Human serum albumin incorporating synthetic heme: Red blood cell substitute without hypertension by nitric oxide scavenging

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Abstract: The administration of extracellular, hemoglobin-based oxygen carriers often elicits an acute increase in blood pressure by vasoconstriction. This side effect is now recognized to be due to the depletion of nitric oxide (endothelial-derived relaxing factor) by the extravasuated hemoglobins. We have recently found that the administration of a recombinant human serum albumin (rHSA)-based oxygen carrier involving synthetic tetraphenyporphinatoiron(II) derivative (FeP) (rHSA-FeP) does not induce such hypertensive action, because of its low permeability through the vascular endothelium. The heart rate responses after the rHSA-FeP injection were also negligibly small. Visualization of the intestinal microcirculatory changes clearly revealed the widths of the venule and arteriole to be fairly constant. The entirely synthetic rHSA-FeP becomes a promising material as a new type of red blood cell substitute. © 2002 Wiley Periodicals Inc. J Biomed Mater Res 64A: 257–261, 2003

Key words: human serum albumin; synthetic heme; red blood cell substitute; hypertension; nitric oxide

INTRODUCTION

Several types of extracellular hemoglobin(Hb)-based oxygen(O_2) carriers are currently used in clinical trials as red blood cell substitutes.[1,2] Especially, diaspirin cross-linked Hb, which is stabilized by a covalent linkage between αα subunits, has been studied in various animal models by many investigators. The administration of such Hb solutions, however, has often elicited an acute increase in blood pressure.[3,4] This side effect is now recognized to be caused by the fact that small Hb molecules extravasuate through the vascular endothelium and deplete the nitric oxide (NO), namely, the endothelial-derived relaxing factor, thus inducing the vasoconstriction.[5–9] It has been also shown that these pressor effects elicited by Hb are accompanied by a decrease in the heart rate (HR).[9,10] Although the precise mechanism of this bradycardia is still a source of controversy, the unfavorable hemodynamic alterations may limit the use of the Hb solutions as blood replacement compositions.

We have recently found that recombinant human serum albumin (rHSA) incorporating a synthetic heme with a covalently linked proximal base, 2-[8-(2-methyl-1-imidazolyl)octanoyloxymethyl]-5,10,15,20-tetrakis[(α,α,α,α-o-pivalamido)phenyl]porphinatoiron(II) (FeP, Scheme 1) [rHSA-FeP], can reversibly bind and release O_2 under physiologic conditions (in aqueous media, pH 7.3, 37°C) in the same manner as Hb and myoglobin.[11] Because serum albumin is the most abundant plasma protein, used extensively in many clinical situations, the advantage of the albumin-based O_2 carrier is significant. It can also be used regardless of blood type, and the preliminary exchange transfusion test with hemorrhagic rats has demonstrated that rHSA-FeP satisfies the initial clinical requirements for an O_2-carrying resuscitative

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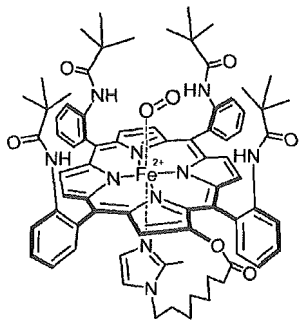

Scheme 1. Structure of synthetic heme with a covalently linked proximal base; 2-[8-(2-methyl-1-imidazolyl)octanoyloxymethyl]-5,10,15,20-tetrakis[(α,α,α,α-*o*-pivalamido)-phenyl]porphinatoiron(II).

fluid.[12] Furthermore, rHSA is now manufactured on a large scale by expression in the methylotropic yeast *Pichia pastoris* as a host cell, and is expected to reach the market within a few years.[13]

Our only source of concern was that the small rHSA-FeP molecules (8 × 3 nm) injected into the blood vessels would be eliminated from the circulation and contribute to the significant consumption of NO in the interstitial space between the endothelium and vascular smooth muscle. In fact, rHSA-FeP strongly binds NO; its NO-binding affinity ($P_{1/2}^{NO} = 1.8 \times 10^{-8}$ Torr) is 9-fold higher compared to that of Hb and sufficiently high to react in amounts as little as 1 μ*M* NO in the wall of the vasculator.[14] In order to clarify the hemodynamic behavior after the administration of this entirely synthetic O_2-carrying hemoprotein, we tested a top-load dose of the rHSA-FeP solution in anesthetized rats.

MATERIALS AND METHODS

Preparation and preservation

An rHSA (25 wt %) was obtained from the WelFide Corporation (Osaka, Japan), and FeP was synthesized as previously reported.[15] The rHSA-FeP solution [FeP/rHSA: 4 (mol/mol); rHSA: 5 g/dL in phosphate-buffered saline (PBS), pH 7.3] and the extracellular hemoglobin (Hb) solution (5 g/dL) were prepared according to our previously reported procedure.[16] The obtained rHSA-FeP solution was sealed in three separate glass vials under nitrogen and stored at 5°C (in a refrigerator), 25°C (in an air-conditioned room), and 40°C (in an incubator), respectively. The solution properties and O_2 binding abilities were then quantitatively evaluated every month for a year.[11]

In vivo measurements of mean arterial pressure and heart rate

Experiments were conducted with 10 male Wistar rats (222 ± 8.0 g) purchased from Nippon Charles River (Yokohama, Japan). All animal studies were reviewed and approved by the Animal Care and Use Committee of Keio University. The rats were anesthetized with sodium pentobarbital (50 mg/kg) and the right jugular vein was cannulated (PE-20 tubing, outer 0.8 mmϕ, inner 0.5 mmϕ) for sample infusion. The left common carotid artery was also cannulated (PE-20 tubing) and connected to a pressure transducer (Polygraph System, Nihon Koden, Tokyo, Japan) for continuous recording of the mean arterial pressure (MAP). The heart rate (HR) was derived from the blood pressure signals. Additional doses of anesthesia were given to some animals during the preparatory procedures to stabilize the anesthetic level. When the preparation of the animals was complete and the hemodynamic parameters (MAP, HR) became stable, each rat was given an intravenously administered single top-load dose of rHSA-FeP (300 mg/kg, 1 mL/min, $n = 5$). The rHSA-FeP solutions were kept at 4°C and warmed to 25°C just before injection into the animals. During the administration, no remarkable acute reaction was observed in the appearance of the rats. The MAP and HR were continuously monitored before (−2 min) and at various times (i.e., 0, 5, 10, 15, 20, 25, 30, 40, 50, and 60 min) after the infusions. The extracellular Hb solution (5 g/dL) was also applied to similarly treated rats for the reference group (300 mg/kg, 1 mL/min, $n = 5$). The PBS solution was found to be inactive on the MAP and HR in our animal model. Data were analyzed using an analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test between the groups. The rats were sacrified at the end of the experiments by pentobarbital overdose. The care and handling of the animals were in accordance with the NIH guidelines.

Intestinal microcirculation

Two male Wistar rats (300 g) were anesthetized with sodium pentobarbital (50 mg/kg). A catheter (PE-20 tubing) was introduced into the left femoral vein for sample administration. After a ventral incision was made in the abdomen, the intestine was extended and placed on a plastic plate. The surface of the intestinal wall was always kept warm and moist by continuous superfusion with saline at 37°C and flow of N_2 gas (O_2 partial pressure was approximately 10 Torr). The rHSA-FeP solution (400 mg/kg, 1 mL/min) was then administered via intravenous infusion. A Nikon Optishot-2 microscope using 20× water-immersion objectives directly monitored the microcirculatory changes in the venules and arteriole. The microscopic images were continuously recorded by a change coupled device (CCD) camera and transferred to a TV-VCR.

RESULTS AND DISCUSSION

The obtained rHSA-FeP exhibited long-term storage stability; the red solution with a physiologic albumin-concentration (5 g/dL) had a shelf life of over a year, in the range of 5–40°C. The changes in the solution

properties (viscosity, pH, specific gravity, albumin and heme concentrations, isoelectric point) and the O_2-binding ability were all less than 5%.

Contrary to our expectations, only a negligibly small change in the MAP was observed after the administration of the rHSA-FeP solution (5 g/dL, 300 mg/kg) [Fig. 1(a)]. If anything, the difference from the baseline (ΔMAP) slowly decreased to −6.8 ± 3.4 mmHg within 20 min and remained constant during the monitoring period. The response was completely the same as that observed following infusion with an equivalent volume of rHSA (5 g/dL) in this experimental setup (results not shown). In contrast, the administration of extracellular Hb solution elicited an acute increase in blood pressure (ΔMAP: 16 ± 1.9 mmHg) that exhibited a graduated decrease throughout the 60-min period of observation. Why does

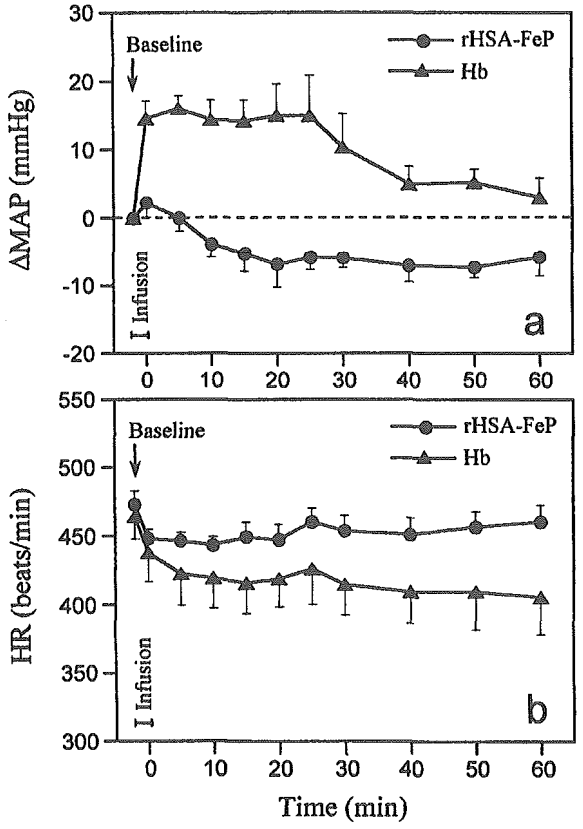

Figure 1. Changes in hemodynamic parameters after the administration of rHSA-FeP solution in the anesthetized rats (significant difference from baseline: $p < 0.05$). (a) Changes of mean arterial pressure before and after the infusion of rHSA-FeP ($n = 5$). All data are shown as changes from the basal values (ΔMAP) just before the infusion and expressed as mean ± standard error. Basal value is 90.1 ± 3.0 mmHg. (b) Time course of heart rate (HR) responses before and after the infusion of rHSA-FeP ($n = 5$). All data are expressed as mean ± standard error.

rHSA-FeP not induce the hypertension? The molecular size is actually the same as Hb, and its association rate constant for NO binding (k_{on}: $1.5 \times 10^7\ M^{-1}s^{-1}$) is high for the rapid scavenging of NO.[14] The answer probably lies in the negatively charged molecular surface of the albumin vehicle. One of the unique characteristics of serum albumin is its low permeability through the muscle capillary pore, which is less than 1/100 that for Hb because of the electrostatic repulsion between the albumin surface and the glomerular basement membrane around the endothelial cells.[17] The isoelectric point of rHSA-FeP (pI = 4.8) is exactly the same as that of rHSA itself, because the FeP molecule without any ionic residue interacts nonspecifically with the hydrophobic cavity of rHSA.[11] In the blood vessels, rHSA-FeP presumably circulates for a longer time compared to Hb without extravasation. On the contrary, the injected Hb molecules immediately dissociated into αβ dimers and appeared in the urine within 15 min after the infusion. These results coincide with the half-life of Hb ($\tau_{1/2}$: 90 min) reported elsewhere.[5] Although the exact circulation half-life of rHSA-FeP is unknown in this model (it should be over 4 h, based on our previous exchange transfusion with anesthetized rats), the rHSA-FeP could not be detected in either the urine or any body fluids during the measurements.

To avoid the depletion of NO by the exravasuated Hb, several researchers have designed large-sized Hb derivatives [e.g., polymerized Hb and polyethylene glycol conjugated Hb (PEGHb)].[2,18] By virtue of the increased molecular size, their extravasations are reasonably attenuated, but the high viscosity of the PEGHb solution may influence to no small extent the plasma volume, shear stress on the capillary wall, and plasma Hb concentration, all of which can affect blood pressure.

Other proposed mechanisms for the hypertensive effect caused by Hb-based O_2 carriers include (1) the constrictive response of arterioles against high O_2 tensions,[19–22] and (2) the decrease in NO production by diminished shear stress on the vascular wall.[23] In the first hypothesis, the O_2-binding affinity of rHSA-FeP ($P_{1/2}^{O_2}$: 32 mmHg at 37°C, similar to the human erythrocyte) is relatively low, leading to excessive O_2 release in the arterioles, thereby inducing vasoconstriction. As described above, the reverse was the case. Doherty et al. also denied this theory as a result of their systematic experiments using recombinant Hb (rHb) with various $P_{1/2}^{O_2}$.[24] The small differences in the O_2-equilibrium curves of the rHb did not affect the magnitude of the pressor response. They concluded that the mechanism for the increase in blood pressure was only depletion of NO by ferrous Hb, not excessive O_2 delivery to the arterioles. In the second hypothesis, the prompt flow as a result of the administration of the rHSA-FeP solution with a low viscosity compared to

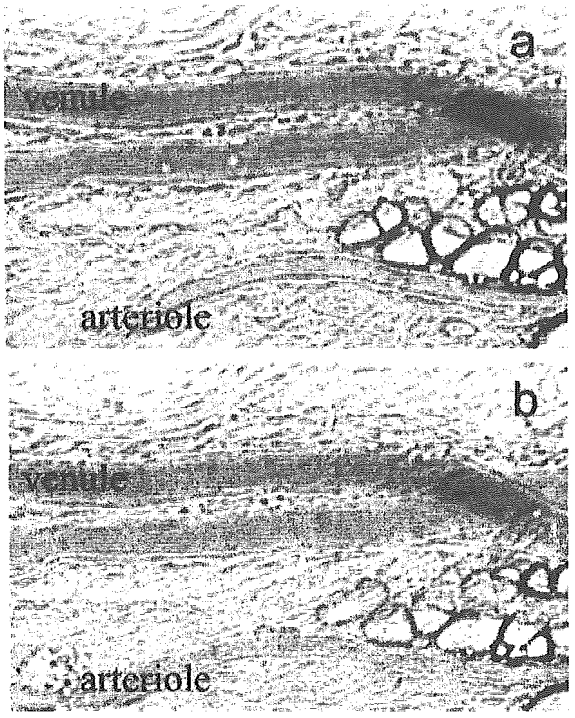

Figure 2. Video prints showing intestinal microcirculation of anesthetized rats, with two venules and an arteriole flow in the images. (a) Observation before the administration of rHSA-FeP. The width of the upper venule is 30–40 μm, and the lower arteriole's width is 10–20 μm. In the venules, white leukocytes are rolling on the vascular wall. (b) Observation at 90 s after the administration of rHSA-FeP solution. The widths of the venules and the arteriole are fairly constant compared to those in image **a**, indicating that vasoconstriction did not occur. The behavior of the leukocyte rolling did not change at all.

that of the whole blood may result in a decrease in the shear stress on wall of the vasculature, which inhibits vasorelaxation. Although the total blood volume increase was less than 11% in our top-load experiments, the negligibly small changes in the blood pressure obviously contradicted these two major hypotheses, which may be responsible only for the hypertensive action by the Hb solutions.

Auto-oxidized ferric Hb or ferric rHSA-FeP bind NO as well, and an additional NO molecule can reduce the nitrosyl complex.[25] However, the NO-binding affinity of ferric rHSA-FeP is too low to form a large amount of the ferric NO-complex *in vivo*.[14] The HR responses after the rHSA-FeP administration were not worth serious consideration (maximum ΔHR; −25 beats/min), and recovered to the baseline within 25 min [Fig. 1(b)]. The Hb group HR, on the other hand, fell significantly (464 ± 16 → 416 ± 22 beats/min), and was further attenuated, finally reaching 406 ± 27 beats/min. Regardless, we observed that the administration of rHSA-FeP did not induce bradycardia, which is quite different from the cases using the modified Hb.

Visualization of the intestinal microcirculation supported the above experimental results on the blood pressure (Fig. 2). The diameters of the venules and arteriole were not transformed at 90 s after the infusion of the rHSA-FeP solution (5 g/dL, 400 mg/kg), where the amplitude of the hypertensive action reached maximum in the Hb group [Fig. 1(a)]. This experimental setting showed an acute vasoconstriction in the intestinal wall with the administration of an equivalent amount of extracellular Hb. Throughout the 90-min microscopic observation, we saw no changes in either the diameters of the vasculators and blood flow rates (3–4 mm/s) or rolling of the leukocytes. Moreover, the platelet numbers were always constant at approximately 750,000. If NO scavenging occurs, platelet aggregation should be induced.

Although more research is required for a full understanding of the biologic and pharmacologic properties of this rHSA-FeP, the present results obviously indicate that the albumin-based synthetic O_2-carrying hemoprotein excludes unfavorable hemodynamic responses observed in the modified-Hb solutions. Thus, rHSA-FeP can not only be utilized as a safe and effective red blood cell substitute, but also as an O_2-carrying medicine suitable for adoption in several clinical applications, such as myocardial infarction, tracheal blockade, preservation of organs for transplantation, and so on.

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Compatibility *in vitro* of albumin–heme (O_2 carrier) with blood cell components

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Abstract: Recombinant human serum albumin including 2-[8-{N-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-*o*-pivaloylamino)phenylporphinatoiron(II) (albumin–heme; rHSA-FeP) is a synthetic hemoprotein that has sufficient capability to reversibly bind and release O_2 under physiological conditions (pH 7.3, 37°C) similar to hemoglobin and myoglobin. In order to use this albumin-based O_2 carrier as a new class of red blood cell substitutes, its compatibility with blood cell components carefully was investigated *in vitro*. After the addition of the rHSA-FeP solution into whole blood at 10, 20, and 44 vol %, the FeP concentration in the plasma phase remained constant for 6 h at 37°C in each group, and no significant time dependence was observed in the numbers of red blood cells, white blood cells, or platelets. The microscopic observations clearly showed that the shapes of the red blood cells had not been deformed during the measurement period. With respect to the blood coagulation parameters (prothrombin time and activated partial thromboplastin time), the coexistence of rHSA-FeP had only a negligibly small influence. Also the blood compatibility under dynamic flow conditions was evaluated using a microchannel array flow analyzer. All these results suggest that the albumin–heme has no effect on the morphology of blood cell components *in vitro*. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 66A: 292–297, 2003

Key words: human serum albumin; albumin–heme; O_2 carrier; blood cell components; red blood cell substitute

INTRODUCTION

Serum albumin is the most abundant plasma protein in the mammal's circulatory system, and it contributes crucial physiologic equilibria by: (1) maintaining the colloid osmotic pressure; (2) transporting many helpful materials around the body; and (3) maintaining blood pH, etc.[1,2] Because of these attributes, serum albumin has been used extensively as a plasma expander in numerous clinical situations. If serum albumin can transport O_2 like hemoglobin (Hb), namely "O_2-carrying albumin," it could be of significant medical importance as a new generation of red blood cell substitute.

We recently have found that synthetic heme, 2-[8-{N-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis (α, α, α, α-*o*-pivaloylamino)phenylporphinato-iron(II) (FeP), efficiently is incorporated into recombinant human serum albumin (rHSA), providing artificial hemoprotein (albumin–heme; rHSA-FeP) that reversibly can bind and release O_2 under physiologic conditions (pH 7.3, 37°C) in the same manner as Hb and myoglobin.[3]

Its physicochemical properties and O_2-transporting ability satisfy the initial clinical requirements as an artificial red blood cell:[3–8] (1) A maximum of eight FeP molecules were incorporated into certain domains of rHSA by hydrophobic interaction.[3–5] The incorporation of FeP does not induce any changes in the highly ordered structure and surface charge distribution of the host albumin. (2) The red-colored rHSA-FeP solution has a long shelf-life of over 1 year, at temperatures of 5°–40°C.[6] (3) The O_2-binding affinity ($P_{1/2}$: 30 Torr at 37°C) is almost the same as that of human blood and independent of the FeP's numbers therein.[7] Although the Hill coefficient is 1.0, the O_2-transporting efficiency (22%) between the lungs (Po_2: 110 Torr) and the muscle tissues (Po_2: 40 Torr) is similar to that of human blood (23%).[3–5,7] (4) In contrast to the fact that the intravenous administration of Hb-based O_2 carriers often elicits an acute increase in blood pressure by vasoconstriction, which is due to the depletion of nitric oxide (endothelial-derived relaxing factor) by

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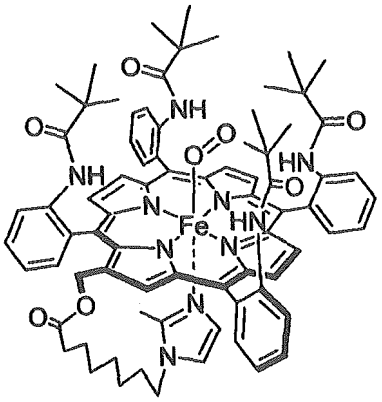
the extravasuated hemoglobin,[9–11] the infusion of rHSA-FeP does not induce such hypertensive action because of its low permeability through the vascular endothelium.[6] (5) Physiological responses to exchange transfusion with albumin–heme into anesthetized rats after hemodilution and hemorrhage (Hct: about 10%) demonstrated its *in vivo* O_2-transporting efficacy as a resuscitative fluid.[12]



To use this albumin-based O_2 carrier for clinical applications, it is necessary to evaluate its biocompatibility with blood under physiological conditions. We report herein for the first time the detailed inspections of the compatibility of rHSA-FeP with blood cell components *in vitro*. The number of the blood cells and the concentration of FeP always were constant for 6 h after mixing with rHSA-FeP, and the blood coagulation was not accelerated. The dynamic measurement using a microchannel flow analyzer suggests that rHSA-FeP has a good blood compatibility even in the narrow vessel model at a high flow rate.

MATERIALS AND METHODS

Preparation of rHSA-FeP

FeP was synthesized as described eleswhere.[13] The recombinant human serum albumin (rHSA, 25 wt %) was a gift from the NIPRO Corp. (Osaka, Japan). The rHSA-FeP solution was prepared according to our previously reported procedure, with some modifications.[3,6] FeP (0.21 g) first was dissolved in 250 mL of ethanol (FeP = 0.6 m*M*), and 10 mL of 25 wt % rHSA were diluted into 1000 mL of phosphate buffer (p.b.) solution (1 m*M*, pH 8.1, rHSA = 37.6 μ*M*).

The ethanolic FeP was bubbled through CO gas for 30 min, and 250 μL of p.b. solution (pH 8.1) of L-ascorbic acid (0.6*M*) was injected to reduce the central ferric ion to the ferrous state. After 5 min, the FeP solution was transferred to a dropping funnel under the CO atmosphere and slowly was added drop-wise (50 mL/h), with continuous stirring, to the rHSA solution in a flat-bottomed flask. After this addition, the rHSA-FeP solution was washed with 10 L of p.b. solution (1 m*M*, pH 7.3) using a Millipore Pellicon permeate membrane (No. P2B010A01) at a speed of 3000 mL/h and concentrated to approximately 280 mL.

The obtained solution was filtered using an ADVANTEC DISMIC 25CS045AS filter (0.45 μm) and further concentrated to approximately 50 mL by an ADVANTEC UHP-76K with a Q0500 076E membrane (cut-off Mw of 50 kDa). Finally, the red-colored solution was again filtered using a DISMIC 25CS045AS, and the ion concentration was adjusted to 0.9 wt % by the addition of NaCl, affording the rHSA-FeP solution [pH 7.4, NaCl 0.9 wt %, rHSA 5 wt %, FeP 3.0 m*M*, FeP/rHSA = 4/1 (mol/mol)].

FeP concentration in the plasma phase

Fresh whole blood was obtained from Wistar rats (250–400 g, male, Saitama Experimental Animals Supply Co., Saitama, Japan) and separately stored in heparinized glass tubes. The rHSA-FeP solution then was added to the blood at 10, 20, and 44 vol % concentrations (total volume of 5 mL each), and the individual sample was incubated immediately at 37°C in a Yamato BT-23 water bath incubator. After 0, 0.5, 1, 2, 3, and 6 h, 100 μL of the sample were drawn out from each tube and centrifuged (10,000 rpm, 5 min). The supernatant was separated and diluted with 1.0 mL of pure water. The amount of FeP was determined by the assay of the iron ion concentration using inductively coupled plasma spectrometry (ICP) with a Seiko Instruments SPS 7000A Spectrometer.

As background data, the blood mixed with rHSA (5 wt %) under the same conditions also was measured. The care and handling of the animals were in accordance with NIH guidelines.

Blood cell numbers

At the time points of 0.5, 1, 2, 3, and 6 h after the mixing, 70 μL of the sample were drawn from each group (rHSA-FeP = 10, 20, and 44 vol %) and diluted with 200 μL of saline solution. The blood cell numbers were counted using a Sysmex KX-21 blood cell counting device. As control groups, the blood suspensions with rHSA (5 wt %) under the same conditions (rHSA = 10, 20, and 44 vol %) also were tested.

Morphology of the red blood cells

One drop of the incubated sample of the blood with rHSA-FeP was observed microscopically at the time points of 0, 0.5, 1, 2, 3, and 6 h after the mixing, using an Olympus IX50 microscope with an IX70 CCD camera.

Prothombin time (PT) and activated partial thromboplastin time (APTT)

Whole blood from the Wistar rats was mixed with rHSA-FeP or rHSA (5 wt %) at concentrations of 10, 20, and 44 vol % in a TERUMO Venojector II plastic tube (VP-C052) con-

taining 0.2 mL of 3.8% sodium citrate. These samples (each 1.8 mL) were centrifuged (3500 rpm, 10 min) and the supernatant (0.5 mL) was immediately frozen at −80°C. The PT and APTT values were determined by BML, Inc. (Tokyo).

Dynamic stability under flowing condition

Venous blood from human volunteers was obtained and stored in 5% heparinized glass tubes. The rHSA-FeP solution was mixed with the blood at 10, 20, and 40 vol % concentrations, and a 200-μL sample was pumped through the microchannel array under a constant suction of 20 cmH_2O in a Hitachi-Haramachi Electronics microchannel array flow analyzer (MC-FAN).[14] A Bloody 6–7 array (groove width, 7 μm; length, 30 μm; depth, 4.5 μm; number of grooves, 8736) was used for all the measurements. The MC-FAN provides a good model of the narrow capillaries (7 μm in width) with appropriate shear stress.

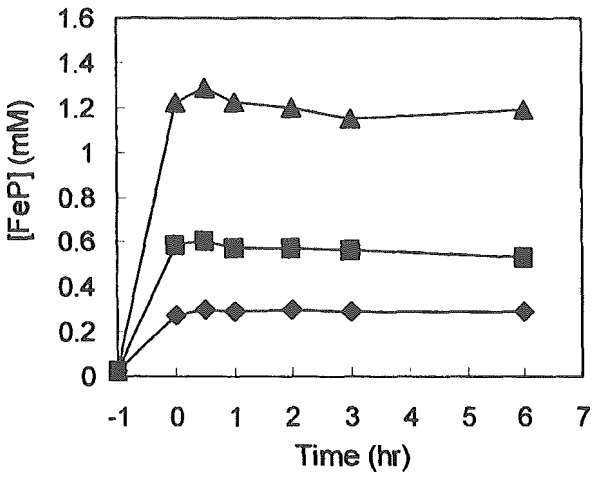


Figure 1. The FeP concentration in the plasma phase of the blood suspension mixed with the rHSA-FeP solution (rHSA = 5 wt %, FeP/rHSA = 4 mol/mol) by 10–44 vol % (◆: rHSA-FeP, 10 vol %, ■: rHSA-FeP, 20 vol %, ▲: rHSA-FeP, 44 vol %).

RESULTS AND DISCUSSION

An association between rHSA and FeP was formed by the hydrophobic interaction, but there was not a covalent bond. If this interaction is not strong enough, the incorporated FePs may dissociate from the hydrophobic cavities of the albumin host and transfer to the other components in the blood stream, such as the membrane of the red blood cells (RBC) or white blood cells (WBC). Such an event would cause some unfavorable influence not only on the function of the blood cells and hemodynamics, but also on the entire physiological reactions in the body.

First, the transference of FeP from the albumin–heme to the blood cells was evaluated by measuring the FeP concentration in the plasma. After centrifugation at 10,000 rpm for 5 min, the blood suspension with rHSA-FeP was separated into two parts, the plasma phase and the blood cell phase. Since rHSA-FeP has a lower density, it should remain only in the supernatant. If the FeP molecule transferred to the RBC or WBC, it would be found in the precipitant, which should lead to a decrease in the FeP concentration in the plasma layer.

Just after the mixing, the FeP concentrations in the supernatant were 0.28, 0.6, and 1.2 mM in the 10, 20, and 44 vol % rHSA-FeP groups, respectively (Fig. 1). These were in good agreement with the prediction values calculated from the dilution ratios, and they remained constant for 6 h at 37°C.

These results imply that no transference of FeP to the blood cell components took place under the physiological conditions. Unfortunately, in this experimental setup, hemolysis gradually occurred after 6 h even in the control group mixed with the same amount of rHSA. This makes it difficult to have an accurate assay of the FeP concentration at the time points over 6 h.

The changes in the number of blood-cell components [RBC, WBC, and platelets (PLT)] were measured for 6 h after having been mixed with the rHSA-FeP solution (Fig. 2). The number of RBCs and WBCs just after the injection of rHSA-FeP was reasonably reduced in proportion to each dilution ratio: ≈90, 80, and 56% of the baseline value in the 10, 20, and 44 vol % rHSA-FeP groups. Furthermore, they remained constant for 6 h at 37°C. The time courses were completely the same as those in the control groups with the rHSA solution.

These observations clearly indicate that the coexistence of a 10–44 vol % of rHSA-FeP did not cause any influence on the number of WBCs and RBCs during the 6-h observation period. This was further proved by the microscopic observation of the morphology change of the RBC. Absolutely no deformation and aggregation of RBC were seen for the blood suspension with the 10, 20, and 44 vol % of rHSA-FeP after 6 h at 37°C (Fig. 3).

In contrast, only the platelet (PLT) numbers showed a further decrease within 30 min after the mixing, a decrease that was approximately 10–22% of the baseline value and independent of the rHSA-FeP ratio. These reduced values did remain stable for another 5.5 h. Interestingly, the same trends were found in the rHSA control groups. Although the reason is not clear at the moment, it could imply that the presence of the FeP molecules is not responsible for these changes. Consequently, we can conclude that there is almost no physicochemical interaction between rHSA-FeP and the blood cell

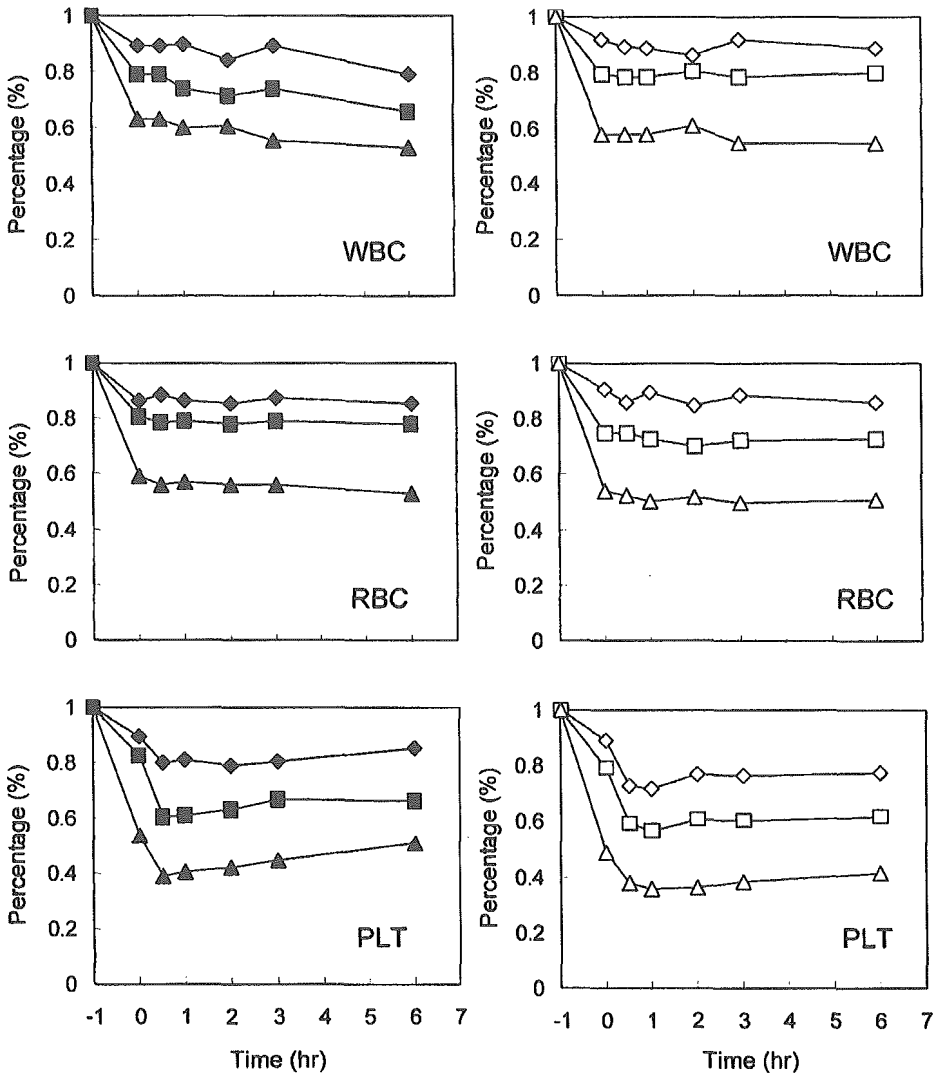


Figure 2. The blood cell numbers of the blood suspension mixed with the rHSA-FeP solution (rHSA = 5 wt %, FeP/rHSA = 4 mol/mol) or the rHSA solution (5 wt %). ♦: rHSA-FeP, 10 vol %; ■: rHSA-FeP, 20 vol %; ▲: rHSA-FeP, 44 vol %.; ◇: rHSA 10 vol %; □: rHSA 20 vol %; and △: rHSA 44 vol %.

components and that the albumin–heme molecule probably acts like normal serum albumin in the blood circulatory system.

The effect of rHSA-FeP on blood coagulation also was investigated by determination of the prothrombin time (PT) and activated partial thromboplastin time (APTT). It is well known that thromboplastin and prothrombin are typical blood coagulation factors, existing in the blood serum, platelet, and WBC, etc. For instance, when a certain amount of Ca^{2+} or phospholipids is added to the blood, the activated thromboplastin induces the inversion of prothrombin to thrombin, which leads to the coagulation of the blood.[15] That is, the decrease in the amount of the prothrombin results in an extension of the blood aggregation time.

The addition of rHSA-FeP by 10–44 vol % to the whole blood had only a negligible influence on PT and APTT (Fig. 4), almost the same changes seen in the control groups with rHSA. The PT and APTT remained at ≈20 and ≈30 s, respectively, independent of the mixing ratio of rHSA-FeP. This indicates that the presence of rHSA-FeP does not obstruct the normal coagulation function of the blood.

Based on these findings, we can summarize that rHSA-FeP has good compatibility with the blood cell components *in vitro*. However, the above experiments all were performed in the static state, which is quite different from the dynamic conditions in the body. We then employed a microchannel device (MC-FAN) to simulate the blood circulatory system and evaluated the dynamic stability of rHSA-FeP with blood. The microchannel arrays created by photolithography and etching are appropriate models of the capillaries and

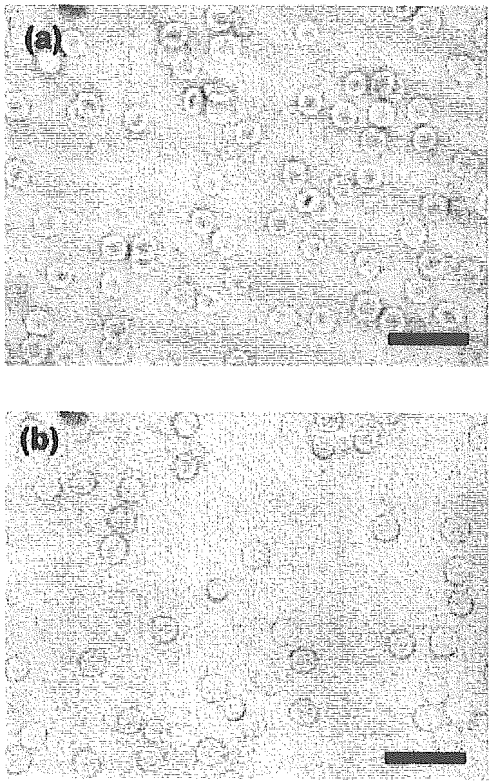


Figure 3. Optical microscopic observations of (a) whole blood of Wistar rat, and (b) the blood suspension mixed with the 44 vol % of rHSA-FeP solution (rHSA = 5 wt %, FeP/rHSA = 4 mol/mol) after incubation at 37°C for 6 h (bar: 20 μm). The shape of the RBC with a diameter of ≈8 μm did not change. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

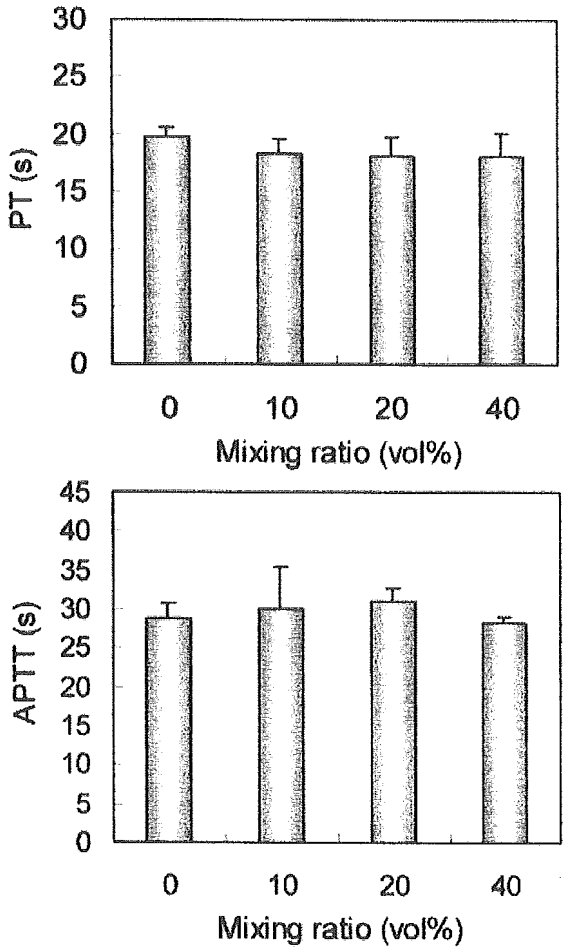


Figure 4. PT and APTT values of the blood suspension mixed with the rHSA-FeP solution (rHSA = 5 wt %, FeP/rHSA = 4 mol/mol) by 10–44 vol %.

enables us to quantitatively and visually study the blood rheology in the microcirculation.

Although the MC-FAN limits blood contact with the artificial capillary walls to a very short period, it seems to be sufficient to see the deformation of the blood cells and the change of the blood rheology under the appropriate shear stress.[14] One hundred μL of whole blood passed through these arrays within ≈40 s (Fig. 5). An increase in the rHSA-FeP concentration ratio significantly reduced the passage time of the same amount of sample. The calculated passage time for each sample (10, 20, and 40 vol % rHSA-FeP) was estimated from the whole blood's value, the rHSA-FeP's value (16.8 s), and its mixing ratio. In accordance with the elevation in the rHSA-FeP ratio, the passage time gradually shortened because the viscosity of the rHSA-FeP solution (1.1 cP) is much lower than that of whole blood (4.4–5.0 cP).[3] While the sample was passing through the microchannel arrays, no decline in the deformation ability of RBC or aggregation of WBC and PLT was observed at all. The flowing mobility of all the sample solutions always seemed to be smooth and constant. These results imply that rHSA-FeP has no influence on the morphology of the blood cells even under the dynamic conditions.

CONCLUSIONS

Although a more functional assay is necessary to firmly establish the biocompatibility of the artificial O_2-carrying albumin, rHSA-FeP, with whole blood, it has a good compatibility with blood cells *in vitro*. The associated FeP is stable and will not transfer to the other blood components. The addition of 44 vol % rHSA-FeP into the blood does not have any influence on the RBC, WBC, and PLT numbers for 6 h at 37°C. The shape of the RBC is always constant, and the coagulation function of the blood is maintained in the presence of rHSA-FeP. These results provide a good foundation for using rHSA-FeP in many clinical evaluations and also promise good biocompatibility *in vivo*. The detailed animal experiments using this novel

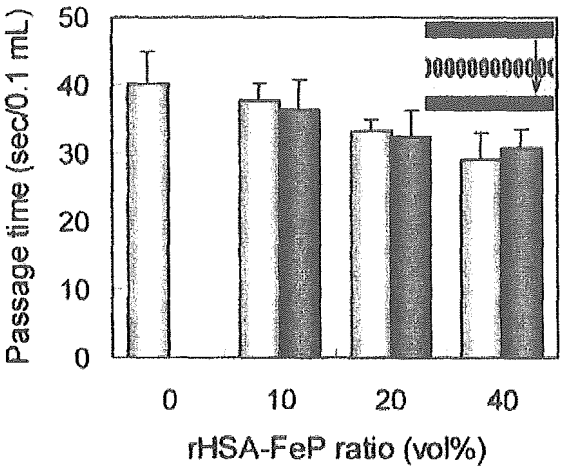


Figure 5. Passage time of 0.1 mL of blood suspension mixed with the rHSA-FeP solution (rHSA = 5 wt %, FeP/rHSA = 4 mol/mol) through the microchannel array vessel model under 20 cmH_2O at 25°C [left (bright area): experimental data; right (dark area): calculated value]. The inset shows a picture of the microchannel array tip (Bloody 6–7,) which has 8736 grooves of 7 μm in width, 30 μm in length, and 4.5 μm in depth. The increase in the concentration ratio of rHSA-FeP, which has a relatively low viscosity, leads to gradual decreases in the passage times of the sample.

O_2-carrying hemoprotein for O_2-therapeutics now are being undertaken.

We thank Professor Yuji Kikuchi, National Food Research Institute (Tsukuba) and Associate Professor Shinji Takeoka, Waseda University, for the MC-FAN measurements and their valuable discussions. Dr. Hiromi Sakai, Waseda University, is gratefully acknowledged for his skillful animal experiments.

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Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats*

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Objective: **Hemoglobin-vesicle (HbV) has been developed to provide oxygen-carrying ability to plasma expanders. Its ability to restore the systemic condition after hemorrhagic shock was evaluated in anesthetized Wistar rats for 6 hrs after resuscitation. The HbV was suspended in 5 g/dL recombinant human serum albumin (HbV/rHSA) at an Hb concentration of 8.6 g/dL.**

Design: **Prospective, randomized, controlled trial.**

Setting: **Department of Surgery, School of Medicine, Keio University.**

Subjects: **Forty male Wistar rats.**

Interventions: **The rats were anesthetized with 1.5% sevoflurane inhalation throughout the experiment. Polyethylene catheters were introduced through the right jugular vein into the right atrium for infusion and into the right common carotid artery for blood withdrawal and mean arterial pressure monitoring.**

Measurements and Main Results: **Shock was induced by 50% blood withdrawal. The rats showed hypotension (mean arterial pressure = 32 ± 10 mm Hg) and significant metabolic acidosis and hyperventilation. After 15 mins, they received HbV/rHSA, shed autologous blood (SAB), washed homologous red blood cells (wRBC) suspended in rHSA (wRBC/rHSA, [Hb] = 8.6 g/dL), or rHSA alone. The HbV/rHSA group restored mean arterial pressure to 93 ± 8 mm Hg at 1 hr, similar to the SAB group (92 ± 9 mm Hg), which was significantly higher compared with the rHSA (74 ± 9 mm Hg) and wRBC/rHSA (79 ± 8 mm Hg) groups. There was no remarkable difference in the blood gas variables between the resuscitated groups; however, two of eight rats in the rHSA group died before 6 hrs. After 6 hrs, the rHSA group showed significant ischemic changes in the right cerebral hemisphere relating to the ligation of the right carotid artery followed by cannulation, whereas the HbV/rHSA, SAB, and wRBC/rHSA groups showed less changes.**

Conclusions: **HbV suspended in recombinant human serum albumin provides restoration from hemorrhagic shock that is comparable with that using shed autologous blood. (Crit Care Med 2004; 32:539–545)**

Key Words: blood substitutes; artificial red cells; liposome; resuscitation; transfusion

A phospholipid vesicle encapsulating concentrated human hemoglobin (Hb) (Hb-vesicle, HbV) can serve as an oxygen carrier whose oxygen-carrying capacity can be formulated to be comparable to that of blood (1–4). HbV are void of blood-type antigens and infectious viruses and are stable and suitable for long-term storage (5). The cellular structure of HbV (particle diameter, ca. 280 nm) has characteristics similar to those of natural red blood cells (RBCs), because both have lipid bilayer membranes that prevent direct contact of Hb with the components of blood and the endothelial lining. Furthermore, Hb encapsulation in the vesicle suppresses hypertension induced by vasoconstriction, a mechanism presumably due to the effect of free Hb that scavenges the endogenous vasorelaxation factors nitric oxide and carbon monoxide (6, 7) consequent to their high affinity with Hb. Once in the circulation, HbV particles are captured by the phagocytes in the reticuloendothelial system (mainly the liver and spleen), and they are metabolized completely within 14 days, with no deposition of iron or lipid (8).

Oxygen-carrying fluids for blood replacement using molecular or encapsulated Hbs have been proposed for volume restoration in hemorrhagic shock (9, 10). We tested the efficacy of HbV suspended in plasma-derived human serum albumin (HSA) in extreme normovolemic hemodilution and found that they are comparable with RBCs (11, 12). In this report, we tested the HbV as a resuscitative fluid for hemorrhagic shock in anesthetized rats. HbV was suspended in recombinant HSA (rHSA), and the efficacy of the resulting HbV/rHSA was compared with that of shed autologous blood and of washed RBCs suspended in rHSA at the same Hb concentration. It has been extensively confirmed that the characteristics of the rHSA are identical with those of conventional plasma-derived HSA

*See also p. 612.

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(13, 14) and that rHSA will soon be approved as a promising plasma expander free from any pathogen from humans.

MATERIALS AND METHODS

Preparation of HbV and Washed RBCs Suspended in rHSA. HbV was prepared under sterile conditions as previously reported (7, 11). Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and the Japanese Red Cross Society (Tokyo, Japan). The encapsulated purified Hb (38 g/dL) contained 14.7 mM of pyridoxal 5'-phosphate (Sigma Chemical, St. Louis, MO) as an allosteric effector at a molar ratio of pyridoxal 5'-phosphate/Hb = 2.5. The lipid bilayer was composed of 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, Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) (NOF, Tokyo, Japan, 0.3 mol% of the total lipid) (15). HbVs were suspended in a physiologic salt solution at [Hb] = 10 g/dL, sterilized with filters (Dismic, Toyo Roshi, Tokyo, Japan, pore size, 0.45 μm), and deoxygenated with N_2 bubbling for storage (5). The content of lipopolysaccharide was <0.1 EU/mL.

Before use, the HbV suspension ([Hb] = 10 g/dL, 8.6 mL) was mixed with a solution of rHSA (25%, 1.4 mL, Nipro, Osaka, Japan) to regulate the rHSA concentration in the suspending medium of the vesicles to 5 g/dL. Under this condition, the colloid osmotic pressure of the suspension is about 20 mm Hg (Wescor 4420 Colloid Osmometer, Wescor, Logan, UT) (11). As a result, the Hb concentration of the suspension was 8.6 g/dL. The viscosities of the suspensions were measured with a capillary rheometer (Oscillatory Capillary Rheometer, OCR-D, Anton Paar GmbH, Graz, Austria). Physicochemical variables of the resulting HbV suspension in comparison with those of the other resuscitative fluids are listed in Table 1.

To prepare washed RBC suspended in rHSA (wRBC/rHSA), blood samples from donor Wistar rats were withdrawn into heparinized syringes and centrifuged to obtain an RBC concentrate. This was washed twice to remove plasma components and buffy coat by resuspension in 5% rHSA and centrifugation (3000 × *g*, 10 mins). The Hb concentration, measured with a cyanometHb method, of the resulting wRBC/rHSA was adjusted to 8.6 g/dL, equivalent to that of HbV/rHSA. The Hb concentration of the shed autologous blood was 13.4 ± 2 g/dL.

Animal Model and Preparation. The experimental protocol was fully approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine. It also complied with the Guide for the Care and Use of Laboratory Animals (16).

Experiments were carried out with 40 male Wistar rats (280 ± 27 g body weight; Saitama Experimental Animals Supply, Kawagoe, Japan). All animals were housed in cages and provided with food and water *ad libitum* in a temperature-controlled room with a 12-hr dark/light cycle. The rats were anesthetized with 1.5%-sevoflurane-mixed air inhalation (Maruishi Pharm., Osaka) with a vaporizer (TK-4 Biomachinery, Kimura Med., Tokyo) throughout the experiment (F_{IO_2} = 21%). Polyethylene catheters (SP-31 tubing, outer diameter 0.8 mm, inner diameter 0.5 mm, Natsume, Tokyo) filled with saline containing 40 IU/mL heparin were introduced through the right jugular vein into the right atrium for infusion and into the right common carotid artery for blood withdrawal. The catheter in the common carotid artery was connected to a Polygraph system (Nippon Koden, Polygraph LEG-1000). The body temperature of the rats was maintained between 37 and 38°C by an isothermal pad (Braintree Scientific, Braintree, MA) during the experiments.

Resuscitation From Hemorrhagic Shock. Hemorrhagic shock was induced by withdrawing 50% of the blood (28 mL/kg, 1 mL/min) from the carotid artery. Systemic blood volume was estimated to be 56 mL/kg body weight (3). Blood was withdrawn into a heparinized syringe and stored for 15 mins at room temperature for the resuscitation with shed autologous blood (SAB). Rats were resuscitated by the infusion of a volume of HbV/rHSA (n = 8), wRBC/rHSA (n = 8), rHSA alone (n = 8), or initially shed autologous blood (n = 8) in 5 min. The volume of the infused resuscitative fluid was identical to the shed volume (i.e., 50% of the blood volume at baseline). To monitor the severity of the shock, eight hemorrhaged rats were not resuscitated with any fluid (nonresuscitated group).

Measurements of Systemic Responses. Systemic variables and blood gases were evaluated before hemorrhage (baseline), after 50% hemorrhage, just after resuscitation, and 1.0, 3.0, and 6.0 hrs after resuscitation. Blood samples were collected in 70 IU/mL heparinized microtubes (125 μL, Clinitubes, Radiometer, Copenhagen) for blood gas analyses and in glass capillaries (Terumo, Tokyo) for hematocrit measurements. A pH/blood gas analyzer (ABL 555, Radiometer, Copenhagen) was used for analysis of Pao_2, $Paco_2$, pH, base excess (BE), and lactate. A recording system (Polygraph System 1000, Nippon Koden, Tokyo) was used for continuous monitoring of mean arterial pressure (MAP) and heart rate (HR). Body temperature was monitored with a thermometer inserted into the anus.

Histopathological Examination and Serum Clinical Laboratory Tests. Six hours after resuscitation, about 5 mL of arterial blood was rapidly withdrawn into heparinized syringes, and the animals were laparotomized and killed by acute bleeding from the abdominal aorta. The liver, spleen, kidney, and then the lung, heart, and brain were resected for a histopathological study. The percentage of the area of ischemic changes (a pyknotic change of nuclei and an edematous change) in the cerebral hemisphere was measured with computer software (IPLab, Fairfax, VA). The blood samples were centrifuged at 3000 × *g* for 5 mins to obtain plasma. The HbV-containing plasma required further ultracentrifugation (50,000 × *g*, 20 mins) to obtain clear plasma avoiding the interference effect of the HbV particles (17). The samples of serum were stored at −80°C before the clinical laboratory tests (BML, Kawagoe). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) serum activities were measured. The organs were fixed in a 10% formalin neutral buffer solution (Wako Chemical, Tokyo) immediately after the resection, and the paraffin sections were stained with hematoxylin/eosin.

Data Analysis. Data are given as the mean ± SD for the indicated number of animals. Data were analyzed using analysis of variance followed by Fisher's protected least significant difference test between the groups. The Student's *t*-test was used for the comparisons with baseline values within each group. The level of confidence was placed at 95% for all the experiments.

Table 1. Physicochemical properties of four resuscitative fluids infused into hemorrhagic-shocked rats; hemoglobin-vesicles suspended in recombinant human serum albumin (HbV/rHSA) compared with shed autologous blood(SAB), washed red blood cells suspended in recombinant human serum albumin(wRBC/rHSA), and recombinant human serum albumin (rHSA)

Variables	HbV/rHSA	SAB	wRBC/rHSA	rHSA
Hemoglobin concentration, g/dL	8.6	13.4 ± 2	8.6	0
Particle diameter, nm	281 ± 11	ca. 7000	ca. 7000	—
P_{50}, torr	32	39[a]	39[a]	—
Colloid osmotic pressure, mm Hg	20	22	20	20
Viscosity, cP at 230/sec	2.8	5.2	2.1	1.1

[a]From Reference 12.

RESULTS

Survival Rate. All the rats in the HbV/rHSA, wRBC/rHSA, and SAB groups survived for 6 hrs after resuscitation until the kill. In the rHSA group, two of the eight rats died between 1 and 6 hrs (Fig. 1). Accordingly, hemodynamic and blood-

gas variables (Figs. 2 and 3) of the rHSA group were divided into the survivor group and the nonsurvivor groups. Therefore, the numbers of rats (n) for the rHSA (survivor) and rHSA (nonsurvivor) groups were six and two, respectively. All the rats in the nonresuscitated group died within 3 hrs.

Systemic Responses to the Hemorrhagic Shock and Resuscitation. MAP of the Wistar rats before hemorrhage was 99 ± 8 mm Hg on the average and declined to 32 ± 6 mm Hg after hemorrhage (Fig. 2*a*). Immediately after resuscitation, the MAP of the SAB group recovered to 110 ± 7 mm Hg, above the baseline value. The value was slightly reduced to 92 ± 9 mm Hg at 1 hr, and the level was maintained for 6 hrs. The MAP of the HbV/rHSA recovered upon retransfusion to 98 ± 8 mm Hg, the baseline level, which was significantly lower than that of the SAB group ($p = .027$). After 1 hr, there was no significant difference between the HbV/rHSA and SAB groups. The HbV/rHSA group showed significantly higher MAP than the rHSA (survivor) ($p = 0.0005$) and wRBC/rHSA ($p = .0032$) groups, whose MAPs at 1 hr were 74 ± 9 and 79 ± 8 mm Hg and remained at this higher level for 6 hrs. The MAP of the nonresuscitated group did not recover and remained at the lowest values. The average HR before hemorrhage was 405 ± 38 beats/min, and there was no significant change after hemorrhage. At 0 hr, the HbV/rHSA ($p = .0215$), SAB ($p = .0085$), and nonresuscitated ($p = .0076$) groups showed slightly lower HR than the basal values; however, there was no noticeable change after that (Fig. 2*b*).

The hematocrit before hemorrhage was 43 ± 2% and was reduced to 36 ± 2% after bleeding due to autotransfusion (Fig. 2*c*). After resuscitation, the hematocrit in the SAB group increased to 42 ± 4%. The hematocrit values in the rHSA (survivor), rHSA (nonsurvivor), and HbV/rHSA groups were significantly reduced to 19 ± 1, 18 ± 1, and 20 ± 2%, respectively ($p < .0001$ vs. baseline), due to the dilution of the blood with the different solutions. The HbV particles remained dispersed in the plasma phase in the glass capillaries for hematocrit measurements. The hematocrit of the wRBC/rHSA group (35 ± 3%) was significantly lower than that of the SAB group ($p < .0001$) corresponding to the lower Hb concentration in the fluid of the wRBC/rHSA groups (8.6 g/dL) than in that of the SAB groups (13.4 ± 2.0 g/dL). The hematocrit of the nonresuscitated group did not change after autotransfusion. The total Hb concentrations in blood after resuscitation with rHSA, HbV/rHSA, wRBC/rHSA, and SAB were estimated to be 6.3, 11, 11, and 13 g/dL, respectively.

Hemorrhagic shock induced metabolic acidosis shown by a decrease in pH from 7.48 ± 0.04 to 7.40 ± 0.09 on the average, a decrease in the BE from 4.5 ± 1.4 to −6.9 ± 3.4 mM, and an increase in lactate from 1.4 ± 0.5 to 6.2 ± 1.4 mM (Fig. 3). As a result, significant compensatory hyperventilation was observed as an increase in Pao_2 of 81 ± 8 torr to 103 ± 6 torr and a decrease in $Paco_2$ of 38 ± 5 torr to 26 ± 5 torr. All the resuscitated groups tended to recover immediately from the hyperventilation after infusion. The pH, BE, and lactate values did not show immediate recoveries after resuscitation but tended to recover at 1 hr. However, they did not return to the baseline level even after 6 hrs ($p < .05$ vs. baseline). There was no significant difference between the HbV/rHSA and SAB group. The nonresuscitated group remained with significant hyperventilation, acidosis, and reduction of BE at 0 hr ($p < .01$ vs. baseline). After that, the Pao_2 decrease and the $Paco_2$ increase were significant in the rats, leading to death. All the variables of the nonresuscitated group were significantly different from those of the HbV/rHSA group at 3 hrs. There was no clear difference between the rHSA (survi-

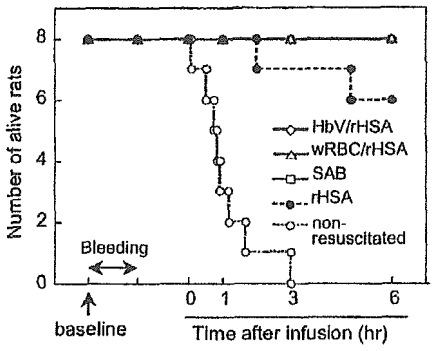

Figure 1. Survival rate of Wistar rats after resuscitation from hemorrhagic shock with infusion of hemoglobin-vesicles suspended in recombinant human serum albumin (*HbV/rHSA*), shed autologous blood (*SAB*), washed red blood cells suspended in recombinant human serum albumin (*wRBC/rHSA*), and recombinant human serum albumin (*rHSA*) alone. The nonresuscitated group did not receive a resuscitative fluid after the hemorrhage.

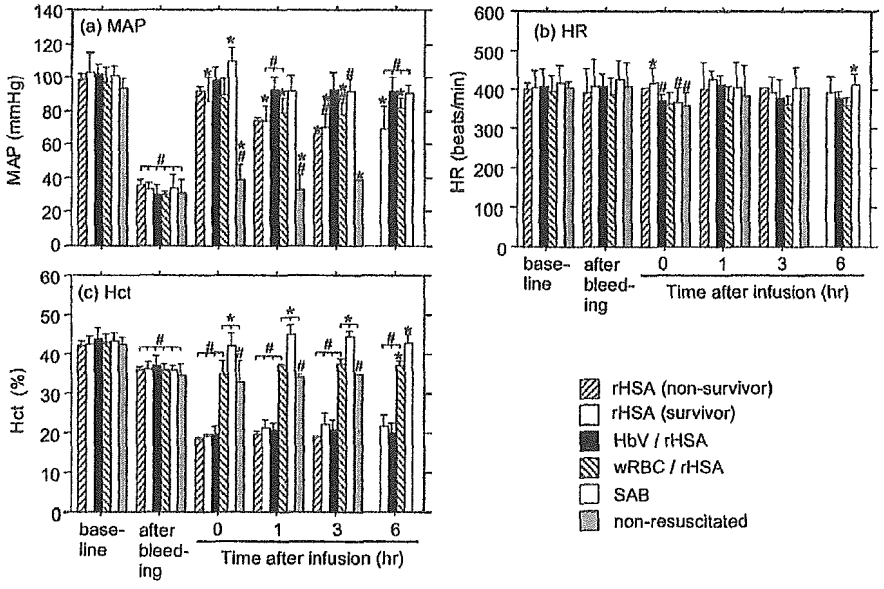

Figure 2. Changes in heart rate (*HR*), mean arterial pressure (*MAP*), and hematocrit (*Hct*) during hemorrhagic shock and resuscitation with infusion of hemoglobin-vesicles suspended in recombinant human serum albumin (*HbV/rHSA*), shed autologous blood (*SAB*), washed red blood cells suspended in recombinant human serum albumin (*wRBC/rHSA*), and recombinant human serum albumin (*rHSA*) alone. The nonresuscitated group did not receive a resuscitative fluid after the hemorrhage and died within 3 hrs (Fig. 1). The number of surviving rats was three at 1 hr. In the rHSA group, two of the eight rats died between 1 and 6 hrs. Accordingly, the rHSA group was divided into the rHSA (survivor) group and the rHSA (nonsurvivor) group until they died. Therefore, the numbers of rats (n) for the rHSA (survivor) and rHSA (nonsurvivor) groups were 6 and 2, respectively. #Significantly different from baseline ($p < .05$); *significantly different vs. the HbV/rHSA group ($p < .05$).

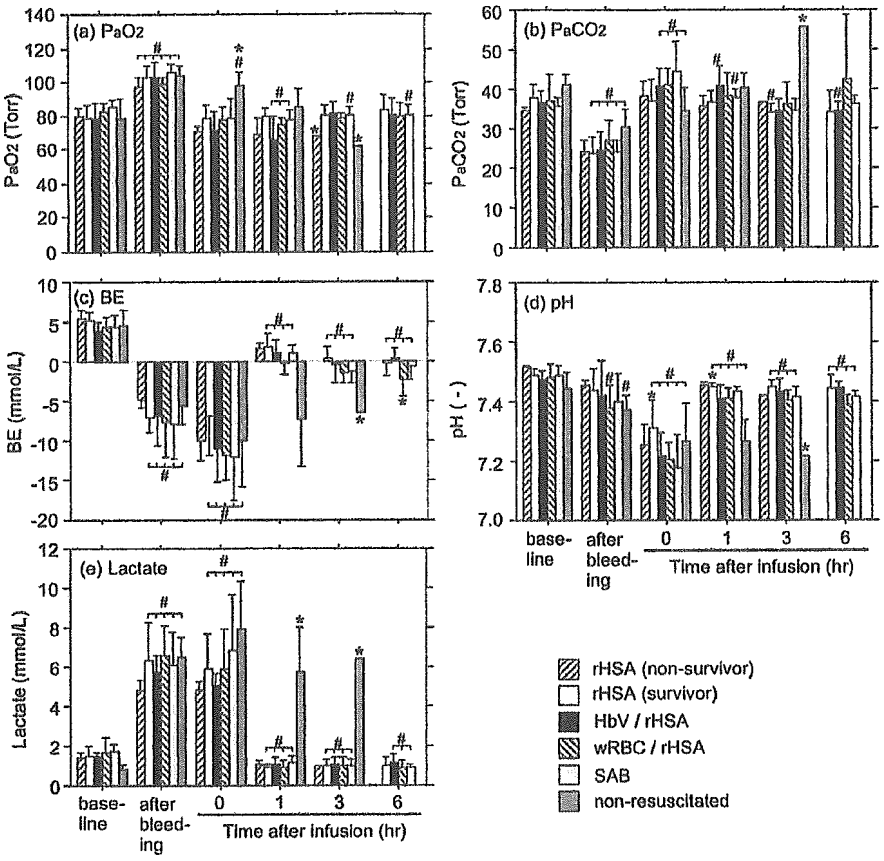

Figure 3. Changes in blood gas variables during hemorrhagic shock and resuscitation with infusion of hemoglobin-vesicles suspended in recombinant human serum albumin (*HbV/rHSA*), shed autologous blood (*SAB*), washed red blood cells suspended in recombinant human serum albumin (*wRBC/rHSA*), and recombinant human serum albumin (*rHSA*) alone. The nonresuscitated group did not receive a resuscitative fluid after the hemorrhage and died within 3 hrs (Fig. 1). The number of surviving rats was three at 1 hr. In the rHSA group, two of the eight rats died between 1 and 6 hrs. Accordingly, the rHSA group was divided into the rHSA (survivor) group and the rHSA (nonsurvivor) group until they died. Therefore, the numbers of rats (n) for the rHSA (survivor) and rHSA (nonsurvivor) groups were 6 and 2, respectively. #Significantly different from baseline ($p < .05$); *significantly different vs. the HbV/rHSA group ($p < .05$). *BE*, base excess.

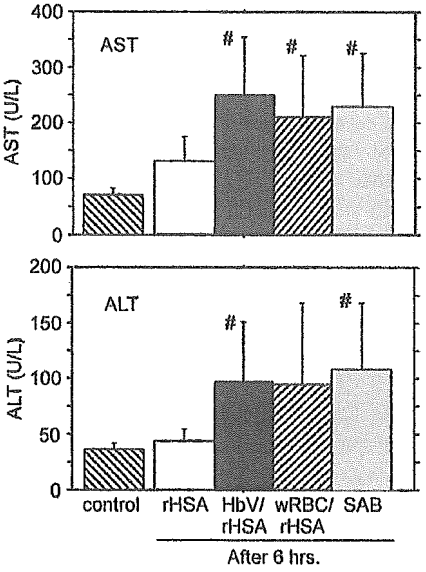

Figure 4. Aspartate aminotransferase (*AST*) and alanine aminotransferase (*ALT*) concentrations 6 hrs after resuscitation with infusion of hemoglobin-vesicles suspended in recombinant human serum albumin (*HbV/rHSA*), shed autologous blood (*SAB*), washed red blood cells suspended in recombinant human serum albumin (*wRBC/rHSA*), and recombinant human serum albumin (*rHSA*) alone. #Significantly different from the control group ($p < .05$).

vor) and rHSA (nonsurvivor) groups in MAP and HR in Figure 2. However, the rHSA (nonsurvivor) group tended to show a slightly lower Pao_2 than the rHSA (survivor) group at 0 and 1 hr and significantly at 3 hrs ($p = .0374$) in Figure 3.

Clinical Laboratory Tests of Blood Serum. Normal Wistar rats showed AST and ALT of 70 ± 13 and 37 ± 5 units/L, respectively (Fig. 4). The HbV/rHSA, wRBC/rHSA, and SAB groups showed significant or nonsignificant increases in AST ($p = .003$, .016, and .005, respectively) and ALT values ($p = .031$, .110, and .025, respectively) compared with the baseline values. On the other hand, the rHSA (survivor) group showed the smallest changes.

Histopathological Examination 6 Hrs After Resuscitation With HbV/rHSA. The hematoxylin/eosin staining of the rat organs demonstrated no significant morphologic abnormalities in the lung, kidney, and liver (data not shown). The red pulp zone of the spleen showed the accumulation of HbV particles as pink-colored dots (8). The myocardium showed focal minimal ischemic changes without apparent necrosis, probably due to the hemorrhagic shock. This histologic finding also was observed in other experimental groups including the rHSA (survivor) group. The cerebral hemisphere on the right side of the rHSA group showed significant ischemic changes, a pyknotic change of the nuclei, and an edematous change (34 ± 3% of the total section area), relating to the ligation of the right carotid artery. However, the other groups that were resuscitated with oxygen-carrying fluids showed minimal changes ($p < .001$ vs. rHSA; HbV/rHSA, 13 ± 5%; SAB, 11 ± 6%; wRBC/rHSA, 11 ± 3%). The nonresuscitated rats that died spontaneously did not show such ischemic changes.

DISCUSSION

One particle of HbV (diameter, ca. 250 nm) contains about 30,000 Hb molecules. HbV acts as a particle in the blood and not as a solute; therefore, the colloid osmotic pressure of the HbV suspension is nearly zero. It requires an addition of a plasma expander for a large substitution of blood such as normovolemic hemodilution to maintain blood volume (18). The candidates of plasma expanders are HSA, hydroxyethyl starch, dextran, or gelatin depending on the clinical setting, cost, countries, and clinicians (19). In this report we tested for the first time the addition of rHSA. The absence of any infectious disease from humans is the greatest advantage of rHSA, which will be soon approved for clinical use in Japan. Moreover, there should be no immunologic and hematologic abnormalities that are often seen with the use of dextran and hydroxyethyl starch (19). The virus inactivation and removal from a human-derived Hb solution can be aggressively

performed in our preparation process of HbV (20, 21). However, to completely avoid unknown infectious diseases, the combination of recombinant Hb-vesicles and recombinant HSA would be the most ideal "artificial red blood cells" in the future.

In our hemorrhagic shock model, all the rats in the nonresuscitated group, which did not receive any fluid, died within 3 hrs, indicating the severity of the shock state. The infusion of the resuscitative fluids resulted in the improvement of all the variables and survival, indicating the importance of the recovery in blood volume. Especially, our principal findings are that the infusion of HbVs suspended in rHSA restores the MAP and blood gas variables including BE and lactate after hemorrhagic shock and that all the rats survived 6 hrs after resuscitation despite the fact that in the rHSA group two rats among eight died within 6 hrs. This clearly shows that the ability of HbV/rHSA as an effective oxygen-carrying resuscitative fluid is comparable with shed autologous blood. After the resuscitation, there were minor differences in blood gas variables between the groups in Figure 3. It would seem that all the animals were hypervolemic in the initial phase of resuscitation, because they experienced autotransfusion during the shock period, although it lasted only 15 mins. This could be one of the reasons there were no significant changes in BE and pH between the groups. Moreover, after the infusion of rHSA, the Hb concentration should not be significantly lower than the transfusion trigger. However, the rHSA group dissociated into the survivor and nonsurvivor groups. There was no remarkable difference between the two, and it was difficult to determine the cause of death. If anything, the rHSA (nonsurvivor) group tended to show the lower Pao_2 values compared with the rHSA (survivor) group ($p = .037$ at 3 hrs), indicating that the respiratory function was not adequate to sustain metabolism under the condition of sevoflurane anesthesia and spontaneous breathing after resuscitation with nonoxygen-carrying fluid. In this case, the combination of the significant hypotension that was seen in all the rats in the rHSA group and the respiratory problem may be one of the causes of incidental death (22). Immediately after resuscitation, the HbV/rHSA group showed a recovery of MAP, Pao_2, and $Paco_2$ that was similar to that of the SAB group. However, the BE, pH, and lactate levels did not show immediate recovery due to the "washing out" of accumulated metabolites including nonvolatile lactate in the peripheral tissues (23). These values recovered 1 hr after resuscitation.

In our previous report (10), we tested HbV suspended in plasma-derived HSA for resuscitation from hemorrhagic shock of conscious small hamsters (ca. 60–70 g body weight) with much lower remaining hematocrit values, maintaining the MAP at 40 mm Hg for 1 hr. Even though the species was different and the observation period after resuscitation was only 1 hr, it seemed that the conscious hamsters showed sufficient compensation of hyperventilation even after resuscitation with HSA alone. In the present study, we could demonstrate the effectiveness of HbV/rHSA in anesthetized rats (280 ± 27 g body weight) as long as 6 hrs after resuscitation, where respiratory function was depressed and compensatory function was not sufficient. This indicates the effectiveness of HbV suspended in a plasma expander for resuscitation from hemorrhagic shock.

It has been reported that resuscitation from hemorrhagic shock with acellular Hb modifications such as polymerized or intramolecularly cross-linked Hb causes the elevation of MAP beyond the baseline values (9, 24, 25), whereas a refined polymerized human Hb that does not contain molecular Hb (<1%) shows no hypertension (26). The hypertension may be presumably due to the high affinity for nitric oxide of molecular Hbs and their smaller size that enables nitric oxide trapping in the proximity of the endothelium (7, 27). However, MAP did not exceed the baseline values after resuscitation with HbV. This is one advantage of cellular HbV in comparison with acellular molecular Hb modifications that may cause vasoconstriction and therefore hypoperfusion of peripheral tissues.

Interestingly, the HbV/rHSA group showed a significantly higher MAP than the wRBC/rHSA group and one that was comparable with that of the SAB group except immediately after resuscitation. It has been extensively confirmed that HbV is not vasoactive and does not induce hypertension (6, 7, 10). Because the total Hb concentrations are identical between the two resuscitative fluids (8.6 g/dL), one of the possible explanations could be related to the more effective oxygen transport by HbV than RBC to the myocardium where the oxygen consumption is significantly large and the oxygen tension gradient is steep. This is speculated from the facts that HbV distributes closer to the endothelial cell layer in the arteriolar blood flow whereas RBCs flow near the axial line (28). Another explanation should be related to the viscosity difference. The viscosity of HbV/rHSA (2.8 cP) is slightly higher than that of wRBC/rHSA (2.1 cP), and this may contribute to the higher vascular resistance and the resulting higher MAP. The slightly higher MAP for the SAB group immediately after infusion may be due to hypervolemia, the trace hemolysis that induces nitric oxide trapping and vasoconstriction, higher viscosity, or clotting during the preservation despite the heparinization.

Histopathological examination of the spleen showed accumulation of HbV in the red pulp zone as previously reported in the study of bolus infusion of HbV in normal rats (8). It was confirmed that HbVs, as foreign particles, were finally captured by the reticuloendothelial system mainly in the spleen and liver, and they were smoothly metabolized within 2 wks. Because the circulation half-life of HbV is about 35 hrs, the spleen had already started to show accumulation of HbV 6 hrs after resuscitation. The lung and kidney did not show any abnormalities such as embolism in the capillaries derived from the aggregation of vesicles (29). In our case, poly(ethylene glycol) modification of the surface of HbV guarantees the homogeneous dispersion and prompt blood flow in microcirculation (11, 30). The complete recovery of the blood gas variables and lactate concentration also supports the normal gas exchanging function of the lung and the excretion and decomposition of metabolites through the kidney and liver, respectively. The myocardium showed a slight influence of ischemic damage for all the groups. The significant difference was observed in the cerebral tissue between the groups receiving oxygen-carrying and noncarrying fluids. The rHSA group showed a significantly larger area with ischemic changes, a pyknotic change of the nuclei, and an edematous change, on the right side; however, other groups receiving oxygen-carrying fluids showed a significantly lower level of changes. We considered that ligation of the right carotid artery and the influence of hypoperfusion induced by the hemorrhagic shock caused ischemic environment in the right cerebral hemisphere, leading to pathologic and irreversible changes of cerebral tissues. The brain tissue was not

Hemoglobin-vesicle suspended in recombinant human serum albumin provides restoration from hemorrhagic shock that is comparable with that using shed autologous blood.

examined in our previous shock study using hamsters with ligation of a carotid artery in the same manner. In the present study, the significantly higher level of ischemic change only in the rHSA group may be caused by the prolonged hypotension and lower oxygen content in blood after resuscitation. Therefore, the cause of death in the rHSA group could be due, in part, to aggravated cerebral damage.

Even though histopathological examination of the liver did not show any abnormalities, the plasma clinical laboratory tests demonstrated elevation of AST and ALT for the HbV/rHSA, wRBC/rHSA, and SAB groups but not for the rHSA (survivor) group. Chemically modified Hbs also were reported to elevate AST and ALT after resuscitation (31, 32). This indicated that the resuscitation with oxygen-carrying fluids might induce ischemia/reperfusion injury that influences liver function (33, 34). However, because AST and ALT values represent the concentration in plasma, the difference in plasma volume between the groups should be considered. The plasma volume ratio should be calculated by subtracting the volumes of RBC and HbV from whole blood. Under the assumption that whole blood volume is equal between the groups, the rHSA group has a 1.35 and 1.20 times larger volume of plasma compared with the SAB group and the HbV/rHSA group, respectively, due to the reduced hematocrit for the rHSA group. Therefore, enzyme concentrations in the rHSA group may possibly be slightly underestimated. Including some antioxidative reagents such as active oxygen scavengers in the resuscitative fluid should be considered to obtain better resuscitation (35, 36).

CONCLUSION

HbV suspended in recombinant HSA at a concentration of only 8.6 g/dL of Hb showed effectiveness for resuscitation from hemorrhagic shock that was comparable to that using shed autologous blood. This acute study encourages us to continue further studies to optimize the physicochemical variables of the HbV suspension such as Hb concentration and oxygen affinity and to look at a longer term survival beyond 6 hrs to weeks using a larger animal model. Some of the polymerized Hbs are now in the final stages of clinical trials (37), and our HbV have to be compared with these materials in terms of safety and efficacy to demonstrate the advantage of cellular structure of HbV.

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A new red blood cell substitute*

Donor red blood cell is the standard replacement when hematocrit falls below the critical level, but it is not without its problems (1). Can red blood cell substitutes with the following properties fulfill some of the special needs for critical care medicine? "There is no need for time-consuming cross-matching or typing in the hospital setting and therefore this can be given immediately on the spot. There is no need for refrigeration since they can be kept at room temperature for >1 yr in the ambulance, in the field, or in the emergency room. There is no potential for infectious agents since they can be sterilized. There is no limit in the amount that can be made available." This has been a dream for many years, but as shown by the article in this issue of *Critical Care Medicine* by Dr. Sakai and colleagues (2) and by research and clinical trials from other centers on other types of blood substitutes, we are coming very close to this possibility.

The dream for a complete red blood cell substitute started in 1964 with the publication of the first attempt to prepare a complete artificial red blood cell (3). This fulfilled the previously mentioned properties except that the artificial cells did not circulate for sufficient length of time to carry out useful functions. As a result, emphasis moved from the overly ambitious attempt at complete red blood cell substitutes to less complex oxygen carriers (4–8). These included perfluorochemicals, polyhemoglobin, conjugated hemoglobin, intramolecularly crosskined hemoglobin, and recombinant human hemoglobin (4–8). Of these, polyhemoglobin is in the most advanced stage of development. Polyhemoglobin is based on the idea of using diacids (3) or glutaraldehyde (9) to cross-link hemoglobin. This basic idea has been developed independently by two groups in the form of glutaraldehyde cross-linked human polyhemoglobin that is in the final stages of phase III clinical trial (10) and glutaraldehyde cross-linked bovine polyhemoglobin that awaiting Food and Drug Administration approval (11). They published a number of articles including the results of their clinical trials (10, 11). Very briefly, they have infused up to 20 units of polyhemoglobin each time into patients during trauma surgery (10) or in other types of surgery (11). They were able by doing this to maintain hemoglobin at the required concentrations (10, 11). These polyhemoglobins can be stored at room temperature for >1 yr and can be sterilized to remove and inactivate infectious organisms or factors. These polyhemoglobins do not contain blood group antigens and thus can be used without cross-matching and typing. Their circulation half-time is >24 hrs. Unlike single hemoglobin molecules, polyhemoglobin preparations that are mostly in the form of large soluble hemoglobin complexes do not cross the intercellular endothelial junctions of the blood vessels to cause vasoconstriction. Polyhemoglobin is an oxygen carrier and does not have the complete function of red blood cells. However, this is already effective in clinical trials as an oxygen carrier for use in perioperative surgery including surgeries related to critical care medicine like trauma surgery, rupture aneurysm repair, and others (10, 11).

Oxygen carrier in solution can more easily perfuse through obstructed vessels in strokes and myocardial infarction. Also, unlike donor red blood cells, these carriers can be used on the spot without the need for cross-matching and typing in severe hemorrhagic shock. However, if the ischemia is severe and prolonged, reperfusion with oxygen-carrying fluids alone may result in ischemia-reperfusion injuries. We found one way to avoid this by cross-linking trace amounts of red blood cell enzymes to hemoglobin, forming a soluble polyhemoglobin-catalase-superoxide dismutase (polyHb-CAT-SOD) complex (12). We tested this in a global cerebral ischemia-reperfusion rat model. Unlike polyhemoglobin that contains no significant enzyme activities, polyHb-CAT-SOD causes no significant ischemia-reperfusion injuries as shown by the preservation of blood-brain barrier and the absence of brain edema (13).

With polyhemoglobin nearly possible for routine clinical use as an oxygen carrier, a number of groups are perusing the next step of the original idea of a complete artificial red blood cell (3). The original short circulation time was improved by research carried out by many groups using small lipid membrane artificial red blood cells (8, 14). The surface modification of the lipid membrane using polyethylene glycol has markedly improved the circulation time to double that of polyhemoglobin (14). As shown by their article in this issue, Dr. Sakai and colleagues (2) have moved the lipid membrane artificial red blood cells to the final stages of animal studies toward clinical trials (2). They have shown the efficacy of these cells in hemorrhagic shock. In other publications, they have studied in detail the safety and efficacy of their lipid membrane artificial red blood cells (8). Another more recent approach toward a complete red blood cell substitute is being develop by our group based on nanotechnology and biodegradable polymer to prepare nano-dimension artificial red blood cells (6, 15). This biodegradable polymer can be readily converted to water and carbon dioxide after use. The nano-dimension artificial red blood cell is a complete artificial red blood cell that contains all the red blood cell enzymes including superoxide dismutase, catalase, carbonic anhydrase, methemoglobin reductase, and others (6, 15). The circulation time is double that of polyhemoglobin (15). However, this is in a much earlier stage of animal studies compared with the lipid membrane artificial cells reported in this issue (2).

In summary, we now have two oxygen carriers in the form of polyhemoglobin that could be very close to routine perioperative use in trauma surgery (10) and other types of surgery (11). For conditions with potential for ischemia reperfu-

*See also p. 539.

Key Words: blood substitutes; oxygen carriers; polyhemoglobin; artificial red blood cells; transfusion; ischemia-reperfusion

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sion injuries, polyHb-CAT-SOD is being developed in animal studies (12, 13). The stage is therefore set for development of complete artificial red blood cells in the form of lipid membrane artificial red blood cells (2) and biodegradable polymeric membrane nano-dimension artificial red blood cells (15). However, in conditions requiring only a simple oxygen carrier (10, 11), there is no need to go to a more complex and more costly system. On the other hand, the more complex systems (2, 12, 13, 15), when ready, will have their roles in those clinical conditions where a simple oxygen carrier cannot fulfill all the requirements.

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Peptidoglycan is an important pathogenic factor of the inflammatory response in sepsis*

Sepsis is the clinical manifestation of the host-derived systemic inflammatory response resulting from invasive infection. A majority of cases of sepsis and septic shock are secondary to infections with Gram-negative bacteria. Gram-positive organisms also account for a significant proportion of cases. The inflammatory process begins at the nidus of infection, where bacteria proliferate and either invade the bloodstream or release various bacterial components, such as endotoxin, peptidoglycan, teichoic acid, and other microbial exotoxins (1). The main therapeutic intervention following sepsis is antibiotic therapy. However, even when effective antibiotics kill bacteria, they do not affect the release of these bacterial toxins (2, 3). The interaction of these microbial cellular components with macrophages, monocytes, or other host cells induces the release of inflammatory mediators that play a major role in the pathophysiology of septic shock (2, 3). This interaction of microbial products and host cells is a major determinant of the innate immune response and represents the first line of defense against pathogens by promoting acute inflammatory responses and evoking early cellular infiltration at the site of infection and tissue injury. The innate immune system has evolved a complex network of receptors, which rapidly identify pathogens based on invariant molecular structures that are shared by a variety of microorganisms. Among these invariant macromolecular structures, peptidoglycans are recognized by cells of the host through specific interactions with cell surface receptors, such as the Toll-like receptors (TLR) (4, 5).

It has been demonstrated that activation of mitogen-activated protein kinase homologs and other kinases mediates the transduction of extracellular signals from the receptor levels to the nucleus and is a pivotal event in the regulation of the transcription events that determine functional outcome in response to stress (6). This signaling cascade is rapid and enables the cells to respond to environmental changes by inducing a prompt production of proinflammatory and anti-inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, IL-10, and interferon-γ (6).

In this issue of *Critical Care Medicine* Dr. Wang and colleagues (7) show that *in vivo* administration in the rat of a purified extract of peptidoglycan of *Staphylococcus aureus* causes liver and renal dysfunction, which correlates with increased tissue gene expression and plasma elevation of cytokines. These events are associated with increased DNA binding activ-

*See also p. 546.

Key Words: sepsis; nuclear factor-κB; Toll-like receptor; signal transduction; peptidoglycan

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