Fig. 2. Capillary hemodynamics in anatomically perfused and ischemic tissues at baseline and 4 h after hemodilution with 6% HES, HbV-HES, and V-HES. Data represent means ± SD. Values for capillary diameter were expressed in percentages of mean in anatomically perfused tissue of control animals at baseline. $*P < 0.05$, $**P < 0.01$ vs. baseline; $\#\#P < 0.01$ vs. other groups; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ vs. HbV-HES.

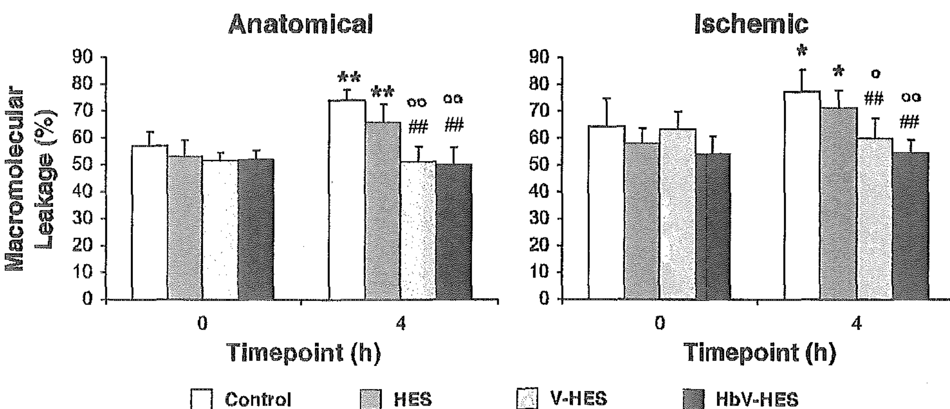

Fig. 3. Macromolecular leakage in anatomically perfused and ischemic tissues at baseline and 4 h after hemodilution with 6% HES, HbV-HES, and V-HES. Data represent means ± SD. $*P < 0.05$, $**P < 0.01$ vs. baseline; $\#\#P < 0.01$ vs. control; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ vs. HES.

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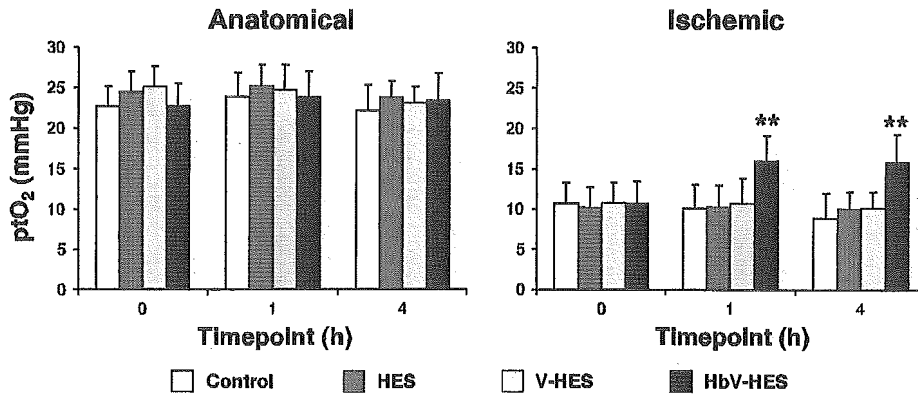

Fig. 4. Partial tissue oxygen tension (P_{tO_2}) in anatomically perfused and ischemic tissues at baseline and 1 and 4 h after hemodilution with 6% hydroxyethyl starch (HES), HbV-HES, and V-HES. Data represent means ± SD. $**P < 0.01$ vs. baseline and other groups.

viscosity. A dependency of FCD on plasma viscosity has been described for conditions of severe hemodilution (2, 3, 33), which has been ascribed to shear stress-induced, nitric oxide-mediated arteriolar vasodilation being required to maintain capillary pressurization (2, 3, 9). However, during the moderate hemodilution applied in the present study, no such arteriolar vasodilation could be observed, which calls for alternative explanations not only for the behavior of FCD but also of capillary RBC velocity and perfusion.

One interpretation may be found in the changes in macromolecular leakage. This parameter allows for a quantitative assessment of capillary leakage, which is an early sign of inflammation appearing in the course of compromised microcirculation such as that due to trauma (31), hemorrhagic shock (5), or ischemia-reperfusion injury (18), and which is paralleled by the activation of the leukocyte-endothelium interaction particularly in the postcapillary venules. Leukocyte adherence, being an early step in this cascade of events, may augment resistance in this vascular segment considerably and thus impair capillary hemodynamics in critically perfused tissues (19). Compared with both the control group and the HES group, macromolecular leakage was significantly reduced in the animals receiving vesicles. Therefore, it may be postulated that the beneficial effect of the vesicles on the capillary hemodynamics was related to a reduction of postcapillary resistance in terms of blunting leukocyte adherence. The capability of leukocytes to adhere to the endothelial wall may be diminished by increasing shear stress (21), which is proportional to linear flow velocity and viscosity of the plasma and inversely proportional to vascular diameter. Provided that our data on plasma viscosity and capillary hemodynamics may be extrapolated to the conditions in the ischemic postcapillary

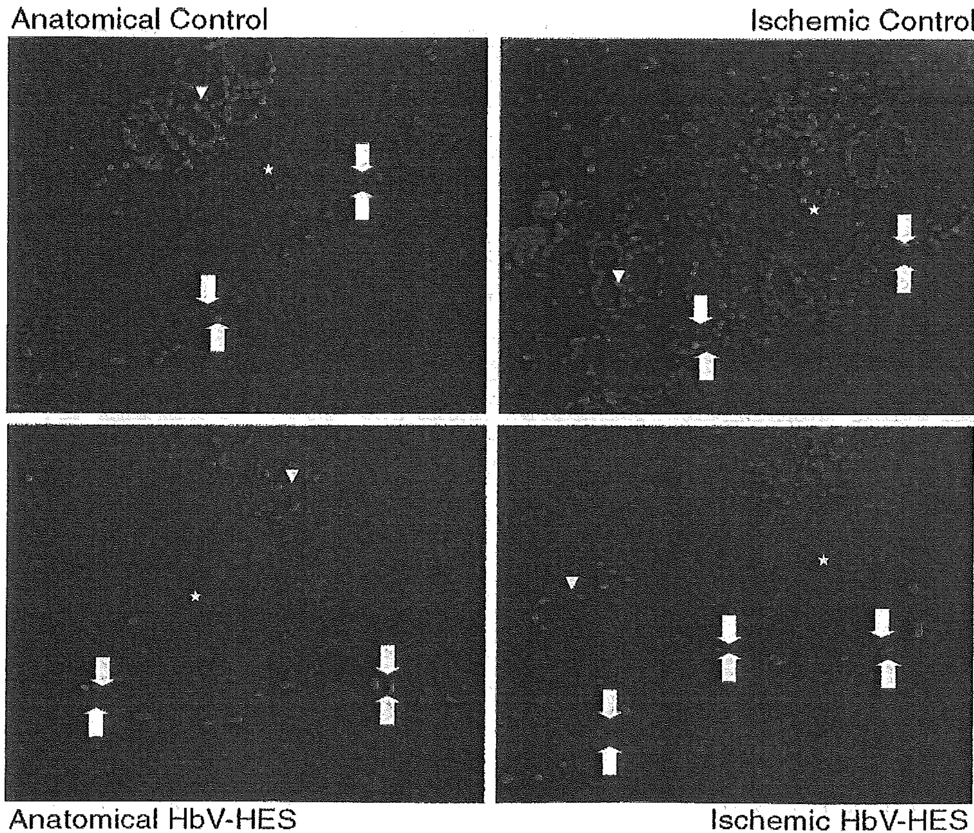

Fig. 5. Transferase-mediated dUTP nick-end labeling (TUNEL) assay of apoptotic cells in anatomically perfused and ischemic tissues 5 h after completion of surgery and 4 h after hemodilution with HbV-HES. Note massive accumulation of red-labeled apoptotic cells in both dermis (☆) and epidermis (arrows) of ischemic tissue and how apoptosis was reduced after hemodilution with HbV-HES. Hair follicles and sebaceous glands are shown (arrowheads).

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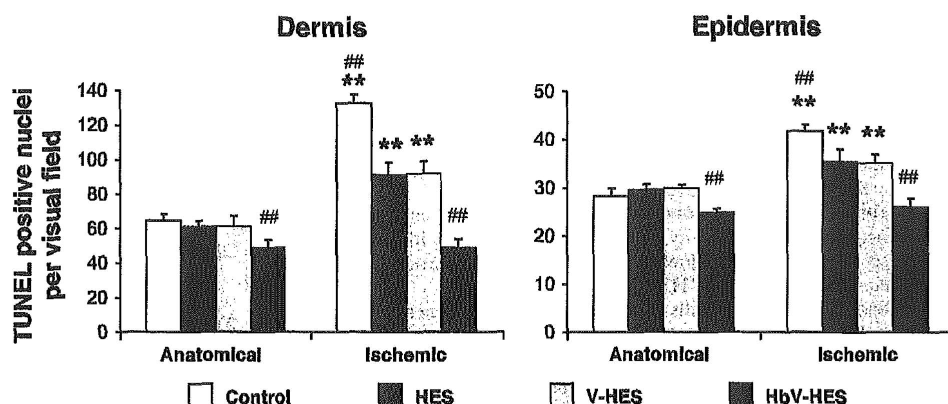

Fig. 6. Density of apoptotic cells in dermis and epidermis of anatomically perfused and ischemic tissues 5 h after completion of surgery and 4 h after hemodilution with 6% HES, HbV-HES, and V-HES. Data represent means ± SD. $^{**}P < 0.01$ vs. anatomically perfused tissue; $^{\#\#}P < 0.01$ vs. other groups.

venules, hemodilution with the vesicle solutions would result in a significant shear stress increase in these vessels compared with baseline and HES, respectively. This mechanism may be of a particular importance in case of ischemia-reperfusion injury after reoxygenation of critically ischemic tissue (16), which may, at least partly, have taken place in the animals receiving HbVs, as evidenced by the improved partial tissue oxygen tension.

In the present preparation, macromolecular leakage appeared to be primarily related to the traumatization of the tissue as a consequence of its surgical manipulation (7), because similar values were obtained in both parts of the flap. However, it is conceivable that the ischemic tissue is more susceptible to changes in postcapillary resistance because of the diminished driving pressure in the collateralized arterioles that are nourished by connecting arterioles, in which perfusion pressures below 30 mmHg were measured, compared with ~45 mmHg in the arterioles feeding the anatomically perfused vasculature (3, 10). With regard to the postulated effect of the vesicles on the postcapillary resistance, this would explain why the vesicle-related improvement of capillary hemodynamics was restricted to the ischemic tissue. Moreover, the vesicle-related increase in capillary perfusion coincided with a decrease in capillary diameters. Given the assumption that the perfusion increase was caused by a reduction of upstream vascular resistance, this would have led to capillary dilation as a result of increased intraluminal capillary pressure (3), whereas intraluminal capillary pressure decreases if vascular resistance is diminished on the postcapillary level. Therefore, the inversely proportional behavior of capillary diameter and perfusion further supports our assumption that the microhemodynamic benefit obtained with the vesicle solutions was predominantly due to its reduction of postcapillary resistance.

Although all capillary hemodynamic parameters in the ischemic tissue were restored to values close to baseline in the anatomically perfused tissue in the V-HES group, this was not sufficient to attenuate hypoxia or hypoxia-induced apoptosis. This suggests that in this group, oxygen delivery to the ischemic tissue is reduced because of a lack of oxygen content in its collateralized, arteriolar inflow, a condition that was presumably circumvented by the presence of Hb in the vesicles because of various reasons. First, the HbVs contribute to a total Hb increase, thus resulting in an enhanced oxygen-carrying capacity not only in terms of arterial oxygen content but also in terms of additional capillary, HbV-related oxygen flow that is not included in the index used to express capillary perfusion in the present study. Second, the high oxygen affinity of the HbVs may have attenuated the unloading of oxygen in the upstream vasculature before reaching the collateralized arterioles, which has been estimated to be as much as 40–50% of the systemic arterial oxygen content (11, 30). This hypothesis is supported by both experimental (15, 25, 30) and theoretical (34) studies, which showed that oxygen delivery may be shifted to the downstream direction if oxygen carriers with high oxygen affinity were infused. Third, because of their small size, HbVs may perfuse capillaries in the compromised microcirculation that are no longer accessible by RBCs. Indeed, HbVs were observed in capillaries showing a cessation of RBC flux (29), which would virtually enhance the density of functional capillaries. Moreover, the occurrence of apoptosis leads to a reduction of oxygen consumption, thus raising partial tissue oxygen tension, provided oxygen delivery remains unchanged. Therefore, the partial tissue oxygen tension increase observed after HbV-HES may underestimate the improvement in oxygen delivery in comparison with the other groups.

In summary, on the basis of the unique constellation in which a HbV solution was compared with a nonoxygen-carrying vesicle solution with identical physicochemical properties, we conclude that the presence of Hb in the vesicles is necessary to obtain an essential improvement of oxygenation and survival in the critically ischemic flap tissue. However, the benefit may, to a certain extent, be ascribed to the rheological changes provided by the vesicles, presumably by reducing postcapillary vascular resistance.

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Acute 40 percent exchange-transfusion with hemoglobin vesicles (HbV) suspended in recombinant human serum albumin solution: degradation of HbV and erythropoiesis in a rat spleen for 2 weeks

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BACKGROUND: Hemoglobin vesicles (HbVs; diameter, 251 ± 81 nm) are artificial O_2 carriers. Their efficacy for acute exchange transfusion has been characterized in animal models. The subsequent profiles of recovery involving the degradation of HbV in the reticuloendothelial system (RES) and hematopoiesis remain unknown, however.

STUDY DESIGN AND METHODS: Isovolemic 40 percent exchange transfusion was performed in 60 male Wistar rats with HbV suspended in 5 g per dL recombinant human serum albumin (HSA; HbV/rHSA, [Hb] = 8.6 g/dL), stored rat RBCs suspended in rHSA (sRBC/rHSA), or rHSA alone. Hematological and plasma biochemical analyses and histopathological examination focusing on the spleen were conducted for the subsequent 14 days.

RESULTS: The reduced hematocrit (Hct) level (26%) for the HbV/rHSA and rHSA groups returned to its original level (43%) in 7 days. Plasma erythropoietin was elevated in all groups: the rHSA group showed the highest value on Day 1 (321 ± 123 mIU/mL) relating to the anemic conditions (HbV/rHSA, 153 ± 22; sRBC/rHSA, 63 ± 7; baseline, 21 ± 3). Simultaneously, splenomegaly occurred in all the groups as HbV/rHSA > rHSA > sRBC/rHSA. Histopathologically, the accumulated HbV in the spleen was undetectable by Day 14, but hemosiderin was deposited in slight quantities for both the HbV/rHSA and sRBC/rHSA groups. Considerable amounts of erythroblasts were apparent in the spleens of both the rHSA and the HbV/rHSA groups.

CONCLUSION: HbVs were phagocytized and degraded in RES, a physiological compartment for the degradation of RBCs, and the elevated erythropoietic activity resulted in the complete recovery of Hct within 7 days in the rat model.

Hemoglobin (Hb)-based O_2 carriers (HBOCs) have been developed progressively for use as a transfusion alternative. Some are now undergoing clinical trials.[1,2] Advantages of HBOCs include the absence of blood-type antigenicity and infectious pathogens and stability for long-term storage when compared with RBC transfusion.[3] Considerably shorter $t_{1/2}$ of the HBOCs in the blood stream (2-3 days) limit their use,[4] but they are applicable for shorter periods of use as: 1) a resuscitative fluid for hemorrhagic shock during an emergency situation temporarily or for bridging until RBCs are available;[5] 2) a fluid for preoperative hemodilution or perioperative O_2 supply fluid for a hemorrhage during elective surgery to avoid or delay allogeneic transfusion;[6] 3) a priming solution for the circuit of an extracor-

ABBREVIATIONS: HBOC(s) = hemoglobin-based O_2 carrier(s); HbV(s) = hemoglobin vesicle(s); MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin count; RES = reticuloendothelial system; sRBC(s) = stored red blood cell(s).

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TRANSFUSION **;**:**-**.

poreal membrane oxygenator during cardiac surgery;[7] and 4) an alternative for use for other potential indications, for example, so-called O_2 therapeutics to oxygenate ischemic tissues.[8,9]

A phospholipid vesicle or liposome-encapsulating concentrated human Hb (Hb-vesicle, HbV) is an HBOC.[10,11] The cellular structure of the HbV (particle diameter, approx. 250 nm) has characteristics that resemble those of natural RBCs because both have lipid bilayer membranes that prevent the direct contact of Hb with blood components and the endothelial lining, thus shielding all side effects of molecular Hb levels.[12,13] Once in circulation, HbV particles are captured by the phagocytes in the reticuloendothelial system (RES or mononuclear phagocytic system) and are metabolized in the physiologically normal pathway after topload infusions.[14-17]

1

We tested the efficacy of HbV suspended in plasma-derived and recombinant human serum albumin (rHSA) in extreme normovolemic hemodilution (80-90% blood exchange) and resuscitation from hemorrhagic shock. They have a comparable O_2-transporting capacity with RBCs.[18-21] We reported results for only a few hours of observation after extensive blood exchange, however.

This study undertakes, for the first time, a longer period of observation (2 weeks) after moderate and clinically relevant isovolemic exchange transfusion of a 40 percent estimated blood volume with HbV suspended in a 5 g per dL rHSA solution.[21] We analyzed plasma biochemical, hematological, and histopathological examinations, particularly addressing the degradation of HbV in RES and erythropoietic activity after the reduced Hct. Splenomegaly was more dominant than hepatomegaly after single and repeated infusions of HbV in our previous studies.[14,15,17] Senescent RBCs are known to be captured and degraded in the spleen.[22] For that reason, we conducted infusion of stored homologous RBCs to compare the relative impacts on the spleen.

MATERIALS AND METHODS

Preparation of HbVs suspended in rHSA

HbVs were prepared under sterile conditions, as reported in previous studies.[23,24] The Hb was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dL) contained 14.7 mmol per L pyridoxal 5′-phosphate (Sigma-Aldrich Corp.., St. Louis, MO) as an allosteric effector at a molar ratio of pyridoxal 5′-phosphate/Hb of 2.5. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd, Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (NOF Corp., Tokyo, Japan), at a molar composition of 5/5/1/0.033. The lipopolysaccharide content, measured with a modified Limulus amebocyte lysate test, was less than 0.1 EU per mL.[25] The physicochemical parameters are P_{50}, 27 Torr; 251 ± 81-nm particle diameter; and less than 3 percent MetHb content. Before use, the HbV suspension ([Hb] = 10 g/dL, 8.6 mL) was mixed with a solution of rHSA (25 g/dL, 1.4 mL; Nipro Corp. Osaka, Japan) to regulate the rHSA concentration in the suspending medium to 5 g per dL. Consequently, the Hb concentration became 8.6 g/dL.[21] Under these conditions, the colloid osmotic pressure and the viscosity (300/sec, 37°C) of the HbV/rHSA were 20 mmHg and 2.9 cP, respectively.

Preparation of stored homologous RBC suspended in rHSA

Blood was withdrawn from donor Wistar rats via the caudal vena cava during ether anesthesia. This was mixed with an RBC preservation fluid, CPDA-1 (C.A. Karmi, Kawasumi Laboratories Inc., Tokyo, Japan) at the volume ratio of 10 percent. The mixture was stored under sterile conditions at 4°C for 1 week because rat RBCs stored for 1 week are reportedly as fragile as the human RBC stored for 4 weeks.[27] After preservation, the stored blood was centrifuged for 10 min at 4000 × g, and then the supernatant and the buffy coat were removed. The sedimented RBCs were resuspended in saline and centrifuged. This procedure was repeated twice. Finally, the RBCs were suspended in a 5 g per dL rHSA solution to prepare stored homologous RBCs suspended in rHSA (sRBC/rHSA). The Hb concentration was regulated at 8.6 g per dL, the same Hb concentration of HbV/rHSA.

2

Exchange transfusion and 2-week observations

Experiments were conducted with 65 male Wistar rats (223 ± 20 g body weight; Saitama Experimental Animals Supply Co., Kawagoe, Japan). During cannulation and exchange transfusion, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (1 mL/kg; Abbott Laboratories, North Chicago, IL). Polyethylene catheters were introduced into the right common carotid artery. Blood withdrawal and sample injection were repeated through one line at 1 mL per 30 seconds. Samples were HbV/rHSA (n = 20), sRBC/rHSA (n = 20), and rHSA only (n = 20). Five rats were used for baseline measurements.

The systemic blood volume was estimated to be 56 mL per kg of the total body weight.[27] Blood was exchanged under the assumption of normovolemia. Therefore, to estimate the necessary amount of HbV, the exchange was assumed to consist of repeating the number of cycles of 1.0-mL withdrawal and sample infusion. The level of exchange, 40 percent, is therefore given as

$$40\% = 100 \times \{1 - [(0.056 \times \text{body weight} - 1.0)/(0.056 \times \text{body weight})]^n\}. \quad (1)$$

The volume exchanged was calculated as $n \times 1.0$ (mL).[28] The sample volume is calculated as 6.0 mL for a rat body weight of 220 g.

After the blood exchange, the catheter was removed, the artery was ligated, and the neck skin was sutured with a stitch. The rats were housed in cages in a barrier room 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-hour 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 with a 1.5 percent sevoflurane-mixed air inhalation. After measuring the body weight, approximately 150 µL of blood was withdrawn from the tail vein via an indwelling needle (24-gauge; Nipro Corp.) for Hct measurement with glass capillaries, and blood cell counts with an automatic blood cell counter (Model KX-21, Sysmex Corp., Kobe, Japan). The animals were laparotomized and approximately 6 mL of blood was withdrawn from the caudal vena cava for the plasma biochemical tests. The organs were resected en bloc and fixed in a 10 percent formalin neutral buffer solution (Wako Pure Chemical Industries Ltd., Tokyo, Japan) and then embedded in paraffin. Four-micrometer sections were stained with the hematoxylin-eosin, Berlin blue, and Giemsa methods.

The collected blood (approx. 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 the exchange transfusion with HbV/rHSA to avoid their interference by HbV particles in the plasma biochemical assays.[29] The obtained transparent serum specimens contained no Hb, indicating that no hemolysis of HbV occurred. They were stored at −80°C until biochemical tests at BML, Inc. (Kawagoe, Japan). Erythropoietin (EPO) was measured with radioimmunoassay. Because the rat EPO shows a high degree of homology with human EPO, the rat EPO cross-reacts in the assay of the antihuman EPO.[30]

3

The experimental protocol was fully approved by the Laboratory Animal Care and Use Committee of School of Medicine, Keio University. It also complied with the *Guide for the Care and Use of Laboratory Animals*.[31]

Statistical analyses

Data are reported as mean ± standard deviation (SD) for all measurements. Differences between the control (baseline) group and a treatment group were analyzed with a one-way analysis of variance followed by Fisher's protected least significant difference test. The changes were considered significant if the p value was less than 0.01.

RESULTS

Body and spleen weights and hematological tests

Rats of all groups well tolerated the 40 percent blood exchange; they survived until their intentional euthanization. The rats survived this intervention because of the normovolemic exchange transfusion while maintaining the blood colloid osmotic pressure with 5 g per dL rHSA as the suspending medium. All rats gained weight until their euthanization (Fig. 1). No noticeable change occurred in their behavior or appearance such as the pilomotor response.

The spleen:body weight ratio increased significantly for the HbV/rHSA group at 1 and 3 days after the exchange. It returned to a level that was comparable to the baseline at 14 days. The rHSA group also showed significant splenomegaly at 3 days, but no splenomegaly at 1 day. At 14 days, the spleen weight reverted to the baseline level. The sRBC/rHSA group also showed moderate, but significant, splenomegaly on Days 1, 3, and 7.

The Hct before the exchange transfusion was approximately 43 percent. It decreased to about 26 percent for the HbV/rHSA and rHSA groups. Both groups showed a monotonic Hct increase; at 7 days, the Hct showed a complete recovery to the baseline level (about 43%) and an overshooting at 14 days (approx. 46%). In the sRBC/rHSA group, the Hct level at 1 day was much higher than that of the other groups because of the sRBC infusion. The Hct level, however, was slightly lower than for the other groups at 7 and 14 days. The mean corpuscular Hb (MCH), mean corpuscular volume (MCV), and mean corpuscular Hb count (MCHC) values remained within normal ranges (data not shown); however, MCH and MCHC of the HbV/rHSA group at 1 and 3 days were not measured because of the presence of HbV. The sRBC/rHSA group showed slightly lowered MCV and MCH levels at 1 day. In contrast to Hct, platelet and white blood cell counts showed nonsignificant decreases at 1 day and then maintained rather steady values. The plasma Hb concentration derived from HbV after the exchange transfusion was estimated as 4.4 g per dL, which decreased, respectively, to 1.8 ± 0.1, 1.1 ± 0.1, and 0 g per dL on Days 1, 3, and 7.

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Plasma biochemical tests

The plasma EPO level, an indicator of an anemic, hypoxic, or stressed condition, increased significantly from 21 ± 3 IU per L in the normal condition to 312 ± 123 IU per L for the rHSA group at 1 day, which was significantly higher than for the HbV/rHSA group (153 ± 22 IU/L) or the sRBC/rHSA group (63 ± 7 IU/L; Fig. 2). After 3 days, they decreased to less than 100 IU/L; at 7 days, they reverted to the baseline level.

Regarding the other routine analytes, apartase aminotransferase showed slight increases on Day 1 for all

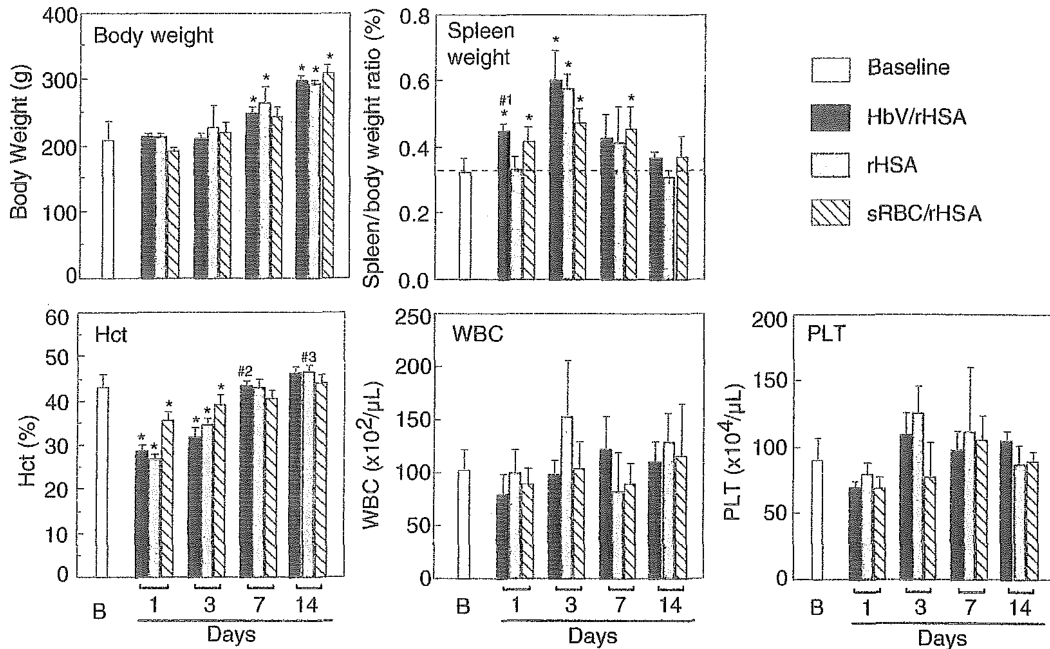

Fig. 1. Changes in body weight, spleen:body weight ratio, and hematological parameters after 40 percent exchange transfusion with HbV/rHSA, rHSA, or sRBC/rHSA. The spleen:body weight ratio (baseline, 0.32 ± 0.04%) increased significantly for the HbV/rHSA group at 1 day (0.45 ± 0.03%) and 3 days (0.60 ± 0.09%). It returned to the baseline at 14 days (0.37 ± 0.02%). The rHSA group also showed significant splenomegaly at 3 days (0.58 ± 0.05%) and returned to 0.31 ± 0.02 percent at 14 days. The sRBC/rHSA group also showed splenomegaly at 1, 3, and 7 days (0.42 ± 0.04, 0.48 ± 0.04, and 0.46 ± 0.06%, respectively). The baseline Hct level was 43 percent; it decreased to about 26 percent for the HbV/rHSA and rHSA groups. At 7 days, they showed complete recovery to approximately 43 percent and then further increased to approximately 46 percent at 14 days. The values are means ± SD. The broken line indicates the baseline value. *Significantly different from the baseline ($p < 0.01$); #1 significantly different from the rHSA group ($p < 0.01$); #2 $p = 0.0288$ versus sRBC/rHSA; #3 $p = 0.0353$ versus sRBC/rHSA. B = baseline.

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groups (HbV/rHSA, 70 ± 5 U/L; rHSA, 69 ± 12; sRBC/rHSA, 72 ± 9; baseline, 60 ± 7), but it reverted to the original level, whereas alanine aminotransferase was stable. Alkaline phosphatase and γ-glutamyltransferase showed significant or nonsignificant reductions for all groups throughout the experiment. Creatine phosphokinase was stable for 14 days. For all groups, creatinine and uric acid were maintained at low levels for 14 days (data not shown). Amylase showed some significant reduction, but did not change markedly for 14 days (Fig. 3). In contrast, lipase showed significant and marked increases for the HbV/rHSA group for 3 days, but it tended to decrease after 7 days.

Regarding plasma lipid components in the HbV/rHSA group, the total cholesterol and free cholesterol showed significant increases with maximum values at 3 days (Fig. 3). Nevertheless, they returned to their original levels at 7 days. The β-lipoprotein tended to decrease after the exchange transfusion, showing significant reductions at 3 and 7 days for the rHSA group. The high-density lipoprotein cholesterol also tended to decrease with a significant reduction at 3 days for the rHSA group. Triglyceride tended to decrease for all groups with a significant difference in the HbV/rHSA group at 1 and 3 days, partly because of ultracentrifugation of the plasma fractions, and in the rHSA group at 7 days. At 14 days, they generally recovered to the baseline level. The phospholipid tended to decrease with significant differences for all groups. Free fatty acid tended to increase at 14 days. The serum bilirubin (<0.1 mg/dL) remained at a low level throughout the experiment.

In electrolyte concentrations, Fe^{3+} showed significant reductions at 1 and 3 days for the HbV/rHSA group, at 3 and 7 days for the rHSA group, and at 3 days for the sRBC/rHSA group, but they returned to the original level at 14 days (Fig. 3).

Histopathological study

Histopathological examination revealed no significant changes in the lung, heart, and kidney in all groups. At 1 and 3 days after infusion, significant amounts of HbV phagocytized by macrophages in the marrow and Kupffer

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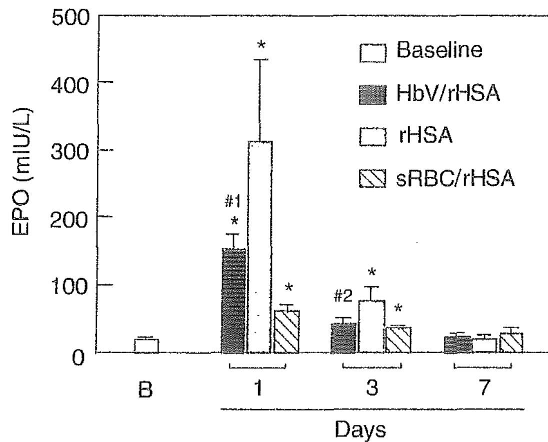

Fig. 2. Plasma EPO activity after 40 percent exchange transfusion with HbV/rHSA or rHSA. All groups showed significant increases at 1 day. However, the HbV/rHSA groups showed a lower level than the rHSA group. The values are means ± SD. *Significantly different from the saline group ($p < 0.01$); [#1]$p = 0.0222$ versus rHSA; [#2]$p = 0.0195$ versus rHSA. B = baseline.

cells in the liver were observed. However, HbV decreased significantly at 7 days and was undetectable at 14 days. At 3 days after infusion, the pancreas in the HbV/rHSA group showed no significant morphological changes in spite of the increased lipase activity.

Sections of the spleen of the HbV/rHSA group, which is stained with Giemsa method, revealed the accumulation of HbV particles in the red pulp zone at 1 and 3 days after the exchange transfusion. The amount of the accumulated HbV decreased at 7 days and then became undetectable at 14 days (Fig. 4). Throughout the period examined in this study, nests composed of erythroblasts and proerythroblasts were formed in the splenic cord, especially at 3 and 7 days, indicating extramedullar erythropoiesis. Nest formation was remarkable for the rHSA group at 3 days. Hematopoietic activity was also observed at 3 days in the marrow of the HbV/rHSA group that contained erythroblastic islets.

The Berlin blue method indicated the presence of hemosiderin in macrophages of the spleen in the HbV/rHSA group at 7 days. This hemosiderin deposition increased until 14 days (Fig. 5). A small amount of hemosiderin was confirmed in the Kupffer cells of the liver at 14 days. Hemosiderin deposition, however, was undetected in the marrow. In addition, in the sRBC/rHSA group, hemosiderin deposition was present in the spleen macrophages at 14 days.

DISCUSSION

A main finding of this study is that the reduced Hct level after the 40 percent exchange transfusion with HbV/rHSA returned to the original level after 7 days; furthermore, the accumulated HbVs in RES became undetectable within 14 days. Significant splenomegaly is attributable to the combination of the accumulation of HbV in the red pulp zone and the considerable presence of nests of erythroblasts in the splenic cord in response to the EPO secretion, but these observations subsided within 14 days.

Extensive studies of circulation kinetics and organ distribution of isotope-labeled HbV clarified that HbV accumulates preferentially in the RES.[11,16] One cause of the splenomegaly is the accumulation of HbV particles in the red pulp zone, as shown in Fig. 4. It subsided completely within 14 days, however. Gradual increases in the plasma cholesterol levels by 3 days after infusion and lack of disruption of the HbV in the plasma suggest that the cholesterols are liberated from the RES after the HbVs are captured by the RES and destroyed in the phagosomes of the macrophages.[14,15] In our previous studies of topload HbV infusions, significant increases in the high-density lipoprotein cholesterol, β-lipoprotein, and phospholipids were observed as surplus amounts.[15,17] In contrast, we observed no such significant increases after the 40 percent blood exchange, only decreases. A large demand of nutrients should pertain for hematopoiesis and so on; also, the lipid components from HbV might be utilized efficiently for proliferation.

During the metabolism of Hb, we would expect a release of bilirubin and iron. They did not increase in the plasma within 14 days, however. The released heme from Hb in HbV might be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells of the liver and the spleen macrophages.[15,31] Bilirubin would normally be excreted in the bile as a normal pathway, and no obstruction or stasis of the bile should occur in the biliary tree. Berlin blue staining revealed considerable deposition of hemosiderin in the liver and spleen, even after 14 days. Normally, iron from a heme is stored in the ferritin molecule.[33] Both ferritin and hemosiderin release iron. They are anticipated to induce hydroxyl radical production followed by lipid peroxidation.[34] The iron release rate from hemosiderin, however, is substantially less than that from ferritin.[35] Consequently, the excess amount of iron would then normally be stored in an insoluble and less toxic form as hemosiderin. Hemosiderosis is often observed in patients who have received repeated blood transfusions because of the shorter $t_{1/2}$ of the stored RBCs. Moderate splenomegaly and hemosiderin deposition were also confirmed in the spleen in the sRBS/rHSA groups of this study, partly because of the accumulation and degradation of stored RBCs with the lowered membrane deformability and shortened circulation $t_{1/2}$.[26] These results indicate that the metabolism of heme from HbV and the iron storage is within the physiological capacity that has been well characterized for the metabolism of senescent RBCs.[35]

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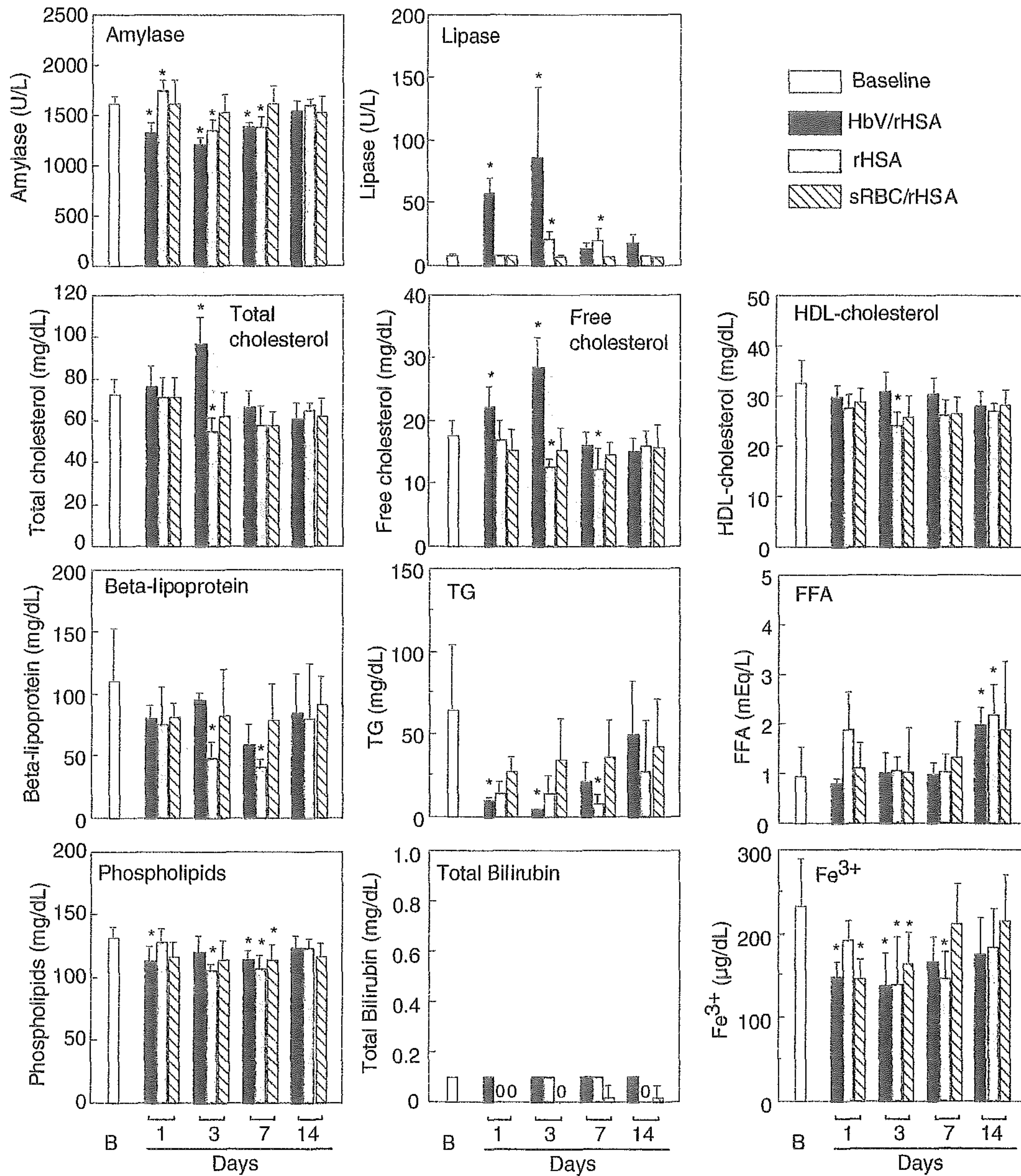

Fig. 3. Plasma biochemical tests representing the metabolism of the components of HbV (lipids and Hb) and pancreatic function after 40 percent exchange transfusion with HbV/rHSA, rHSA, or sRBC/rHSA. The values are means ± SD. *Significantly different from the saline group ($p < 0.01$). TG = triglyceride; FFA = free fatty acid; B = baseline.

Interestingly, not only the HbV/rHSA and sRBC/rHSA groups, but also the rHSA group, showed a significant splenomegaly at 3 days, even though the rHSA group showed no symptoms on Day 1. In rats, extramedullary hematopoiesis induced by hypoxia is localized predominantly in the spleen.[37,38] We observed extensive nests of erythroblasts in the splenic cords, especially at 3 days. It is not plausible that the rHSA as an xenogeneic protein accumulates in the spleen macrophages, according to the fact the ^{125}I-labeled rHSA in a rat showed no specific distribution to the spleen.[39,40] Therefore, the splenomegaly for the rHSA group is attributed to the erythropoiesis stimulated by the significant increase in the plasma EPO level.

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COLOUR FIG.

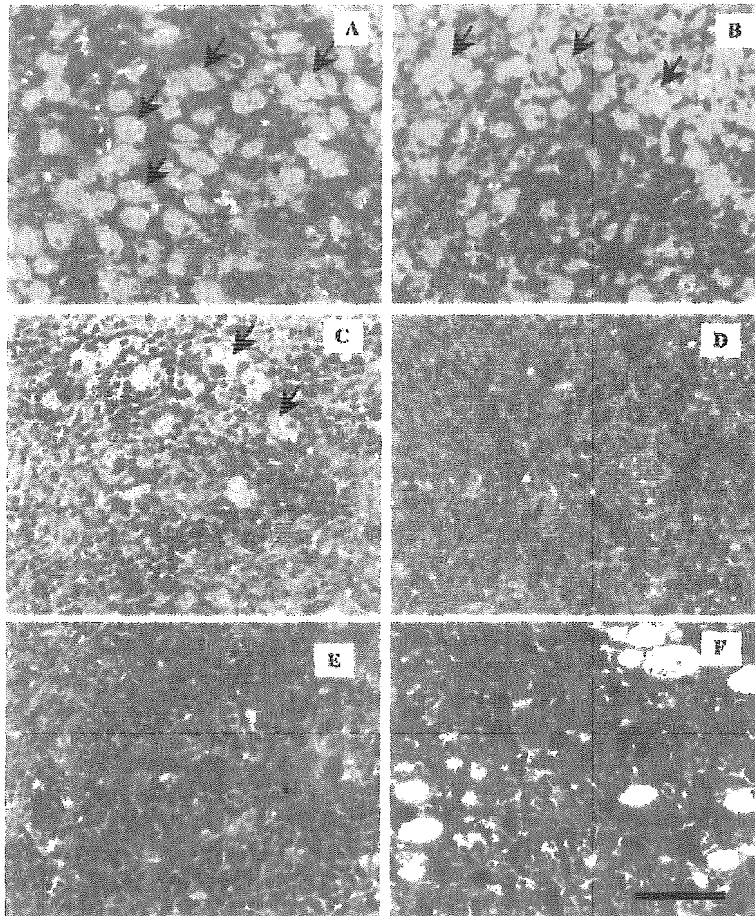

Fig. 4. Histology of rat spleen and marrow after exchange transfusion with HbV/rHSA or rHSA alone. (A-C) Respective images of the spleen of the HbV/rHSA group at 1, 3, and 7 days. Accumulated HbV particles are visible as light-blue areas (black arrows). Nests of erythroblasts are visible as dark blue cells (red arrows). The domain of the HbV particles decreased significantly at 7 days. (D) Spleen of the HbV/rHSA group at 14 days. HbV particles had disappeared, whereas the erythroblast nests remained, as indicated by the red arrows. (E) Spleen of the rHSA group at 3 days. The erythroblast nest formation is remarkable. (F) Marrow of the HbV/rHSA group at 3 days. Hematopoietic activity is visible. Bar = 50 µm (Giemsa method).

Plasma EPO release from the kidney strongly reflects an anemic condition, depending on the O_2-carrying capacity of the circulating blood.[41,42] The highest EPO level was seen in the rHSA group, indicating that its anemic condition was the severest. Because of the short $t_{1/2}$ and MetHb formation,[43] the HbV/rHSA also showed a significant increase in the EPO level. It was considerably lower than that of the rHSA group, however. The sRBC/rHSA group also showed a moderate increase in the EPO level probably caused by the reduced Hct by the exchange transfusion. Accordingly, the splenomegaly for the HbV/rHSA and sRBC/rHSA groups is also partly attributable to the nests of erythroblasts for erythropoiesis that was sufficient for recovery from the reduced Hct. Interestingly, both HbV/rHSA and rHSA groups tended to show higher Hct values than the sRBC/rHSA group at 7 and 14 days, probably because of the enhanced erythropoiesis caused by the higher levels of EPO excretions than for the sRBC/rHSA group. The MCH, MCV, and MCHC levels were normal overall, supporting our inference of normal erythropoiesis.

Routine plasma biochemical tests showed that the hepatic function was maintained despite the large amount of HbV that were captured and degraded by Kupffer cells. Significant reductions were seen in the amylase activity, whereas a transient increase in lipase activity was observed consistently in our previous topload infusion experiments; this should be due to the up regulation of lipase in response to the infusion of phospholipid vesicles.[15,17,44]

In conclusion, all rats tolerated the 40 percent exchange transfusion with HbV/rHSA and showed complete Hct recovery within 7 days. Although transient splenomegaly and the hemosiderin deposition were confirmed, no excess iron was found in the blood. The recycling or excretion of iron as well as lipid components should be on the physiological pathway that is known for the degradation of senescent RBCs. Although some aspects remain unresolved, the present results offer important information on the safety and handling of HbV during preoperational or perioperational infusion in a clinical setting.

ACKNOWLEDGMENTS

The authors acknowledge K. Sou and Mr Y. Masada (Waseda University) for HbV sample preparation; Mr H. Abe, Ms. T. Yamaguchi, and Mr S. Kurasaki (Department Pathology, Keio University) for their excellent histopathological techniques; and M. Suematsu, PhD (Department Biochemistry, Keio University), and Dr M. Murata (Department Intern. Medical, Keio University), for meaningful discussion of phagocytic and

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COLOUR FIG.

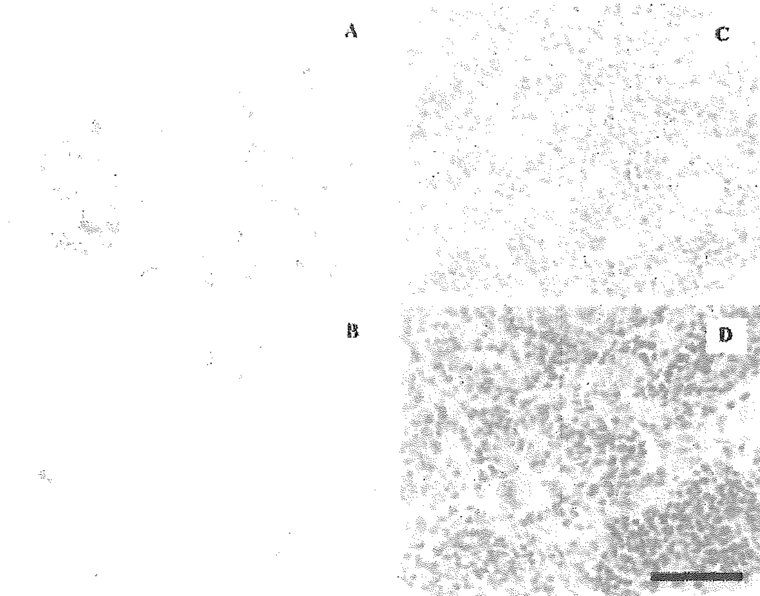

Fig. 5. Histology of rat spleen, liver, and marrow 14 days after exchange transfusion with HbV/rHSA or sRBC/rHSA. Spleen (A), liver (B), and marrow (C) of the HbV/rHSA group. The spleen and liver contained slight hemosiderin deposition, but not the marrow. The spleen of the sRBC/rHSA group (D) also contained slight hemosiderin deposition. Bar = 50 μm (Berlin blue method).

hematopoietic activities. The rHSA was obtained from Nipro Corp.

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Performances of PEG-modified hemoglobin-vesicles as artificial oxygen carriers in microcirculation

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Abstract. Hemoglobin-Vesicles (HbV; diameter, 250 nm) are artificial O_2 carriers encapsulating purified and concentrated human Hb solution in phospholipid vesicles, and their safety and efficacy, as a transfusion alternative, have been studied. In this paper, we summarized the characteristics of HbV that have been clarified by the microcirculatory observations.

Keywords: Blood substitutes, liposome, microcirculation, EDRF, oxygenation

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1. Introduction

Hemoglobin (Hb)-based O_2 carriers (HBOCs) have been developed for use as a transfusion alternative and some of them are now in the process of clinical trials [1]. The advantages of the HBOCs are the absence of blood-type antigenicity and infectious pathogens, and stability for long-term storage when compared with the RBC transfusion [2]. A phospholipid vesicle or liposome encapsulating concentrated human Hb (Hb-vesicle, HbV) has been developed as an O_2 carrier [2–7]. The cellular structure of the HbV (particle diameter, ca. 250 nm) has characteristics similar to those of natural RBCs, since both have lipid bilayer membranes that prevent the direct contact of Hb with the components of blood and the endothelial lining [8]. The reasons for the Hb encapsulation in red blood cells (RBCs) should be: (1) a decrease in the high viscosity of Hb and a high colloidal osmotic pressure; (2) prevention of the removal of hemoglobin from the blood circulation; and (3) preservation of the chemical environment in the cells such as the concentration of phosphates (2,3-DPG, ATP, etc.) and other electrolytes. Moreover, during the long history of the development of Hb-based O_2 carriers (HBOCs), many side effects of molecular Hb have become apparent. These side effects of molecular Hb would imply the importance of the cellular structure.

Our *in vivo* studies of HbV have revealed the sufficient O_2 transporting efficiency comparable to RBCs [9–12], the safety in terms of blood compatibility [13], and prompt degradation in the reticuloendothelial system [14–17], all of which make us confident about advancing to the further development of HbV.

In this paper, we focus on the performances of our polyethylene-glycol (PEG)-modified HbV from the viewpoint of hemorheology and microcirculation.

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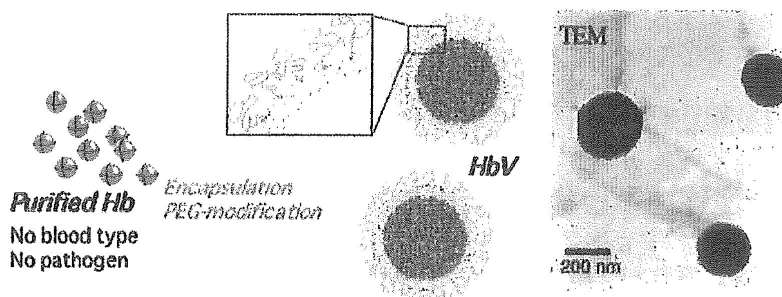

Fig. 1. Hemoglobin-vesicles (HbV) encapsulate the ultrapurified and concentrated human Hb solution (35 g/dl) with phospholipid bilayer membrane, and the surface is modified with polyethylene glycol chains. The well-regulated particle size (about 250 nm) was confirmed by TEM. One particle contains about 30,000 Hb molecules and about 6000 PEG chains were fixed on the surface.

2. Impact of PEG-modification of HbV

The rheological property of an artificial oxygen carrier is important because the infusion amount should be significantly large and that may affect the blood viscosity and hemodynamics. One HbV contains about 30,000 Hb molecules so that the suspension of HbV does not have colloid osmotic pressure (COP) (Fig. 1). The HbV suspended in 5 g/dl HSA at [Hb] = 10 g/dl shows comparable COP and viscosity to the blood.

We tested he function of PEG-modified and unmodified HbV as a blood replacement in the subcutaneous microvasculature of awake hamsters during severe hemodilution in which 80% of the red blood cell mass (70 ml/kg) was substituted with suspensions of the vesicles in 5% HSA solution [18,19]. Both materials yielded normal mean arterial pressure, heart rate, and blood gas parameters, which could not be achieved with albumin alone. Subcutaneous microvascular studies showed that PEG-modified HbV/HSA significantly improved microhemodynamic conditions (flow rate, functional capillary density, vessel diameter, and oxygen tension) relative to unmodified HbV/HSA. PEG-modified HbV was homogeneously dispersed in the plasma phase while the unmodified HbV showed aggregation in venules and capillaries. Even though it was confirmed *in vitro* that the aggregates dissociated reversibly at higher shear rates, it is unlikely that they would dissociate in vessels where the flow rate or shear stress was low. Aggregation and decreased flow rate may constitute a vicious circle that reinforces negative effects on blood flow. PEG reduced vesicular aggregation and viscosity, improving microvascular perfusion relative to the unmodified type. From this result, PEG modification is important for HbV in microvascular blood flow.

3. Interaction with NO and CO

As clinical trials of the chemically modified Hbs are extended to include larger numbers of individuals, it becomes apparent that the principal side effect consistently reported in the administration of acellular Hb solutions is hypertension presumably because of vasoconstriction. Hypertension, a well-defined reaction of the acellular intramolecularly cross-linked Hb (XLHb), was proposed to be beneficial in the treatment of hypotension concomitant to hemorrhagic shock [20]. However, vasoconstriction reduces blood flow, lowering functional capillary density, and therefore affecting tissue perfusion and oxygenation [21,22]. Nitric oxide (NO) scavenging by Hb due to intrinsic high affinity of NO to Hb is the mechanism presumed to cause vasoconstriction and hypertension [23,24].

We analyzed the relationship between the constriction of resistance vessel and hypertension after administration of acellular Hb and the extent to which the effect is dependent on the size of acellular Hb molecules modified by polymerization, polymer conjugation, and cellular liposome encapsulation [6,25]. Conscious Syrian golden hamsters with dorsal skinfold preparation were used. After the top load infusion of Hb products (7 ml/kg) into arterial catheter into jugular vein, mean arterial pressure, and heart rate were monitored through jugular arterial catheter, and microvascular responses were monitored by an intravital microscopy. The Hb products included intra-molecularly crosslinked Hb (XLHb), PEG-conjugated pyridoxalated Hb (PEG-PLP-Hb), hydroxyethylstarch-conjugated XLHb (HES-XLHb), glutaraldehyde-polymerized XLHb (Poly-XLHb) and HbV. Their molecular diameters were 7, 22, 68 and 224 nm, respectively. The top load infusion of 7 ml/kg of XLHb (5 g/dl) caused the immediate increase of MAP, which was 34 ± 13 mmHg higher 3 hrs after infusion. There was a simultaneous decrease in diameter of A_0 vessels ($79 \pm 8\%$ of basal value), which caused blood flow to decrease throughout the microvascular network. The diameter of smaller arterioles did not change significantly. Infusion of O_2 carriers of greater molecular size resulted in lesser vasoconstriction and hypertension with HbV showing the smallest changes. Infusion of human serum albumin was used as control and produced no microvascular or systemic effects. Constriction of resistance arteries was found to be correlated to the level of hypertension, and the responses proportional to the molecular dimensions of Hb-based O_2 carriers. Since the results correlate with molecular size it is likely that the effects are related to the diffusion properties of the different hemoglobin molecules.

The liver is a major organ that detoxifies excess amount of heme by the action of heme oxygenase (HO). HO decomposes protoheme IX to generate biliberdin-IXa and CO. Under normal conditions, liver contains at least two OH isozymes for physiologic degradation of the heme: HO-1 and HO-2. One of the important roles of the HO reaction is to generate CO that serves as an endogenous regulator that is necessary for maintaining microvascular blood flow [26]. Since Hb strongly binds with CO (about 200 times stronger than O_2), it is necessary to confirm the effects of HbV in hepatic microcirculation in comparison with stroma free Hb solution. Suematsu et al. studied the perfusion of a rat liver with an acellular Hb solution and HbV, and found out that the Hb solution increased vascular resistance by 30% [27]. The smaller acellular Hb molecules (7 nm) extravasate across the fenestrated endothelium with a pore size of about 100 nm, and reach to the space of Disse. Heme is excessively metabolized by hemeoxygenase-2 to produce CO and bilirubin. Even though CO acts as a vasorelaxation factor in the liver, the excess amount of Hb in the space of Disse rapidly binds CO, resulting in the vasoconstriction and the increase in vascular resistance. On the other hand, Hb-vesicle (250 nm) is large enough to maintain in the sinusoid, and the vascular resistance is maintained.

These results indicate the importance of the size of the oxygen carriers, and the size of HbV is appropriate for the maintenance of microvascular blood flow.

4. Oxygen releasing behavior of HbV and oxygen therapeutics

We measured the O_2 release from HbV perfused through an O_2 permeable fluorinated ethylenepropylene copolymer tube (inner diameter, 28 μm), that was exposed to a deoxygenated environment [28] (Fig. 2). The addition of HbV to RBC did not influence on the O_2-releasing rate. On the other hand, the addition of 50-vol% acellular Hb solution to RBC significantly enhanced the rate of deoxygenation. This outstanding difference in the rate of the O_2 release between the HbV suspension and the acellular Hb solution should mainly be due to the difference in the particle size (250 vs. 8 nm) that affects their

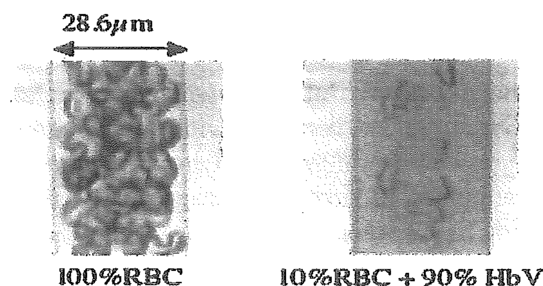

Fig. 2. Flow patterns of RBCs mixed with HbVs suspended in human serum albumin in a narrow tube (diameter, 28 μm) [28]. RBCs tended to flow in the centerline, while the HbV particles were homogeneously dispersed in a suspension medium. The individual particles could not be seen at this magnification. However, semitransparent elements were seen in the suspension medium, indicating the presence of HbV. This experimental model, developed by Maeda et al., was used to analyze the O_2 releasing behavior of HbV and RBC. [Hb] = 10 g/dl; centerline flow velocity, 1 mm/s.

diffusion for the facilitated O_2 transport. It has been suggested that the faster O_2 unloading from the HBOCs is advantageous for tissue oxygenation [29]. However, this concept is controversial regarding the recent findings since an excess O_2 supply would cause autoregulatory vasoconstriction and microcirculatory disorders [22,30]. We confirmed that HbV does not induce vasoconstriction and hypertension, due to not only the reduced inactivation of NO as an endothelium-derived vasorelaxation factor, but also possibly the moderate O_2 releasing rate similar to RBC as confirmed in this study.

One characteristic of HbV is that the O_2 affinity (P_{50}) of Hb can be easily regulated by the amount of coencapsulated allosteric effector, pyridoxal 5′-phosphate [19]. It has been clarified by Erni et al. that oxygenation of an ischemic skin flap, where one branch of feeding arteriole was ligated, was improved by infusion of HbV with a high O_2 affinity (low P_{50}) [31,32]. To clarify the underlying mechanism of ischemic tissue oxygenation, we prepared two HbVs with different P_{50}s (8 and 29 mmHg, termed HbV_8 and HbV_{29}, respectively), and observed their O_2 releasing behavior from an occluded arteriole in a hamster skinfold window model [33]. Conscious hamsters received HbV_8 or HbV_{29} at the dose rate of 7 ml/kg bw. In the microscopic view, an arteriole (diameter: 53.0 ± 6.6 μm) was occluded transcutaneously by a glass pipette on a manipulator and the reduction of the intra arteriolar O_2 tension (pO_2) 100 μm down from the occlusion was measured by the phosphorescence quenching of pre-infused Pd-porphyrin. The baseline arteriolar pO_2 (50–52 mmHg) decreased to about 5 mmHg for all the groups. Occlusion after HbV_8 infusion showed slightly slower rate of pO_2 reduction in comparison with that after HbV_{29} infusion. The arteriolar O_2 content was calculated at each reducing pO_2 in combination with the O_2 equilibrium curves of HbVs, and it was clarified that HbV_8 showed significantly slower rate of O_2 release in comparison with HbV_{29} and was a primary source of O_2 (maximum fraction, 0.55) overwhelming red blood cells when the pO_2 was reduced (e.g., <10 mmHg) in spite of a small dosage of HbV.

Accordingly, the result of improved oxygenation of the ischemic skin flap, observed by Erni et al., could be explained by low P_{50} HbVs retaining O_2 in the upstream vessels and delivering it to the ischemic tissue via collateral arterioles, even when these may have significantly slower blood flow. Moreover, an advantage of small HBOCs including HbV is that they are homogeneously dispersed in the plasma phase and therefore can deliver O_2 more homogeneously to the periphery than RBCs because microvascular Hct is heterogeneous particularly in pathological states. In such conditions HbV with a higher O_2 affinity (lower P_{50}) should show a slower O_2 unloading which would be effective for oxygenating ischemic tissues. This result supports the possible utilization of Hb-based O_2 carriers with lower P_{50} for oxygenation of ischemic tissues.

In summary, observation of microcirculation is important for the development of artificial oxygen carriers because it is the site where oxygen is unloaded to the target tissues. From the international collaborative evaluation studies of HbV, we have clarified the rheological property, advantages of the cellular structure, and the performances of HbV not only as a transfusion alternative but also for oxygen therapeutics.

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Carbon Monoxide From Heme Oxygenase-2 Is a Tonic Regulator Against NO-Dependent Vasodilatation in the Adult Rat Cerebral Microcirculation

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Abstract—Although the brain generates NO and carbon monoxide (CO), it is unknown how these gases and their enzyme systems interact with each other to regulate cerebrovascular function. We examined whether CO produced by heme oxygenase (HO) modulates generation and action of constitutive NO in the rat pial microcirculation. Immunohistochemical analyses indicated that HO-2 occurred in neurons and arachnoid trabecular cells, where NO synthase 1 (NOS1) was detectable, and also in vascular endothelium–expressing NOS3, suggesting colocalization of CO- and NO-generating sites. Intravital microscopy using a closed cranial window preparation revealed that blockade of the HO activity by zinc protoporphyrin IX significantly dilates arterioles. This vasodilatation depended on local NOS activities and was abolished by CO supplementation, suggesting that the gas derived from HO-2 tonically regulates NO-mediated vasodilatory response. Bioimaging of NO by laser-confocal microfluorography of diaminofluorescein indicated detectable amounts of NO at the microvascular wall, the subdural mesothelial cells, and arachnoid trabecular cells, which express NOS in and around the pial microvasculature. On CO inhibition by the HO inhibitor, regional NO formation was augmented in these cells. Such a pattern of accelerated NO formation depended on NOS activities and was again attenuated by the local CO supplementation. Studies using cultured porcine aortic endothelial cells suggested that the inhibitory action of CO on NOS could result from the photo-reversible gas binding to the prosthetic heme. Collectively, CO derived from HO-2 appears to serve as a tonic vasoregulator antagonizing NO-mediated vasodilatation in the rat cerebral microcirculation. (*Circ Res.* **2005;97:e104-e114.**)

Key Words: carbon monoxide ■ heme oxygenase ■ diaminofluorescein ■ nitric oxide ■ NO synthase ■ vascular tone

Carbon monoxide (CO) has attracted much interest since implicated as a gaseous messenger for neural and vascular systems.[1–3] This diatomic gas displays considerable similarities as well as differences with NO, an established gaseous mediator.[4] In view of gas-generating mechanisms, CO is synthesized by heme oxygenase (HO), the enzyme executing oxidative cleavage of protoheme IX into biliverdin IXα. This reaction is similar to that of NO synthase (NOS) in that both require NADPH as an electron donor and molecular oxygen as cosubstrates. HO resembles NOS because both involve constitutive and inducible isozymes; the latter is induced by a similar spectrum of stressors such as hypoxia and cytokines.[5,6] Another common property is a vital role played by the heme in catalytic reactions of these enzymes. Whereas in the NOS, the heme is incorporated within the protein interior, HO is unique because the substrate (ie, protoheme IX) also serves as a catalytic center constituting oxygen activation. In this respect, its enzyme–substrate complex but not HO alone forms a transient heme protein. Considering such properties of gas-generating reactions, one gas can interfere with generation of another through multiple mechanisms. First, the two reactions could compete for using NADPH and molecular oxygen. Second, enhanced HO reaction could reduce the amount of heme in cells,[7] causing a reduction in the enzymatic activity of NOS. Finally, in vitro, CO and NO can bind to the heme of NOS and of the HO–substrate complex, respectively; therefore, it could inhibit the reactions.[8–10]

Such a property that the two gases bind to the ferrous heme with high affinity could be targeted not only to gas-generating enzymes but also to other receptor proteins possessing the heme. Consequently, it provides both gases a point to interact with each other to effect functions of receptor proteins in vivo.[11,12] One example of this cross-interaction of the two gases on one receptor is soluble guanylate cyclase (sGC), to which heme either NO or CO can bind to increase its activity,

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