PPP groups showed marked impairment in these variables (Table 2). The time after which APTT measurement was no longer recorded (75 sec in this case) was exceeded in all groups (data not shown).

Analyses of whole blood coagulation activity

Administration of H12-(ADP)-liposomes/PPP as well as PRP alone significantly shortened the CT in comparison to that observed in the PPP and the H12-(PBS)-liposomes/PPP groups (Table 2). Supplementation of fibrinogen to H12-(ADP)-liposomes/PPP did not significantly affect CT in rabbits (Table 2). Although H12-(ADP)-liposomes/PPP and PRP groups tended to show an increase in the CR compared to that of the PPP or H12-(PBS)-liposomes/PPP groups, supplementation of fibrinogen to H12-(ADP)-liposomes/PPP remarkably augmented CR (Table 2), suggesting that fibrinogen potently affected CR in the Sonoclot analyses. Representative data in each group are shown in Fig. 4.

Electron microscopic examinations

Clot formation adjacent to the injured site in the liver was observed after administration of H12-(ADP)-liposomes/PPP (Fig. 7A). These clots involved both PLTs and fibrin (Fig. 7B). Electron microscopic assessment of the lesion revealed liposomes (approx. 0.2 to 0.4 μm in diameter) around the PLTs or fibrin deposits (Fig. 7B, indicated by arrows), suggesting the presence of H12-(ADP)-liposomes in the lesion.[9] In the uninjured hepatic lobe, a few liposomal particles were found in the sinusoidal space; however, they did not accumulate PLTs or fibrin (Fig. 7C, indicated by left arrow). Kupffer cells as well as splenic macrophages phagocytosed liposomal particles (Fig. 7C, indicated by right arrow; Fig. 7D, indicated by arrows), suggesting degradation of H12-(ADP)-liposomes by reticuloendothelial system; however, no thrombi were found in the spleen.

TABLE 2. Hematologic variables and coagulation factors or activities in rabbits 20 minutes after liver injury*

Variable	H12-(ADP)-liposomes/PPP (n = 10)	PRP (n = 10)	PPP (n = 10)	H12-(PBS)-liposomes/PPP (n = 6)	Fibrinogen + H12-(ADP)-liposomes/PPP (n = 10)
MAP (mmHg)	59 ± 20	59 ± 11	41 ± 12†	39 ± 10†	62 ± 11
Hb concentrations (g/dL)	5.5 ± 2.2	4.8 ± 2.4	3.0 ± 0.9‡	3.8 ± 1.3	5.3 ± 1.5
PLT counts ($\times 10^9$/L)	55 ± 14	70 ± 21§	54 ± 10	48 ± 14	53 ± 10
Fibrinogen concentration (mg/dL)	<55	<55	<55	<55	93 ± 22
AT III activity (%)	39 ± 9	40 ± 10	26 ± 8‡	24 ± 9‡	39 ± 8
PT (sec)	14 ± 3	12 ± 2	>30	>30	14 ± 1
CT (sec)	270 ± 57	306 ± 77	401 ± 149‡	461 ± 196‡	251 ± 112
CR (sec)	2.9 ± 1.6	2.5 ± 1.3	1.8 ± 1.1	1.6 ± 0.3	6.2 ± 3.1‖

* Hematologic variables and coagulation factors were measured in rabbits 20 minutes after liver injury (followed by administration of H12-(ADP)-liposomes/PPP, PRP, PPP, H12-(PBS)-liposomes/PPP, or H12-(ADP)-liposomes/PPP plus fibrinogen). Data are reported as mean ± SD.
† $p < 0.01$.
‡ $p < 0.05$ versus H12-(ADP)-liposomes/PPP or PRP.
§ $p < 0.05$ versus H12-(ADP)-liposomes/PPP, PPP, H12-(PBS)-liposomes/PPP, or H12-(ADP)-liposomes/PPP plus fibrinogen.
‖ $p < 0.05$ versus H12-(ADP)-liposomes/PPP, PRP, PPP, or H12-(PBS)-liposomes/PPP.

Histologic examinations

Neither macro- nor microthrombi were found in the lung, liver (uninjured lobe), kidney, or spleen in the rabbits 24 hours after administration of H12-(ADP)-liposomes or PRP or PPP (Fig. 8). We also carefully examined the tissue specimens from the H12-(ADP)-liposomes/PPP plus fibrinogen group; however, no thrombi were detected in their organs (Fig. 8). Moderate septum thickness in the lung was observed in the PPP group but not other groups (Fig. 8).

DISCUSSION

It is difficult to control exsanguinating hemorrhage from an injured organ in patients with acute thrombocytopenia. Treatment for coagulopathy as well as surgical interventions including damage control surgery is important for their intensive care. Even with damage control intervention, such patients can die due to complications from severe coagulopathy. In the present rabbit model, loss of blood volume reached approximately 45% to 50% of systemic circulation in the PPP group and 35% to 40% of that in the H12-(ADP)-liposomes/PPP or PRP groups (Fig. 6B). Those levels were sufficient to achieve shock criteria Classes IV and III, respectively.[13] Balloon compression alone failed to rescue rabbits with thrombocytopenia from severe hemorrhage, although it likely offered critical damage control. The rabbits' residual PLT counts might have been too low to achieve effective hemostasis because when we deflated the balloon after a 5-minute compression, exsanguinating hemorrhage was observed again in the rabbits with thrombocytopenia (in the absence of agent administration). Therefore, effective treatment against pernicious coagulopathy is required.

PLT transfusion is believed to be quite effective against severe coagulopathy. In line with this, PRP administration after balloon compression rescued half of the rabbits with thrombocytopenia from lethal hemorrhage (Fig. 5). However, PLT concentrates are often not readily available (at least in hospitals, if not prehospital), because of their short shelf life.[14,15] Currently, the US military uses apheresis PLTs prepared on site in its Role 3 hospitals (combat support hospital) in Afghanistan and these are the only Role 3 hospitals currently deployed in active combat.[16]

We previously reported that pretreatment with H12-(ADP)-liposomes effectively prevented noncompressible liver hemorrhage and rescued all rabbits with acute thrombocytopenia. However, in clinical settings, hemostatic treatments are usually performed in response to ongoing exsanguinating hemorrhage in patients. Damage control surgery is also often performed and accompanied by hemostatic treatment. Therefore, we attempted to simulate those conditions in the present model. Infusion of H12-(ADP)-liposomes or PRP showed effective hemostatic potential even after hemorrhage was initiated and it rescued more than half of the rabbits with thrombocytopenia (Fig. 5). However, neither H12-(ADP)-liposomes nor PRP alone without balloon compression rescued any of the rabbits with thrombocytopenia from hemorrhage. Those results suggest that treatments for coagulopathy using PLTs or a PLT substitute as well as damage control intervention are indispensable for the rescue of patients experiencing severe bleeding.

In our previous study, pretreatment with H12-(PBS)-liposomes significantly improved the survival of rabbits with thrombocytopenia from hemorrhage, whereas H12-(PBS)-liposomes were not effective in the present model. In the present study, hemorrhagic injury preceded administration of H12-(PBS)-liposome, whereas in the previous study, hemorrhagic injury followed administration of H12-(PBS)-liposome. Blood loss due to hemorrhage in the

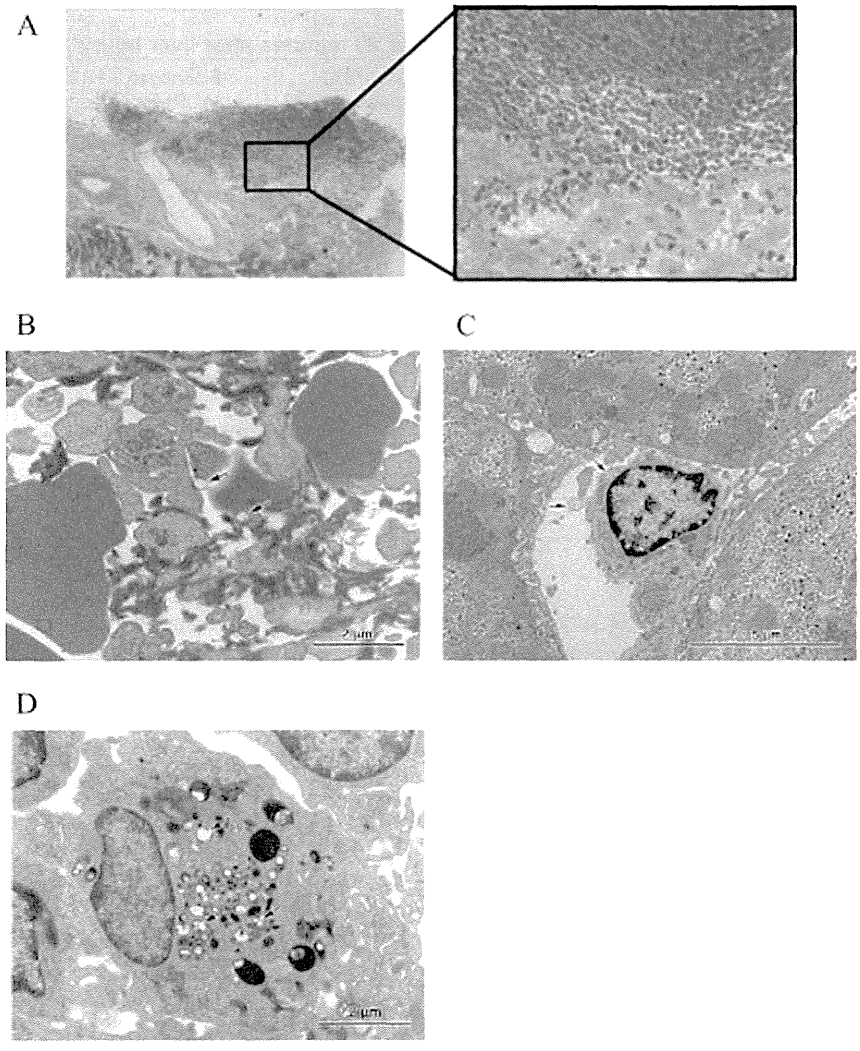


Fig. 7. Microscopic (A) and electron microscopic (B) observations of the penetrating liver injury site in rabbits. Electron microscopic observation of the uninjured hepatic lobe (C) and spleen (D) in rabbits. Specimens were obtained 1 hour after injury from rabbits with thrombocytopenia administered H-12(ADP)-liposomes/PPP. Liposomes are indicated with arrows.

current model was more severe than that of our previous study (bleeding volume from the liver injury site was severalfold larger). These differences between the present and previous studies might have affected the efficacy of H12-(PBS)-liposome. Because ADP plays a key role in both initiating and sustaining integrin αIIbβ3 activation necessary for the development of stable PLT-PLT adhesion contacts,[17] we designed the H12-coated liposomes to contain ADP and release it at the bleeding site.[18] The ADP might be required to induce PLT activation at the bleeding site followed by prompt aggregation of PLTs, resulting in effective hemostasis even in the severe hemorrhagic condition encountered in the present model.

Nevertheless, administration of H12-(ADP)-liposomes was unable to rescue 40% of the rabbits from hemorrhage in this study. In contrast, H12-(ADP)-liposomes achieved 100% survival of rabbits with thrombocytopenia in our previous study.[9] PRP treatment rescued only one-half of the rabbits in this study, whereas PRP pretreatment rescued all subject rabbits in the previous study. Considering this difference in the survival of PLT-transfused rabbits, one can ask why H12-(ADP)-liposome/PPP supported lower survival in the present report than the previous study (60% vs. 100% survival). The reasons are, in fact, not entirely clear, but the marked severity of hemorrhage in the present model might have contributed to the poor survival rates.

To further improve the survival of H12-(ADP)-liposome–treated rabbits, we combined fibrinogen concentrates with H12-(ADP)-liposomes/PPP because there is a consensus that the level of fibrinogen required for effective clot formation to overcome hemorrhaging is at least 100 mg/dL blood.[2,4] However, even though fibrinogen levels were corrected to approximately 100 mg/dL and the CRs markedly improved from 2.1 to 6.2, enhanced survival was not observed. At present, the reason for the failure of fibrinogen supplementation to improve survival from coagulopathy is not known. Theoretically, fibrinogen might contribute to the coagulation step, not to the primary PLT aggregation step. Therefore, further experiments are needed to explore the role of fibrinogen and other coagulation factors in this particular animal model.

Finally, in the present model, we examined the effect of adding fibrinogen concentrate to PPP for administration to the rabbits with thrombocytopenia. However, none of the rabbits survived the experimental conditions (data not shown). Interestingly, blood that accumulated in the peritoneal cavity from the injured liver showed a clot formation. This was not observed in the rabbits receiving PPP alone. In addition, clot formation was not observed at the site of bleeding in the liver in rabbits treated with fibrinogen concentrates added to PPP. Those results suggest that it is important to form the PLT clot in the bleeding site to achieve effective hemostasis. Notably, H12-(ADP)-liposomes effectively formed PLT thrombi in the bleeding site, resulting in effective hemostasis.

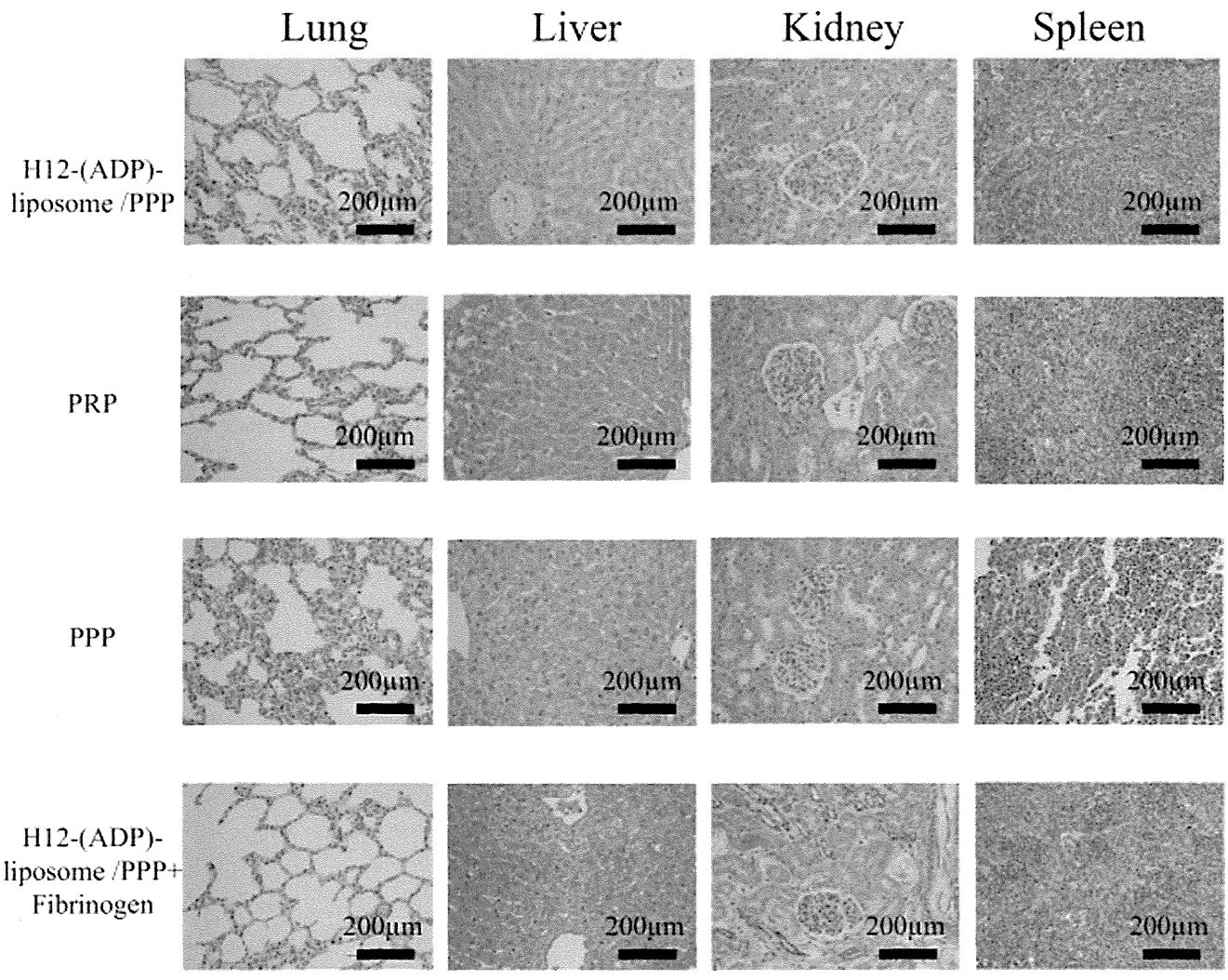


Fig. 8. Histologic findings 24 hours after liver hemorrhage in rabbits with thrombocytopenia. Lung, liver, spleen, and kidney samples were obtained from the rabbits 24 hours after liver injury.

Limitations

Despite achieving hemostasis, several rabbits eventually died even in the H12-(ADP)-liposomes/PPP and the PRP group (Fig. 6A). We noted that the deceased rabbits showed severe anemia. In this study, to exclude the effects of allogeneic RBC transfusion, we utilized autologous blood. However, in clinical settings of acute hemorrhage, allogeneic transfusion of RBCs is the norm. Considering these issues, we will next study the hemostatic efficacy of H12-(ADP)-liposome on exsanguinating hemorrhage in rabbits with acute thrombocytopenia using allogeneic RBCs transfused from other rabbits.

In this study, bleeding was tamponaded immediately after the liver trauma and within 5 minutes, the artificial PLTs were transfused. These brief time periods might well have been required to assure sufficient survival of the experimental model, but certainly do not reflect clinical reality, especially on the battlefield. In conclusion, H12-(ADP)-liposomes might be a safe and effective therapeutic tool during damage control surgery for trauma patients with acute thrombocytopenia with massive bleeding.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

REFERENCES

1. Bolliger D, Görlinger K, Tanaka KA. Pathophysiology and treatment of coagulopathy in massive hemorrhage and hemodilution. Anesthesiology 2010;113:1205-19.
2. Lier H, Böttiger BW, Hinkelbein J, et al. Coagulation management in multiple trauma: a systematic review. Intensive Care Med 2011;37:572-82.
3. Miller RD. Massive blood transfusions: the impact of Vietnam military data on modern civilian transfusion medicine. Anesthesiology 2009;110:1412-6.

4. American Society of Anesthesiologists Task Force on Perioperative Blood Transfusion and Adjuvant Therapies. Practice guidelines for perioperative blood transfusion and adjuvant therapies: an updated report by the American Society of Anesthesiologists Task Force on Perioperative Blood Transfusion and Adjuvant Therapies. Anesthesiology 2006;105:198-208.
5. Holcomb JB. Optimal use of blood products in severely injured trauma patients. Hematology Am Soc Hematol Educ Program 2010;2010:465-9.
6. Perkins JG, Cap AP, Spinella PC, et al. An evaluation of the impact of apheresis platelets used in the setting of massively transfused trauma patients. J Trauma 2009;66:S77-84; discussion S84-5.
7. Gonzalez EA, Moore FA, Holcomb JB, et al. Fresh frozen plasma should be given earlier to patients requiring massive transfusion. J Trauma 2007;62:112-9.
8. Takeoka S, Okamura Y, Teramura Y, et al. Function of fibrinogen gamma-chain dodecapeptide-conjugated latex beads under flow. Biochem Biophys Res Commun 2003; 312:773-9.
9. Nishikawa K, Hagisawa K, Kinoshita M, et al. Fibrinogen γ-chain peptide-coated, ADP-encapsulated liposomes rescue thrombocytopenic rabbits from non-compressible liver hemorrhage. J Thromb Haemost 2012;10:2137-48.
10. Okamura Y, Takeoka S, Eto K, et al. Development of fibrinogen gamma-chain peptide-coated, adenosine diphosphate-encapsulated liposomes as a synthetic platelet substitute. J Thromb Haemost 2009;7:470-7.
11. Burch JM, Franciose RJ, Moore EE. Trauma. In: Brunicardi FC, editor. Schwartz's principles of surgery. 8th ed. New York: McGraw-Hill; 2005. p. 162-5.
12. Suzuki H, Okamura Y, Ikeda Y, et al. Ultrastructural analysis of thrombin-induced interaction between human platelets and liposomes carrying fibrinogen γ-chain dodecapeptide as a synthetic platelet substitute. Thromb Res 2011;128:552-9.
13. Demling RH, Gates JD. Medical aspects of trauma and burn care. In: Goldman L, Ausiello D, editors. Cecil textbook of medicine. 22nd ed. Philadelphia: Saunders; 2004. p. 642-3.
14. Blajchman MA. Substitutes for success. Nat Med 1999;5: 17-8.
15. Slichter SJ. Evidence-based platelet transfusion guidelines. Hematology Am Soc Hematol Educ Program 2007:172-8.
16. Macdonald VW. Blood products advanced development. Cryopreserved platelets (CPP). 2010 [cited 2014 Aug 08]. Available from: http://mhsrs.amedd.army.mil/ConferenceArchive/2010/blood/B2.pdf
17. Jackson SP. The growing complexity of platelet aggregation. Blood 2007;109:5087-95.
18. Okamura Y, Katsuno S, Suzuki H, et al. Release abilities of adenosine diphosphate from phospholipid vesicles with different membrane properties and their hemostatic effects as a platelet substitute. J Control Release 2010;148:373-9.

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preventable 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。

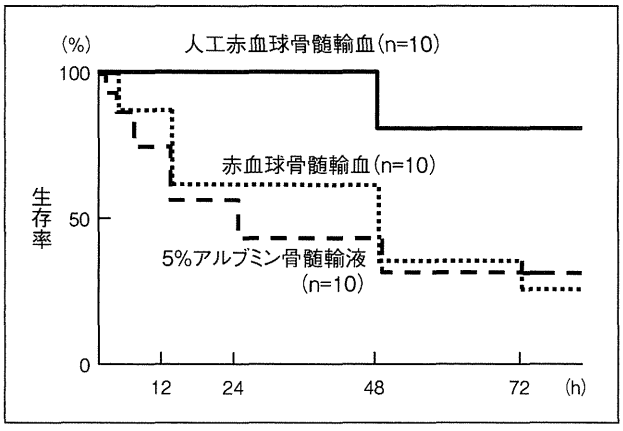


Fig.1. 人工赤血球の骨髄輸血による出血性ショックマウスの救命蘇生効果 (文献3より引用)

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。

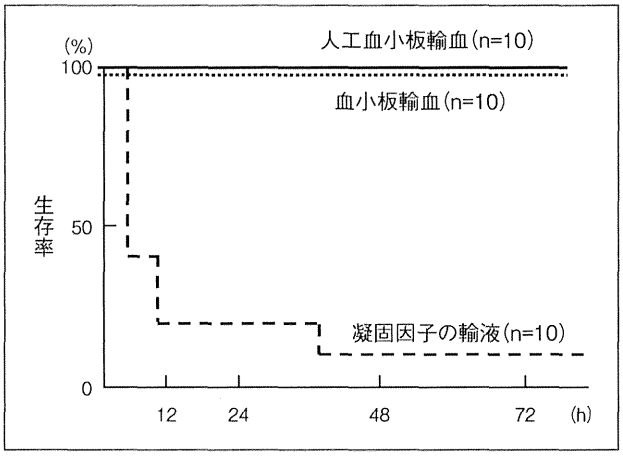


Fig.2. 人工血小板の急性血小板減少家兎での肝臓出血に対する止血救命効果 (文献5より引用改変)

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。

引用文献

1) Bateman C : Hope for reducing SA's trauma deaths? S Afr Med J 94 : 728-729, 2004.
2) Natanson C, Kern SJ, Lurie P, et al. : Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death : a meta-analysis. JAMA 299 : 2304-2312, 2008.
3) Shono S, Kinoshita M, Takase B, et al. : Intraosseous transfusion with liposome-encapsulated hemoglobin improves mouse survival after hypohemoglobinemic shock without scavenging nitric oxide. Shock 35 : 45-52, 2011.
4) Okamura Y, Takeoka S, Eto K, et al. : Development of fibrinogen gamma-chain peptide-coated, adenosine diphosphate-encapsulated liposomes as a synthetic platelet substitute. J Thromb Haemost 7 : 470-477, 2009.
5) Nishikawa K, Hagisawa K, Kinoshita M, et al. : Fibrinogen γ-chain peptide-coated, ADP-encapsulated liposomes rescue thrombocytopenic rabbits from non-compressible liver hemorrhage. J Thromb Haemost 10 : 2137-2148, 2012.