によって高次脳機能を保持している可能性が示唆された。

E．健康危険情報

該当なし

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別添5

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FLUID RESUSCITATION WITH ARTIFICIAL OXYGEN CARRIERS IN HEMORRHAGED RATS: PROFILES OF HEMOGLOBIN-VESICLE DEGRADATION AND HEMATOPOIESIS FOR 14 DAYS

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ABSTRACT—Polyethylene glycol (PEG)–modified hemoglobin (Hb) vesicles (HbVs) are artificial oxygen carriers encapsulating a concentrated Hb solution in phospholipid vesicles. In our previous studies, HbV showed a sufficient resuscitative effect comparable to that of red blood cells in hemorrhagic shock animal models during several hours' observation. However, the profiles of the recovery, including hematopoiesis and elimination of HbV, remain unknown. This study conducted 14-day observations of Wistar rats after hemorrhagic shock and fluid resuscitation with HbV suspended in recombinant human serum albumin. Shock was induced by 50% blood withdrawal from a femoral artery. The rats showed hypotension, metabolic acidosis, and hyperventilation. After 15 min, they received HbV or shed autologous blood through a femoral vein. Both groups showed rapid recovery of hemodynamic and blood gas parameters. No meaningful difference was found between groups. After decannulation and awakening, the rats were housed in cages. The reduced hematocrit of the HbV group returned to the original level in 7 days. Plasma enzyme levels were slightly higher in both groups at 1 day because of systemic reperfusion injury. Splenomegaly was considerable in the HbV group because of the HbV accumulation and extramedullar hematopoiesis, but it subsided within 14 days. Along with the HbV elimination in the spleen and liver, immunohistochemistry with anti-PEG antibody revealed that PEG-conjugated lipid had disappeared within 14 days. In conclusion, HbV showed a sufficient resuscitative effect comparable to that of red blood cell transfusion. Phagocytized HbV disappeared within 14 days. Elevated hematopoiesis contributed to complete hematocrit recovery within 7 days.

KEYWORDS—Hemorrhagic shock, blood substitutes, transfusion alternative, hemoglobin, liposome, resuscitation, reticuloendothelial system, erythropoiesis

ABBREVIATIONS—β-LP—β-lipoprotein; ALT—alanine aminotransferase; AST—aspartate aminotransferase; BE—base excess; BP—arterial blood pressure; BUN—blood urea nitrogen; CRE—creatinine; GM-CSF—granulocyte macrophage-colony-stimulating factor; HBOCs—hemoglobin-based oxygen carriers; HbV—hemoglobin vesicles; Hct—hematocrit; HSA—human serum albumin; IFN-γ—interferon γ; LDH—lactate dehydrogenase; metHb—methemoglobin; $PaCO_2$—arterial blood carbon dioxide tension; PaO_2—arterial blood oxygen tension; PEG—polyethylene glycol; PEG-DSPE—1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG; RBCs—red blood cells; RES—reticuloendothelial system; rHSA—recombinant human serum albumin; SAB—shed autologous blood; UA—uric acid; T-Chol—total cholesterol; WBC—number of white blood cells

INTRODUCTION

Blood transfusion is an indispensable practice that has contributed substantially to continued improvement of human health and welfare. However, some unresolved issues threaten the achievement of safer transfusions: the possibility of contamination of pathogens such as human immunodeficiency virus and hepatitis virus, or other unknown viruses, even after the costly nucleic acid amplification testing; mismatching of blood types; and numerous immunological difficulties. Guidelines for safer blood transfusion have been revised repeatedly, such as the reduction of a transfusion trigger, the critical hemoglobin (Hb) level, to 6 g dL^{-1} to minimize unnecessary transfusion strictly or to avoid allogeneic transfusion as long as possible to prevent such side effects (1). In this respect, Hb-based oxygen carriers (HBOCs) are superior to allogeneic transfusion because they are free of blood-type antigens, pathogens such as human immunodeficiency virus and hepatitis viruses, and immunological and other blood-bone side effects. The stability of HBOCs for a long-term storage over several years is also advantageous, which far surpasses the limited storage period of packed red blood cells (RBCs), which is limited to a mere 3 weeks in Japan (2).

A phospholipid vesicle or liposome encapsulating concentrated human Hb (Hb-vesicle [HbV]) is one example of an HBOC (3, 4). The cellular structure of the HbV (ca. 250-nm particle diameter) has characteristics resembling those of natural RBCs because both have lipid bilayer membranes that prevent the direct contact of Hb with blood components and

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the endothelial lining, thereby eliminating some side effects of molecular Hbs (5, 6). In previous reports, we clarified that the injected HbVs were finally captured by macrophages in the reticuloendothelial system (RES or mononuclear phagocytic system) and degraded promptly, subsequently disappearing within 2 weeks (7–10). However, those experiments were conducted with healthy rats receiving bolus or repeated topload injections or isovolemic exchange transfusion with less surgical intervention. In exchange hemodilution experiments, hematocrit (Hct) reverted to the original level during 1 week after exchange transfusion with HbV, indicating that the hematopoietic activity was preserved despite the accumulation of HbV in RES, including bone marrow (10). Although we previously confirmed the oxygen transport capacity of HbV as a resuscitative fluid for hemorrhagic shock, the observation period was only several hours (11–14); what would happen during the succeeding days or weeks was not known.

Given this background, the present work is intended to clarify the subsequent profiles of recovery involving the degradation of HbV in RES and hematopoiesis in addition to the oxygen transporting capabilities of HbV and its impact on organ functions compared with resuscitation with shed autologous blood.

MATERIALS AND METHODS

Preparation of HbV

For use in this study, HbV was prepared as reported in previous studies (15, 16). The Hb was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g dL^{-1}) contained 14.7 mM of pyridoxal 5′-phosphate (Sigma Chemical Co., St. Louis, Mo) as an allosteric effector to regulate P_{50} to 25 to 28 torr. The lipid bilayer was a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate at a molar ratio of 5/5/1 (Nippon Fine Chemical Co. Ltd., Osaka, Japan), along with 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethyleneglycol) (PEG-DSPE, 0.3 mol%; NOF Corp., Tokyo, Japan). The HbVs were suspended in a physiologic salt solution at [Hb] = 10 g dL^{-1} and [lipids] = 6.8 g dL^{-1}, and deoxygenated in vials for long-term storage (17). The HbV suspended in saline does not show an oncotic effect, just as RBCs do not. For a large substitution of blood, HbV must be suspended in or coinjected with a plasma substitute to prevent extravasation of the injected fluid and to maintain blood volume. We often use plasma-derived or recombinant human serum albumin (HSA) because the compatibility of HbV and HSA has been confirmed well from rheological points of view (18). Furthermore, in Japan, recombinant HSA (rHSA) will become clinically available in 2008. In this study, the HbV suspension ([Hb] = 10 g dL^{-1}; 8.6 mL; occupied volume, 40%) was mixed with an rHSA (25%, 1.4 mL; Nipro Corp., Osaka, Japan) to regulate the concentration of rHSA in the suspending medium to 5 g dL^{-1} and the colloid osmotic pressure to approximately 19 to 20 torr, a physiological colloid osmotic pressure value. Consequently, Hb was 8.6 g dL^{-1}; HbV bound oxygen in an aerobic condition. Before use, the suspension was filtered (0.45-μm pore size, Dismic; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) to ensure a homogeneous dispersion state.

Animal model of resuscitation from hemorrhagic shock

The Laboratory Animal Care and Use Committee of the School of Medicine, Keio University, approved the entire experimental protocol. The protocol complies with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council-National Academy of Sciences (Washington, DC: National Academy Press, 1996).

Experiments were carried out using 48 male Wistar rats (274 ± 26 g body weight). The rats were anesthetized using 1.5%-sevoflurane-mixed air inhalation (Maruishi Pharmaceutical Co., Osaka, Japan) with a vaporizer (Model TK-4 Biomachinery; Kimura Medical Instrument Co. Ltd., Tokyo, Japan) throughout the experiment (fraction of inspired oxygen, 21%), whereas spontaneous breathing was maintained. Polyethylene catheters (SP-31 tubing; outer diameter, 0.8 mm; inner diameter, 0.5 mm; Natsume Seisakusho Co. Ltd., Tokyo, Japan) filled with a saline solution containing 40 IU mL^{-1} heparin were introduced into the left femoral artery and vein. The arterial catheter was connected to a polygraph system (LEG-1000; Nihon Kohden Corp., Tokyo, Japan).

The systemic blood volume was estimated as 56 mL kg^{-1} body weight (19). Withdrawing 50% of the blood (28 mL kg^{-1}, 1 mL min^{-1}) from the femoral artery into a heparinized syringe (Fig. 1) induced hemorrhagic shock. The rats were kept hypotensive for 15 min (MAP < 40 mmHg). They were resuscitated by infusion of HbV (n = 24) or shed autologous blood (SAB group; n = 24) at a rate of 1 mL min^{-1}. The volume of the infused resuscitative fluid was equal to the shed volume: 50% of the blood volume at baseline. Systemic parameters were observed for 6 h, and then the catheters were removed. The femoral artery and vein were ligated, and the skin was sutured with a stitch. The rats were housed in cages in a room with a barrier against infection at the animal experimental facility of Keio University. Rats were provided *ad libitum* access to food and water in a temperature-controlled environment with a 12-h dark/light cycle.

Five rats were selected randomly from each group at 1, 3, 7, and 14 days for sequential measurements. At each time point, the rats were anesthetized using 1.5%-sevoflurane-mixed air inhalation. After measuring the body weight, approximately 150 μL blood was withdrawn from the tail vein via an indwelling needle (24-gauge; Nipro) for an Hct measurement using glass capillaries and for blood cell counts. A catheter was introduced into the right femoral artery for monitoring systemic hemodynamics and blood gas parameters. The animals were then laparotomized, and approximately 6 mL of blood was withdrawn from the caudal vena cava for plasma biochemical tests. The organs were resected en bloc, weighed, and fixed in a 10% formalin neutral buffer solution (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) for histochemical examination.

Measurements

Systemic hemodynamics and blood gases were evaluated before hemorrhage (baseline); after hemorrhage; immediately after resuscitation; and 1, 3, and 6 h after resuscitation with the left femoral artery; and at 1, 3, 7, and 14 days with the right femoral artery. Blood samples were collected in 70-IU mL^{-1} heparinized microtubes (125 μL; Clinitubes; Radiometer A/S, Copenhagen, Denmark) for blood gas analyses and in glass capillaries (Terumo Corp., Tokyo, Japan) for Hct measurements. A pH/blood gas analyzer (either model ABL 555 or ABL 700; Radiometer A/S) was used for analyses of arterial blood oxygen tension (PaO_2), carbon dioxide tension ($PaCO_2$), pH, lactate, base excess (BE), and glucose. A recording system (Polygraph System 1000; Nihon Kohden Corp., Tokyo, Japan) was used for continuous monitoring of the arterial blood pressure (BP) and heart rate.

Soon after the measurement of Hct using the glass capillary and centrifugation (12,000 rpm, 5 min), the supernatant (×30 μL) was carefully collected in a plastic tube for measurement of the cytokine levels, HbV concentrations, and methemoglobin (metHb) contents of HbV. The specimens for cytokine measurements were stored in a freezer at −80°C. The concentrations of HbV in the plasma and metHb contents were measured spectroscopically according to a process described in a previous report (17). The collected blood (ca. 6 mL) was centrifuged (5,000*g*, 10 min) to separate the plasma, which was then ultracentrifuged (50,000*g*, 20 min) to sediment the HbV particles from the plasma at 1 and 3 days after resuscitation with HbV to avoid interference by HbV particles in the plasma biochemical assays (20). The obtained transparent serum specimens contained no Hb, indicating that no hemolysis of HbV occurred. The plasma samples of both the HbV and SAB groups and those from 10 healthy rats as the control group were stored at −80°C until biochemical tests were conducted at BML, Inc. (Kawagoe, Japan).

Measurements of cytokine

Plasma cytokine levels were measured using a Bioplex Protein Array system (Bio-Rad Laboratories Inc., Hercules, Calif) according to the manufacturer's instructions. This is a multiplexed, particle-based, flow cytometric assay that uses anticytokine monoclonal antibodies linked to microspheres incorporating distinct proportions of two fluorescent dyes (21). The assay enables quantification of several mediators in a sample volume as small as 15 μL. Our assay was customized to detect and quantify rat IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-α, interferon (IFN) γ, and granulocyte macrophage–colony-stimulating factor (GM-CSF). For each cytokine, eight standards were between 2 and 32,000 pg mL^{-1}.

Histopathological examination

The organs were fixed in a 10% formalin neutral buffer solution (Wako). The paraffin sections were stained using hematoxylin/eosin and by Berlin blue method. Immunohistochemical analyses of spleen and liver tissues were performed to detect the released PEG chains that are conjugated on the HbV surface.

Subsequently, 4-μm-thick paraffin sections were treated with 0.3% H_2O_2 in methanol for 20 min. After blocking nonspecific binding with an antibody diluent (S2022; DakoCytomation, Glostrup, Denmark), they were incubated overnight at 4°C with rabbit monoclonal antibody [PEG-B-47] against methoxy PEG (1/200 dilution with a diluent, ab51257; Abcam PLC, Cambridge, England). They were then incubated for 45 min at room temperature with antibodies against mouse and rabbit immunoglobulins conjugated to the amino acid polymer (no dilution, Histofine Simple Stain MAX-PO(MULTI), Code: 414191; Nichirei Corp., Tokyo, Japan). Negative control was performed without the primary antibody against PEG.

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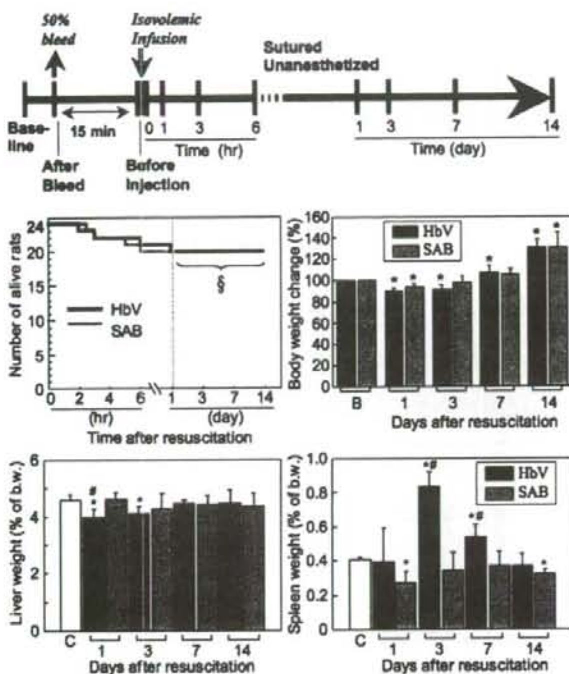

FIG. 1. **Scheme illustrating the experimental protocol (top). Number of rats that after resuscitation from hemorrhagic shock with HbV or SAB (middle left).** The 20 rats survived hemorrhagic shock and resuscitation for 6 h in an anesthetized condition. The catheters in the femoral artery and vein were removed, and the rats were unanesthetized and housed in cages. [§]During this period, all the rats survived until the planned killing (survival rate, 100%) after nonanesthesia for up to 14 days. Among 20 rats, 5 rats each were killed for examination at 1, 3, 7, and 14 days. **Body weight changes (%) of Wistar rats after resuscitation from hemorrhagic shock with HbV or SAB (middle right).** Mean ± SD. No significant difference was found between the groups (n = 5). $^*P < 0.05$ vs. baseline. B, Baseline. **Weight ratios of liver and spleen to body weight at 1, 3, 7, and 14 days after resuscitation with HbV or SAB (bottom).** Mean ± SD. $^*P < 0.05$ vs. baseline; $^\#P < 0.05$ vs. SAB (n = 5). C, Control group. Five healthy rats were used for the control group.

Color was developed using 3,3′-diaminobenzidine (16.7%; Sigma) in 0.05 M Tris-HCl, pH 7.4, containing 0.04% H_2O_2. Nuclei were stained with hematoxylin.

Our separate studies clarified that the PEG molecule itself (MW, 5,000) is very soluble in both water and organic solvents, and it easily disappears from the tissue sections during the staining procedures. On the other hand, PEG-DSPE remains in the sections probably because released PEG-DSPE would be anchored to any cell membranes or proteins.

In vivo *data analysis*

The *in vivo* data are given as the mean ± SD for the indicated number of animals. Data were analyzed using StatView (ver. 5.0; Abacus Concepts, Inc., Berkeley, Calif). For systemic parameters, time-related differences compared with the baseline within each group were assessed using a paired *t* test. Differences among the groups at the same time point were assessed using ANOVA, followed by Fisher protected least significant difference. Unpaired *t* tests were used for comparison of plasma enzyme levels between the groups. Differences were inferred as significant when $P < 0.05$.

RESULTS

Survival rate and body weight increase

Among the 24 rats that received HbV as a resuscitative fluid, 20 rats survived the 6-h anesthetized condition. The catheters in the femoral artery and vein were removed. The rats were unanesthetized and housed in cages. All rats, after awakening from anesthesia, survived up to 14 days later until their planned death (Fig. 1). This is a very similar tendency to that of the rats that received SAB. The body weights of both the HbV and SAB groups (baseline, 274 ± 21 and 287 ± 41 g, respectively) had decreased by 10% at 1 day after resuscitation. However, the rats then tended to gain weight, as measured at 3 days; finally, their weight increased to approximately 130% of the baseline levels. No significant difference was found between the two groups.

Organ weights

The spleen weight ratio to the body weight had increased significantly in the HbV group by 3 days (Fig. 1). Splenomegaly was attributable mainly to the accumulation of HbV; the maximum ratio was observed at 3 days. However, it had reverted to the baseline level by 14 days. The spleen of the SAB group showed no such changes; however, some shrinkage was observed at 1 day probably because of autotransfusion. The liver-weight-to-body-weight ratios of both groups showed no such remarkable changes. However, the HbV group showed significant reduction by 1 and 3 days after resuscitation.

Systemic parameters

Arterial blood pressure before hemorrhage was 84 ± 8 mmHg on the average; it decreased significantly to 25 ± 6 mmHg immediately after hemorrhage. It increased slightly to 39 ± 12 mmHg after 15 min (immediately before resuscitation; Fig. 2). After resuscitation, both the HbV and SAB groups showed immediate recovery beyond the baseline level. The SAB group showed significantly higher BP than HbV. At 1 h, BP was reduced to approximately 75 mmHg and remained stable for 6 h. Overshooting probably occurred because of hypervolemia at the initial phase caused by the combination of autotransfusion, isovolemic injection, and monitoring of peripheral, not central, blood pressure. BP was stable for the succeeding 14 days in both groups. The heart rate (baseline, 414 ± 41 beats per minute) tended to show a slight reduction during shock and at 0, 3, and 6 h after resuscitation. However, both groups showed stable values for the 6 h of anesthesia and for the succeeding 14 days.

The values of PaO_2 (baseline, 81 ± 8 torr) increased to 109 ± 8 torr on the average. In addition, $PaCO_2$ (baseline, 40 ± 5 torr) decreased to 28 ± 5 torr after hemorrhage because of compensatory hyperventilation. The lactate level increased from 1.2 ± 0.4 to 5.3 ± 1.5 mM immediately after hemorrhage and to 8.0 ± 2.2 mM after 15 min. Metabolic acidosis (pH 7.26 ± 0.09) became evident 15 min after hemorrhage (baseline, 7.44 ± 0.03). The BE (baseline, 2.7 ± 1.4 mM) had decreased significantly to −11.7 ± 4.2 mM by 15 min after hemorrhage. The blood glucose level also increased from 9.8 ± 0.7 mM as the baseline level to 23.0 ± 2.4 mM 15 min after hemorrhage. Immediately after resuscitation, PaO_2 and $PaCO_2$ reverted to the baseline level. However, pH, lactate, and glucose levels showed a complete but delayed recovery 1 h after resuscitation. Furthermore, BE showed incomplete recovery 1 h after resuscitation; complete recovery was achieved at 1 day. These parameters were stable

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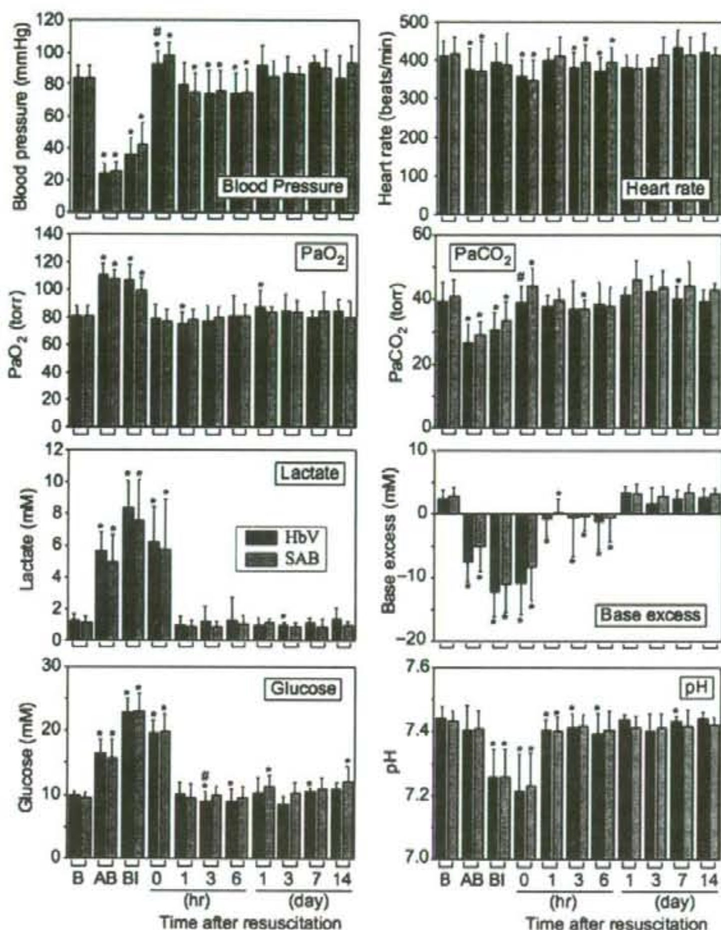

FIG. 2. **Hemodynamic and blood gas parameters during and after hemorrhagic shock and resuscitation with HbV or SAB for 6 h in anesthetic condition and the succeeding 14 days.** Mean ± SD. *$P < 0.05$ vs. baseline. #$P < 0.05$ vs. SAB (B, 6 h; n = 24; 1–14 days; n = 5). AB indicates after bleeding; B, baseline; BI, before injection.

after the anesthetic condition and 14 days of observation. No meaningful difference was found between the groups.

Hematocrit had decreased from the baseline, 40% ± 2%, to 30% ± 3% at 15 min after blood withdrawal because of the dilution of the remaining blood by autotransfusion (Fig. 3). After resuscitation with SAB, Hct increased to 36% ± 2% immediately after resuscitation; it further increased to 41% ± 3% 1 h after resuscitation. It sustained a higher level for 14 days. Immediately after resuscitation with HbV, Hct was further reduced to 20% ± 7% as a result of the dilution of blood; it remained stable for 6 h. Subsequently, it tended to increase and reverted to the original level 7 days after resuscitation and to a slightly higher level at 14 days. The number of platelets decreased markedly after resuscitation with HbV, as did Hct, probably as a result of the dilution of blood, but it returned to the baseline level 3 days after resuscitation, which was earlier than the recovery of Hct. The number of white blood cells (WBCs) showed a slight reduction after resuscitation with HbV, but it increased at 6 h. Large deviations were apparent in WBC; no statistically significant difference was found. The Hb concentration derived from HbV immediately after resuscitation was 3.6 ± 0.8 g dL^{-1}. It did not change until 6 h (3.6 ± 0.5 g dL^{-1}). However, it had decreased to 2.8 ± 0.6 g dL^{-1} at 1 day, 1.6 g dL^{-1} at 3 days, and to an undetectable level at 7 days. The metHb content of the HbV fraction elevated to 37% ± 3% at 6 h and 83% ± 4% at 1 day.

Plasma biochemical parameters

One day after resuscitation, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) tended to increase in both groups in comparison to the baseline levels (Fig. 4). However, they tended to return to the baseline level 3 days after resuscitation. The total protein concentration showed minimal changes. Amylase levels decreased significantly for both groups in comparison to the baseline levels. However, lipase levels displayed no such changes. Creatinine (CRE), uric acid (UA), and blood urea nitrogen (BUN) exhibited marked changes that were not statistically significant. Both groups showed reduction of

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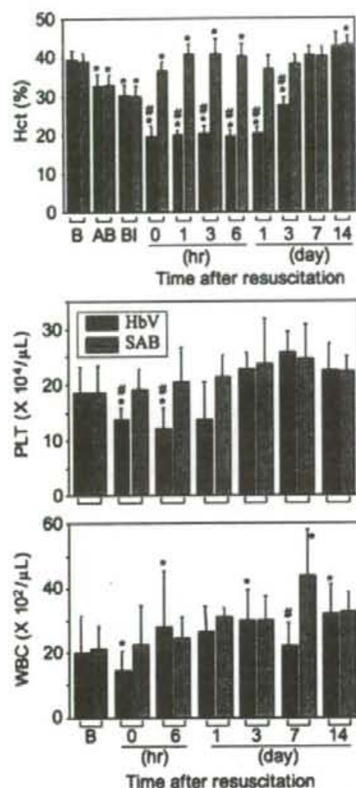

Fig. 3. **Hematocrit and the numbers of platelets and WBCs during and after hemorrhagic shock and resuscitation with HbV or SAB for 6 h in anesthetic condition and the succeeding 14 days.** Mean ± SD. *P < 0.05 vs. baseline. #P < 0.05 vs. SAB (B, 6 h, n = 24; 1–14 days; n = 5). AB indicates after bleeding; B, baseline; BI, before injection.

plasma Fe^{3+} level for 3 days and reverted to the baseline level in 14 days. The return was faster for the SAB group than for the HbV group. The total bilirubin level remained at low levels throughout the experiment (<0.1 mg dL^{-1} detection limit). The plasma total cholesterol (T-Chol) and β-lipoprotein (β-LP) levels had increased significantly for the HbV group at 1 and 3 days after resuscitation, but the phospholipid levels were decreased in both groups. The increased T-Chol and β-LP returned to the baseline levels at 7 days. The SAB group showed no such changes. Erythropoietin activity in the HbV group was significantly higher than that of the SAB group at 1 and 3 days.

Plasma cytokine levels

Neither group showed an increase in IL-1α, IL-2, IL-4, IL-6, IFN-γ, or GM-CSF in comparison to the baseline levels (Fig. 5). Slight increases were apparent for IL-1β for the SAB group (<20 pg mL^{-1}), IL-10 for both groups (<50 pg mL^{-1}), and TNF-α for the HbV group (<50 pg mL^{-1}) after resuscitation. However, they were much lower than those in a septic shock model induced using an LPS injection (IL-1β, 8,000 pg mL^{-1}; TNF-α, 4,000 pg mL^{-1}; IL-10, 1,200 pg mL^{-1}), which were confirmed in our unpublished data and in a previous report (22).

Histological examination

Sections of the spleen and liver of the HbV group showed accumulation of HbV particles in the spleen macrophage in the red pulp zone and liver Kupffer cells 1 day after resuscitation, as portrayed in Figure 6. At 3 days, many nests of erythroblasts were observed in the red pulp zone in the spleen, indicating aggravated extramedullar erythropoiesis. At 7 days, Kupffer cells phagocytizing HbV were not detectable. However, many spleen macrophages phagocyting HbV were observed. At 14 days, HbV disappeared in the spleen. The Berlin-blue method indicated the presence of hemosiderin in macrophages of the spleen in the HbV group at 14 days but not at 1, 3, and 7 days (data not shown). Hemosiderin deposition was undetected in the liver. No other abnormal morphological changes were revealed by our examinations.

Figure 7 portrays immunohistochemical staining of spleen and liver tissues with anti-PEG antibody. For the liver, PEG was detectable 1 day after resuscitation. The strongest deposition was observed at 3 days, but it was not detectable at 14 days. The spleen, on the other hand, showed no staining at 1 day; the strongest deposition was observed at 7 days. It was speculated that PEG of PEG-DSPE became detectable during degradation of HbV. At 14 days, no staining was detected in the spleen.

DISCUSSION

Our primary finding in this study is that HbV showed a similar resuscitative ability to that of SAB, as evidenced by the similar survival rate and levels of systemic parameters after resuscitation for 14 days. The reduced Hct reverted to the original level in 7 days, and the phagocytized HbV seemed to disappear during the observation period.

Experimental hemorrhage induced significant hypotension, hyperventilation, lactacidemia, glycemia, and acidosis. Glycemia is induced by accelerated glycogenolysis in the liver because of catecholamine secretion elicited via baroreceptors in hemorrhagic shock (23, 24). In both HbV and SAB groups, these parameters reverted completely to their respective baseline levels within 1 h after resuscitation, although both groups included four death cases among the 24 rats. Hemorrhagic shock and resuscitation induce ischemia, hypoxia, and reperfusion injury, all of which influence organ functions. Many precedent articles have described the elevation of plasma enzyme levels, AST, ALT, and LDH after resuscitation with HBOCs or transfusion (13, 25–29). Therefore, such elevation would unquestionably occur after resuscitation with HbV or SAB. The plasma enzymatic activities of amylase and lipase, which reflect the function of pancreas, and the levels of CRE, UA, and BUN, which reflect the renal function, showed some significant changes. Nevertheless, they did not seem to constitute meaningful changes in this experiment. The results of the elevated AST, ALT, and LDH raise the issue of the resuscitation treatment. Resuscitation with an oxygen-carrying fluid induces oxidative damage at an early phase; blood volume recovery is primarily important. Slight increases in IL-10 and TNF-α were confirmed, although the levels were very small in comparison

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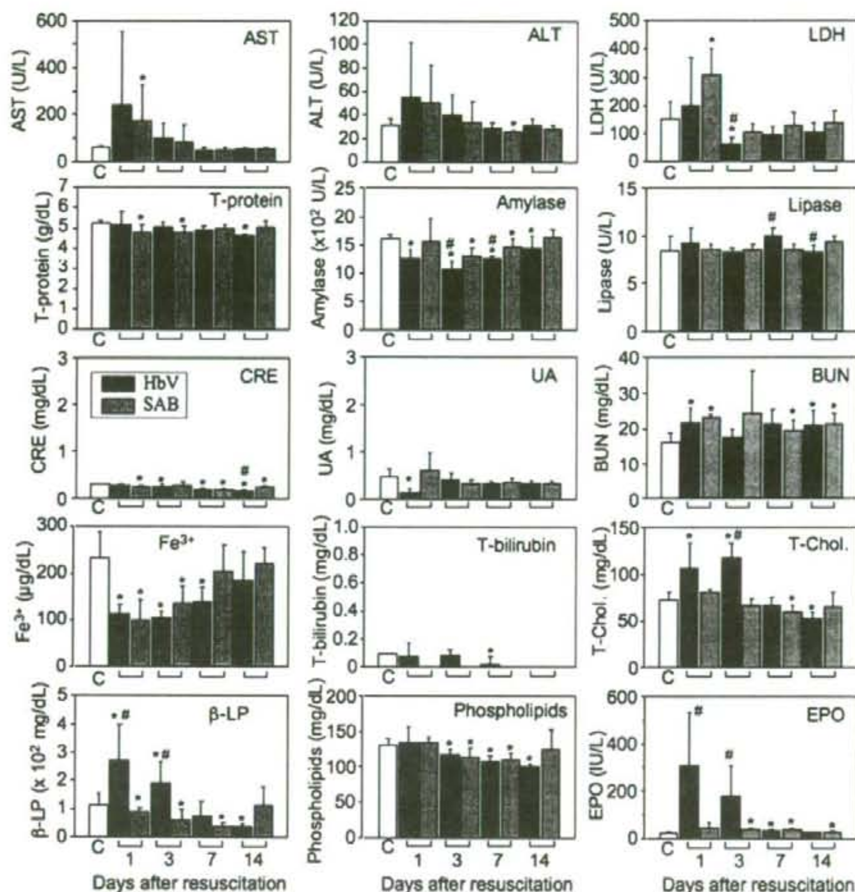

FIG. 4. **Plasma enzyme levels at 1, 3, 7, and 14 days after resuscitation with HbV or SAB.** Mean ± SD. $^*P < 0.05$ vs. control group (n = 5). $^\#P < 0.05$ vs. SAB group. C, Control group. Ten healthy rats were used for the control group. EPO indicates erythropoietin; T-bilirubin, total bilirubin; T-protein, total protein.

to the significant elevation that can result from endotoxemia (data not shown), which is probably a response to light inflammatory damage induced by reperfusion.

The liver is an important organ for degradation of HbV in RES. We anticipated that such an oxidative situation might reduce the capacity of degradation of HbV in RES. Pathological examination of the liver showed evidence of Kupffer cells phagocytizing HbV; it disappeared within 7 days in the liver. In the spleen, substantial accumulation of HbV was confirmed in macrophages in the red pulp zone in the same manner as that in previous studies of bolus injection, daily repeated injections, and exchange transfusion (7, 9, 13). It is reported that PEG-conjugated Hb, one HBOC developed in the United States, also accumulates in the spleen; foamy or vacuolated macrophages were observed (29, 30). Spleen macrophages serve an important role of eliminating a series of HBOCs from blood circulation (31). It must be clarified that no components are deposited in the tissue for a long time. In our previous studies of topload infusion of HbV (20 mL kg^{-1}, [Hb] = 10 g dL^{-1}; 2 g Hb kg^{-1}) and 40% blood exchange (22.4 mL kg^{-1}, i.v., [Hb] = 8.6 g dL^{-1}; 1.9 g Hb kg^{-1}) in healthy rats, the HbV disappeared within 7 days (9, 10). However, in the present study, HbV remained using the time of measurement at 7 days but had disappeared at 14 days. One reason for the slow degradation might be that the dosage is calculated as 28 mL kg^{-1} ([Hb] = 8.6 g dL^{-1}; 2.4 g Hb kg^{-1}), which is slightly greater than that used in the precedent reports. Another reason is that phagocytosis would be compromised to some degree in this shock model, although HbV disappeared at 14 days. The possibility exists that the distribution of HbV is different in this shock model from the distribution shown for topload injection to normal healthy rats. Our collaborators are studying details of the biodistribution.

The gradual increase in the plasma total cholesterol levels by 3 days after resuscitation suggests that the cholesterols are liberated from the RES after the HbVs are captured by the RES and destroyed in the phagosomes of the macrophages (7, 8). In our previous studies of topload HbV infusions, significant increases in the high-density lipoprotein–cholesterol, β-LP, and phospholipids were observed as surplus amounts (8, 9). In contrast, we observed no such increase resembling those that were apparent after the 40% blood exchange and the present resuscitation studies—only

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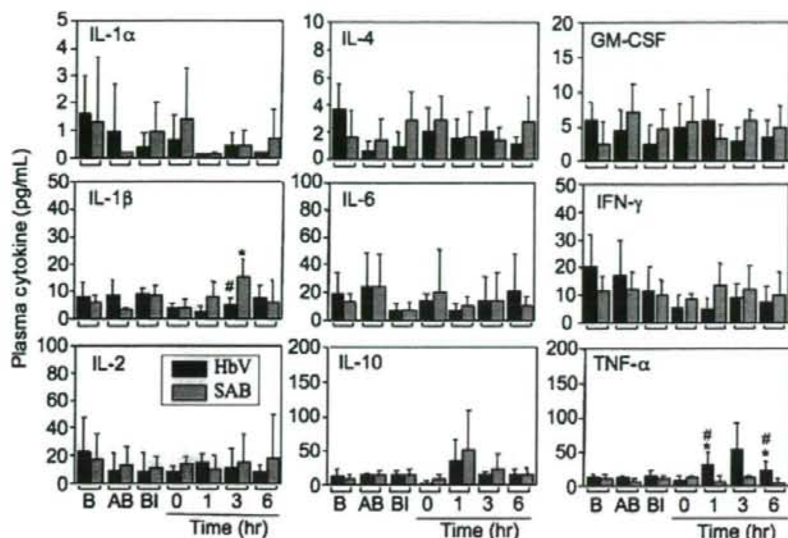

Fig. 5. **Plasma cytokine levels during hemorrhagic shock and a 6-h resuscitation period.** Neither group showed an increase in IL-1α, IL-2, IL-4, IL-6, IFN-γ, or GM-CSF in comparison to the baseline levels. Slight increases were apparent for IL-1β for the SAB group at 3 h, IL-10 for both groups at 1 h, and TNF-α for the HbV group at 3 h after resuscitation. However, they are much lower than those obtained using a septic shock model induced with an LPS injection (IL-1β, 8,000 pg mL^{-1}; TNF-α, 4,000 pg mL^{-1}; IL-10, 1,200 pg mL^{-1}), as described in unpublished data and an earlier report (22). Mean ± SD. $^*P < 0.05$ vs. baseline. $^{\#}P < 0.05$ vs. SAB.

decreases (10). A large demand for nutrients is expected to pertain for hematopoiesis; in addition, the lipid components from HbV can be used efficiently for proliferation. The plasma bilirubin levels and Fe^{3+} showed no abnormal increases, although hemosiderin deposition was detected in the spleen, but not in the liver, of the HbV group at 14 days. Because of the shorter half-lives of the stored RBCs, hemosiderosis is often observed in patients who have received repeated blood transfusions. These results indicate that no obstruction or stasis of the bile is expected to occur in the biliary tree, and that the metabolism of heme from HbV and the iron storage is within physiological capacity (10).

The HbV surface is modified with PEG chains by using the PEG-conjugated phospholipid PEG-DSPE. They stabilize the dispersion state during preservation and in blood circulation. It is expected that PEG-DSPE is hydrolyzed at the crosslink between the PEG chain and DSPE; the released PEG would be excreted through the kidney (32). The weight percentage (wt%) of PEG in the total chemical components of HbV (Hb and lipids) is estimated as only 1 wt%, which is considerably less than that of PEG-Hb (32 wt%); the latter comprises one Hb and six PEG strands (33). However, our previous studies of HbV did not confirm whether PEG disappears from RES. We attempted immunohistochemical analyses using rabbit anti–methoxy-PEG antibody for the first time to collect some information. Kupffer cells showed staining with the anti–methoxy-PEG antibody 1 day after resuscitation, but not in the spleen macrophages. The strongest staining was observed 7 days after resuscitation in the spleen. These results indicate that PEG chains conjugated on the surface of HbV are not recognized using the antibody in a similar manner as the excluded volume of PEG chains on the surface of HbV prevents the access of macromolecules, plasma proteins, and enzymes. During the degradation of HbV, the PEG chains of PEG-DSPE became recognizable with the antibody. At 14 days, PEG was undetectable presumably because the conjugate between the PEG and DSPE was hydrolyzed and PEG would be excreted through the kidney. A detailed biodistribution study is necessary to show complete excretion.

Transient but substantial accumulation of HbV in RES raises concerns of the impact on the ability of the RES to respond to infectious challenge. We previously measured the phagocytic activity by carbon clearance measurement after injection of HbV to normal rats (7). The phagocytic activity decreased transiently 1 day after HbV infusion (20 mL kg^{-1}) by approximately 40%, but it recovered and was enhanced at 3 days, showing a maximum of about twice the quiescent level at 7 days. It then returned to the normal value at 14 days. The initial transient decreased activity indicates a partly, but not completely, suppressed defensive function of the body. It does not seem to cause any irreversible damage to the phagocytic organs. These results are demonstrated only in healthy rats, although rats in hemorrhagic shock or septic shock and those with repeated infusions might react differently in these pathological situations. These results must be clarified through ongoing safety evaluations.

The reduced Hct in the HbV group returned to its original level at 7 days because of the aggravated hematopoiesis, which was evident from the large amount of nests of erythroblasts in the red pulp zone in the spleen. At the same time, the phagocytized HbV was degraded and disappeared. Because of the rapid metHb formation and short circulation half-life, the HbV group became anemic, and erythropoietin excretion was enhanced, which might have facilitated hematopoiesis in the spleen. In rats, extramedullary hematopoiesis induced by hypoxia is localized predominantly in the spleen

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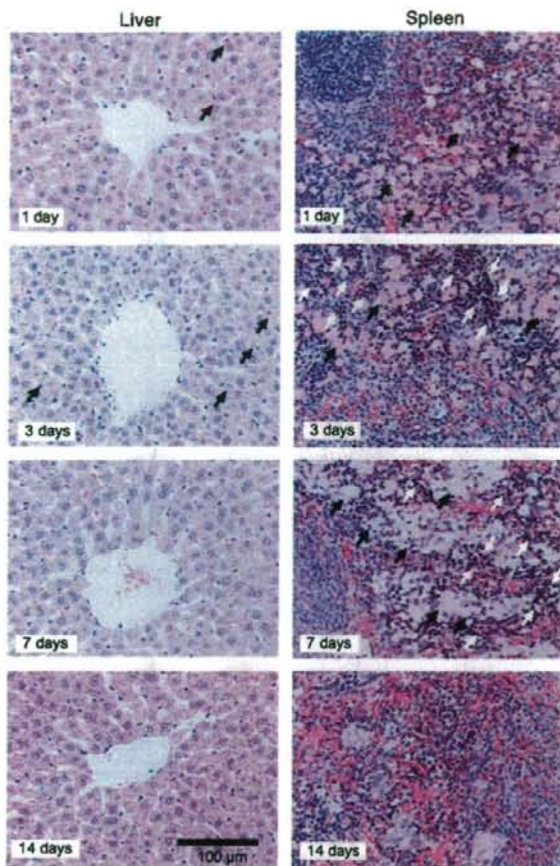

FIG. 6. **Histology of rat liver and spleen of HbV group at 1, 3, 7, and 14 days after resuscitation**. Hematoxylin and eosin staining. Black arrows indicate the presence of spleen macrophages and liver Kupffer cells phagocytizing HbV particles. Hemoglobin vesicles disappeared completely at 14 days. The white arrows in spleens indicate nests of erythroblasts in the red pulp zone, reflecting enhanced extramedullar erythropoiesis.

(34, 35). Intravenous administration of HbV can restore hemorrhagically shocked animals in a critical condition (emergency care). However, the functional half-life of HbV is much shorter than that of RBC. For that reason, additional infusion of HbV or RBC would be necessary to minimize the anemic period. Regarding the safety of repeated infusion of HbV, we previously tested massive doses of HbV to healthy rats using repeated infusions (10 mL kg^{-1} d^{-1} × 14 d). Although the experimental model was not relevant to the present study and clinical situation, all rats survived, and no severe toxicity was confirmed except splenohepatomegaly, hemosiderin deposition, and lipidemia (8). However, we must confirm the safety of repeated infusions of HbV in a hemorrhagic shock model.

In fact, HbV contains no enzymes such as catalase, superoxide dismutase, or metHb-reducing enzymes that are originally present in RBCs. During our rigorous purification process of the Hb solution from outdated RBCs (virus inactivation by heating and virus removal by nanofiltration) (16), we eliminated all unstable enzymes, aiming at utmost safety from infection. The advantages of HbV are that it is pathogen-free and blood-type-antigen-free; moreover, it can withstand long-term storage of a few years, none of which can be achieved by the conventional blood transfusion systems. As a result, the metHb formation is fast, and functional half-life is short. This profile is common to all HBOCs, and we must select suitable clinical uses. In this study, we examined the possible use of HbV as a resuscitative fluid for hemorrhagic shock temporarily, as during an emergency situation, or for bridging until RBCs are available. Other possible uses might be (1) as a fluid for preoperative hemodilution or as a perioperative oxygen supply fluid for a hemorrhage during elective surgery to avoid or delay allogeneic transfusion; (2) as a priming solution for the circuit of an extracorporeal membrane oxygenator during cardiac surgery; and (3) as an alternative for use for other potential indications, for example, so-called oxygen therapeutics to oxygenate ischemic tissues (2).

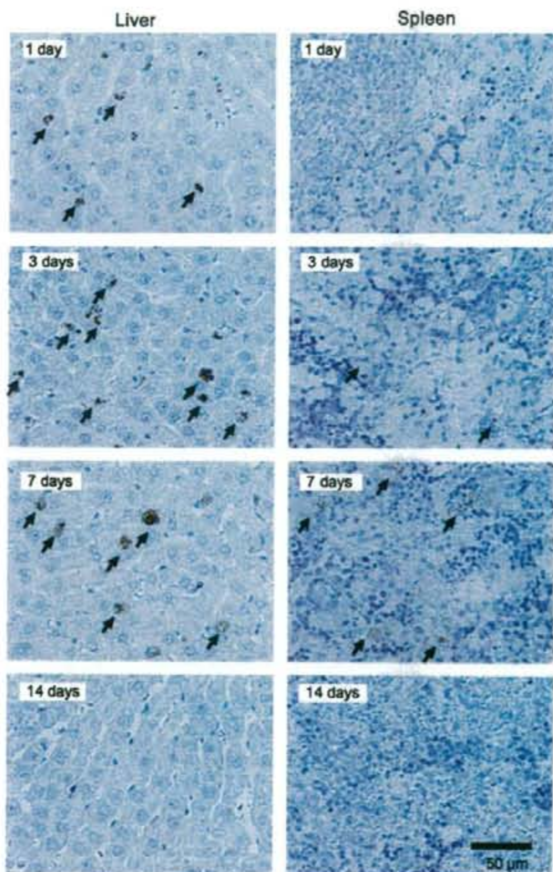

FIG. 7. **Immunohistochemical staining with anti–methoxy-PEG antibody**. Black arrows indicate stains in the liver Kupffer cells and spleen macrophages. Staining was detectable at 1 day in the liver, but not in the spleen, although the amount of HbV was much larger in the spleen. In the spleen, the strongest staining was observed at 7 days after resuscitation. These results show that the anti–methoxy-PEG antibody had difficulty in recognizing PEG on the surface of HbV; also, degradation of HbV in the spleen macrophage is much slower than that in the Kupffer cells.

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In summary, resuscitation with HbV suspended in rHSA showed rapid recovery of hemodynamic and blood gas parameters. They were stable for 14 days. The profiles were identical to those of SAB. The HbV group gained body weight, and their reduced Hct returned to its original level by 7 days because of elevated hematopoiesis. Significant splenomegaly was observed in the HbV group at 3 days because of HbV accumulation. However, it subsided within 14 days. Histopathologically, a significant amount of HbV accumulated in the spleen macrophages, with complete disappearance within 14 days, and HbV induced no overt toxicity. Based on results of this study, we began similar studies using beagle dogs and observed detailed hemodynamic parameters in addition to long-term survival and safety outcomes. Those studies' results will be reported elsewhere.

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Enhanced radiation response of a solid tumor with the artificial oxygen carrier 'albumin-heme'

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Tumor-cell hypoxia is one of the main factors inducing radioresistance. Enhanced tumor oxygenation has previously been achieved in an animal model using the synthetic heme-based oxygen carrier 'albumin-heme' (recombinant human serum albumin-Fe cyclohexanoil heme; rHSA-FeP). The present study was done to determine whether rHSA-FeP enhances the radiation response in an experimental tumor model. Male Donryu rats and LY80, a variant of the syngenic liver ascites tumor, were used. A total of 1×10^6 cells were injected into the subfascial tissue of the right thigh. The rats were divided randomly into five groups: sham (tumor implantation and sham operation); rHSA-FeP; irradiation; rHSA + irradiation; and rHSA-FeP + irradiation. Six days after, under general anesthesia, intra-arterial administration of 10 mL/kg of either 5% rHSA solution or oxygenated rHSA-FeP solution at 2.5 mL/min was done and a dose of 20 Gy was given. There were significant differences in tumor growth between the sham and irradiation groups, and between the sham and rHSA-FeP + irradiation groups. Tumor growth delay was observed and differences were significant between the sham and irradiation groups, and between the irradiation and rHSA-FeP + irradiation groups. In the present study, rHSA-FeP itself had a slight effect on tumor growth without irradiation. Enhancing the effect of rHSA-FeP on the radiation response is responsible in part for the oxygen-carrying property of rHSA-FeP. In conclusion, rHSA-FeP is a candidate radiation-enhancing drug. Arterial infusion of rHSA-FeP may serve as a local oxygenation method that enhances the radiation effect. (*Cancer Sci* 2008; 99: 1274–1278)

Resistance to radiation therapy is observed in many types of tumors and can be due to several causes.[1,2] Tumor cell hypoxia and tumor cell repopulation are the main factors causing radioresistance. Oxygen mediates the majority of biological effects of sparsely ionizing radiation, and the response of cells to radiation depends strongly on the availability of oxygen.[3] Various methods to deliver oxygen to cancer tissue have been studied, including inhalation of high oxygen-content gas,[4] the use of an artificial oxygen carrier,[5-7] and the use of agents that manipulate tumor blood flow.[8] Some of these methods have been studied in the clinical setting.[9,10]

Tumor circulation is characterized by tortuous capillaries, compressed vessels, a scant capillary network, arterio-venous junctions, and plasma channels. It is difficult to deliver adequate oxygen to hypoxic cancer tissue as the tortuous circulation causes limited flow of red blood cells. We developed a synthetic heme-based molecular oxygen carrier, albumin-heme (rHSA-FeP), which is recombinant human serum albumin (rHSA) incorporating a Fe (II)tetra(phenyl)porphyrin derivative (FeP) (Fig. 1). We found that this molecule has the potential to transport oxygen *in vivo*.[11] As for the tumor tissue oxygen status, a hypoxic environment is usual in experimental tumor models as well as in clinical cases. Thus, we analyzed the tissue oxygen tension in the transplanted tumor in a previous report.[12] LY80 was evaluated using the Pd coproporphyrin phosphorescence decay method and we found that intra-arterial injection of rHSA-FeP can increase tissue oxygen tension 2.4 times higher than rHSA solution.

In the present study, we studied whether rHSA-FeP could enhance the response to radiation therapy in an experimental tumor model.

Materials and Methods

Animal and tumor. Five-week-old male Donryu rats (Crj-Donryu; Nippon Charles-River, Yokohama, Japan), with an average weight of 120–150 g, were used in the present study. The rats (two to three per cage) were maintained on a bed of pulp paper in a ventilated, temperature-controlled (23 ± 1°C), specific pathogen-free environment with a 12:12 h L:D cycle and access to food and water ad libitum.

The LY80 tumor cell, a variant of the Yoshida sarcoma, was used for this study. LY80 was established in 1966 by Hiroshi Satoh and has been maintained by successive intraperitoneal transplantation. This tumor was kindly donated by Dr Katsuyoshi Hori of Tohoku University. The characteristics of this tumor have been described by Tanda *et al.*[13] Briefly, this tumor was a subline of ascites hepatoma. Once transplanted intraperitoneally, tumor cells grew in ascites and caused peritoneal dissemination as well as systemic spread. Solid tumors may be obtained by subcutaneous transplantation of tumor cells.

All experimental protocols were reviewed by the Committee on the Ethics of Animal Experiments at our university and were carried out in accordance with the Guidelines for Animal Experiments issued by Keio University School of Medicine, Experimental Animal Center, and law no. 105 and notification no. 6 issued by the Japanese Government. The ethical guidelines that were followed meet the guidelines for animal handling issued by the United Kingdom Co-ordinating Committee on Cancer Research, 1998.

Tumor cell implantation. LY80 cells growing in the peritoneal effusion of a donor rat were collected, suspended in phosphate-buffered saline, and adjusted to a concentration of 10^7 cells/mL. Recipient rats were anesthetized with diethyl ether (Wako Pure Chemical Industries, Osaka, Japan). A small skin incision was made on the lateral side of the inguinal ligament of the right thigh. The tumor cell suspension was drawn into a 1-mL graduated syringe (Nipro, Osaka, Japan), and a 30-gauge syringe needle (Becton Dickinson, Franklin Lakes, NJ, USA) was used to inject 100 μL of the cell suspension directly under the subfascial tissue of the biceps muscle, avoiding the femoral artery and vein. The incision wound was then closed with one layer of thin silk thread, and the animals were allowed to recover.

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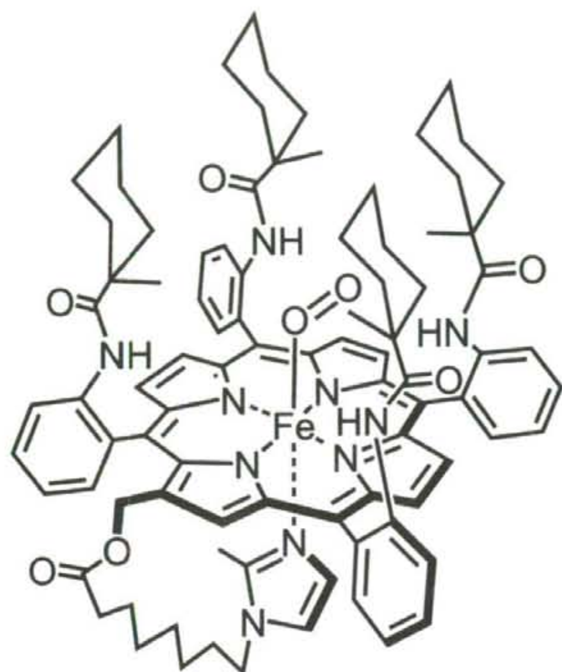

Fig. 1. The chemical structure of FeP: 2-(8-[N-(2-methylimidazolyl)] octanoyloxymethyl)-5,10,15,20-terakis(α,α,α,α-o-[1-methylcyclohexanamido] phenyl)porphinatoiron(II).

Table 1. Characteristics of albumin-heme

Characteristic	rHSA-FeP
Osmotic pressure ratio	0.97
Chlorine (mM)	141.30
pH	7.22
Viscosity (cP)	1.18
Met ratio (%)	0.0
Endotoxin (EU/mL)	<0.6
$P_{1/2}$ (Torr) (37°C)	34.0
FeP (mM)	2.94
rHSA (%)	4.79

EU, endotoxin unit; $P_{1/2}$, half saturation tension at pH7.4; rHSA, recombinant human serum albumin.

Artificial oxygen carrier. rHSA-FeP is an artificial oxygen carrier composed of rHSA incorporating FeP (Fig. 1).[14,15] Its characteristics have been described previously.[14] Briefly, rHSA was obtained from Nipro (Osaka, Japan), and FeP was synthesized according to our previous reported procedure in Tsuchida's laboratory.[15] We named this material 'albumin-heme'. The physical, chemical, and solution properties of rHSA-FeP used in the present study are shown in Table 1.

Irradiation of tumor-bearing rats. Radiation was delivered to the tumor-bearing rats using a radiation unit (MBR-1520R-3; Hitachi Medicotechnology, Hitachi, Japan). A specially designed lead cage was used to deliver radiation only to the area with the tumor as the rest of the body was shielded. The radiation power output was 150 kV at 20 mAmp. A 1.0-mm aluminum filter was used to filter forward-scattered radiation. Dose rate was measured using a Fricke dosimeter. The dosimeter was placed inside the shield at the spot where the tumor was to be located.

The radiation dosage was determined based on the results of a preliminary experiment. Tumor-bearing rats were given 5, 10, 20, or 40 Gy radiation, and tumor growth was observed. The growth rate of tumors given 5 or 10 Gy was not significantly decreased compared to the sham group. However, 20 Gy radiation decreased the tumor growth rate significantly compared to the sham group, and 40 Gy irradiation eradicated the tumors. No dermal reaction was observed among any animal that was given less than 20 Gy irradiation. Therefore, 20 Gy was the appropriate dosage to use in the present study. It took around 8 min to irradiate a tumor.

Six days after tumor cell inoculation, when the tumor grew to a diameter of 10 mm (estimated weight 0.4 g), the rats were divided randomly into five groups: sham group (tumor implantation and sham operation); rHSA-FeP group; irradiation group; rHSA + irradiation group; and rHSA-FeP + irradiation group.

The animals were anesthetized by intraperitoneal injection of pentobarbital (75 mg/kg; DaiNippon Pharmaceutical, Osaka, Japan) prior to catheterization. Intra-arterial catheterization was done using the following technique. A small incision was made on the neck, and the left carotid artery was dissected. A polyethylene catheter (SP31, 0.5 mmID; Natsume Seisakusho, Tokyo, Japan) filled with saline was inserted to a length of 9 cm; in preliminary studies, we had confirmed that the end of the catheter was located approximately 1.0 cm upward of the bifurcation of the descending aorta.

After the polyethylene catheter was inserted, the rat was placed in the irradiation cage, which was then properly positioned with respect to the radiation source and with the shield. The rHSA-FeP or rHSA solution was injected at a rate of 2.5 mL/kg/min for 4 min. Immediately after administration of the material, the irradiation chamber was closed and 20 Gy irradiation was given.

After irradiation the carotid catheter was removed, the left carotid artery was ligated, the wound was sutured, and the animals were allowed to recover. After full recovery, animals were returned to their cages. Animals in the sham operation group were catheterized but received neither materials nor irradiation. They were returned to the cage after removal of the carotid catheter.

Monitoring of animal. Animals were monitored every day. Tumor size and bodyweight were measured and recorded. Movement was also observed. Tumor weight was calculated according to following formula:[16]

$$\text{estimated tumor weight (g)} = A \times B^2/2/1000,$$

where A is the longer diameter of the tumor (mm) and B is the shorter diameter of the tumor (mm).

When the estimated tumor weight reached 30 g (below 12% of the animal's weight), the animal was removed from the study and killed with an excess volume of pentobarbital sodium injected intraperitoneally. The date of removal from the study was recorded.

Tumor suppression rate. Tumor size was measured six days a week, and the tumor weight was calculated. The tumor suppression rate (TSR) was calculated according to the following formula:

$$TSR_n\ (\%) = T_n/C_n \times 100,$$

where T_n is the mean tumor weight at n days after tumor implantation in the radiation, rHSA-FeP, rHSA + radiation, and rHSA-FeP + radiation groups and C_n is the mean tumor weight in the sham group at n days after tumor implantation.

Statistics. The results are expressed as mean ± standard difference. Differences between the groups were assessed using the Tukey–Kramer test. The Kaplan–Meier method was applied to access tumor growth delay, and differences between groups were analyzed using the Log rank test.

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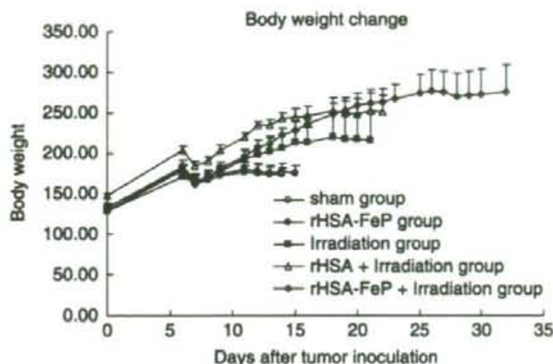

Fig. 2. The bodyweights of rats bearing LY80. The rats were inoculated subcutaneously with 1×10^6 tumor cells on day 0. Radiation treatment was given on day 6. Each value represents the mean ± SD of six rats. There were significant differences between the non-irradiated and irradiated groups after day 12. rHSA, recombinant human serum albumin.

Results

Change of bodyweight. Bodyweight is a parameter that can indicate the severity of disease. The sham group stopped gaining weight 10 days after tumor inoculation; bodyweight decreased thereafter. Although gain of weight ceased, food intake didn't decrease and animals didn't seem distressed. In contrast, animals that received treatment continued to gain bodyweight, except for a temporary 3-day decrease after irradiation (Fig. 2).

Tumor growth curve and tumor suppression rate. There were significant differences between the sham and irradiation groups after day 9. There were also significant differences between the sham and rHSA-FeP + irradiation groups after day 7. A significant difference between the irradiation and rHSA-FeP + irradiation groups was seen after day 19 (Fig. 3).

We compared the TSR on days 7, 12, and 14. The tumor suppression rate is shown in Table 2. On day 7, the TSR was 65.5% in the rHSA-FeP group, 64.3% in the irradiation group, 47.9% in the rHSA + irradiation group, and 39.8% in the rHSA-FeP + irradiation group. We didn't find any significant differences between the groups on day 7. On day 12, the TSR was 69.0% in the rHSA-FeP group, 8.5% in the irradiation group, 8.0% in the rHSA + irradiation group, and 0.6% in the rHSA-FeP + irradiation group. There were significant differences between the sham and irradiation groups, the rHSA-FeP and rHSA-FeP + irradiation groups, and the irradiation group and the rHSA-FeP group, whereas there were no significant differences between the sham and rHSA-FeP groups or the irradiation and rHSA + irradiation groups. On day 14, the TSR was 75.6% in the rHSA-FeP group, 12.8% in the irradiation group, 8.8% in the rHSA + irradiation group, and 0.93% in the rHSA-FeP + irradiation group (Table 2). There were significant differences between the sham and irradiation groups, the sham and rHSA-FeP + irradiation groups, and the rHSA + irradiation and rHSA-FeP + irradiation groups. No differences were observed between the sham and rHSA-FeP groups or the irradiation and rHSA + irradiation groups.

Tumor growth delay. The numbers of animals remaining in the study was recorded and analyzed by the Kaplan–Meier method. Differences between groups were analyzed using the log-rank test. There were significant differences between the sham and irradiation groups ($P < 0.01$) and the irradiation and rHSA-FeP + irradiation groups ($P < 0.03$). There was no difference between the irradiation and rHSA + irradiation groups (Fig. 4).

Table 2. Tumor suppression rate

Group	D7	D12	D14
Sham	100	100	100
Albumin-heme	65.5	69.0	75.6
Irradiation	64.3	8.5	12.8
rHSA + irradiation	47.9	8.0	8.8
Albumin-heme + irradiation	39.8	0.6	0.93

rHSA, recombinant human serum albumin.

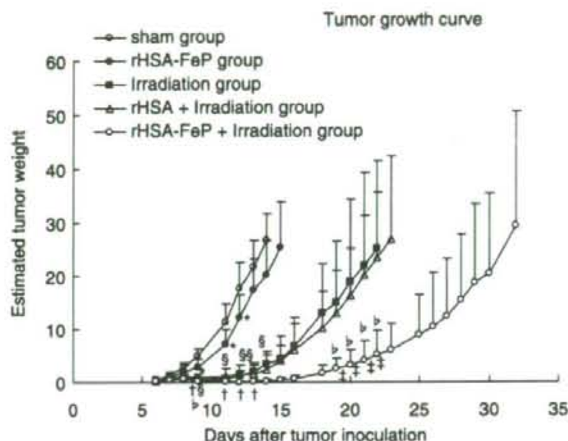

Fig. 3. The tumor weights in rats bearing LY80. Tumor cells were transplanted on day 0. Radiation treatment was carried out on day 6. Each value represents the mean ± SD of six rats. There were significant differences between the sham and recombinant human serum albumin (rHSA)-FeP groups on days 9, 11, and 12 (*$P < 0.05$), and between the sham and irradiation groups on days 9, 11, 12, 13, and 14 (§$P < 0.01$). There were also significant differences between the sham and rHSA-FeP + irradiation groups on days 7, 9, 11, 12, 13, and 14 (†$P < 0.01$). Significant differences between the irradiation and rHSA-FeP + irradiation groups were seen on days 19, 20, 21, and 22 (‡$P < 0.05$), and between the rHSA + irradiation and rHSA-FeP + irradiation groups on days 20, 21, and 22 (♭$P < 0.05$).

Discussion

Radiotherapy is a very potent cancer treatment. However, solid tumors may develop radioresistance by cell repopulation, hypoxia, DNA repair, and production of cytoprotective cytokines. Of these factors, hypoxia is the major cause of radiation resistance; thus, releasing hypoxia is a strategy to enhance the effect of radiation.

Several studies have shown that hypoxic tumors may be radioresistant.[10,17,18] Experimental and clinical studies have proven that oxygenation of tumor tissue increases radiosensitivity.[19,20] Currently, oxygen inhalation is one of the main methods of increasing tumor tissue oxygen tension; mixed-gas inhalation, such as carbogen, is also used. The concept of using an artificial oxygen carrier has long been discussed. Several such products were found to increase tumor tissue oxygen tension (PtO_2).[5,21] Several reports have suggested that artificial oxygen carriers act as radiosensitizers.[22,23] However, in previous papers either oxygen or carbogen inhalation was necessary to enhance radiosensitivity.

In the present study, we used rHSA-FeP, a unique heme-based artificial oxygen carrier, to enhance the effect of radiation on the tumor by increasing the oxygen tension in the tumor tissue. rHSA-FeP has unique characteristics in that the solution's isoelectric

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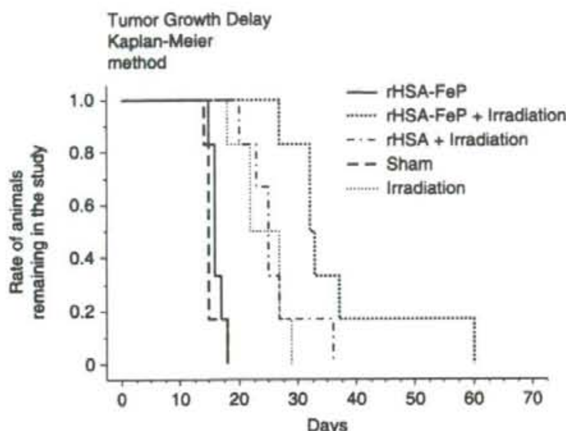

Fig. 4. Tumor growth delay was analyzed using the Kaplan–Meier method. When the estimated tumor weight reached 30 g (approximately 12% of bodyweight), animals were removed from the study. The date of removal was recorded and analyzed. Significant differences between the sham and irradiation groups and the irradiation and recombinant human serum albumin (rHSA)-FeP + irradiation groups were observed ($P < 0.03$). There were no significant differences between the sham and rHSA-FeP groups, or between the irradiation and rHSA + irradiation groups.

point and osmolarity are comparable to native albumin,[15] and it has the potentiality to carry oxygen.[11] rHSA-FeP is synthesized by the association of totally synthetic heme and recombinant human albumin.[15] Recently, rHSA has been developed and manufactured on an industrial scale. rHSA-FeP was reported to be metabolized like the albumin molecule, and the heme component is metabolized in the liver.[11] With rHSA-FeP comprising up to 20% of the blood volume, no toxic effects were noted in an animal study.[24] Thus, rHSA-FeP could be a useful fluid for resuscitation.[15] Because rHSA-FeP is dissolved in the plasma fraction, it can pass through the tortuous vascular system of tumors, whereas the cellular component of blood has difficulty passing through. Therefore, rHSA-FeP is considered to be useful for the delivery of oxygen to hypoxic tumor areas.

In a previously published paper, we reported that rHSA-FeP can increase PtO_2;[12] however, the increase of PtO_2 in the tumor was observed for 300 s, followed by a gradual decrease throughout the monitoring period. This solid tumor showed consistent hypoxia throughout the tumor, therefore we thought that the hypoxic fraction in this tumor is composed of chronically (diffusion-limited) hypoxic fractions.

In the present study, a syngenic tumor model was applied. It is believed that solid tumor growth is orchestrated by various environmental factors such as fibroblast recruitment, angiogenesis, lymphogenesis, immunological reaction, and invasion to the surrounding tissue. Xenograft study help to clarify the effect of targeting certain tumors with specific molecules, however, may not be applicable when studying biological response to radiation.[25] Syngenic systems are beneficial for clarifying the physiological effects of a treatment on the tumor and body.[26,27]

Anemia has been reported to reduce the response to radiation. The results of a clinical study showed that even though human tumors are heterogeneous in terms of oxygen status, the hemoglobin level was an important factor for the radiation response.[28] A low hemoglobin level may reflect a reduced oxygen supply to the tumor, and thus be associated with tumor tissue hypoxia. In the present study, the animals were not anemic, but the artificial oxygen carrier may have delivered an extra supply of oxygen to the tumor.

The current study showed that rHSA-FeP had no effect on tumor growth without irradiation. However, the combination of rHSA-FeP with irradiation resulted in regression of tumor growth. Time to reach 30 g (TR30) became longer when irradiation was added. Moreover, it revealed that in the rHSA-FeP + irradiation group, TR30 was much longer compared to the irradiation group. These phenomenon indicate that rHSA-FeP enhanced the irradiation effect both spatially and temporally.

Even if the rise in oxygen tension in the tumor tissue is limited, it seems important to achieve the maximum radiation effect within the period when the tumor PtO_2 is elevated from the hypoxic conditions. Although there seemed slightest change in the surrounding normal tissue in this experiment, conventional high-dose radiation causes side effects such as skin and soft tissue fibrosis and ulcers. To prevent these side effects and achieve good tumor control, stereotaxic irradiation may be used when rHSA-FeP is the irradiation enhancer.

Linberg and coworkers reported that the administration of polyethylene glycol (PEG)-conjugated hemoglobin increased the surface tissue oxygen tension of UMR-106 osteogenic sarcoma within 2 h of injection.[6] Nevertheless, the delivery of oxygen was rather limited, with only a 78% increase from baseline. The differences observed in the degree of oxygenation of the hypoxia by the albumin-heme and PEG-conjugated hemoglobin could be due to the characteristics of each product (e.g. molecular size, viscosity, surface charge, colloid osmotic pressure, and oxygen-binding affinity). The high viscosity and colloid osmotic pressure of the PEG-conjugated hemoglobin should influence the plasma volume and shear stress on the capillary wall. Perfluorochemical emulsion and modified hemoglobin solution have been reported to improve tumor hypoxia.[21-23] Teicher and colleagues reported that ultrapurified stabilized bovine hemoglobin solution with a high concentration of oxygen inhalation enhanced the radiation response and increased tumor growth delay.[7] In previous studies, radiation-enhancing effects were observed when large amounts of oxygen-carrying material or high concentrations of oxygen were used. In the present study, albumin-heme with air breathing showed an enhanced radiation effect. These materials could be candidates for enhancing radiotherapy and chemotherapy.

Conclusion

Albumin-heme (rHSA-FeP) appears to enhance the effect of radiation in an animal tumor model. Thus, the intra-arterial administration of this artificial oxygen carrier followed by a bolus of radiation could be a potential candidate for clinical study.

Acknowledgments

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