arterial pressure and renal cortical O_2 tension, and finally all the rats died of anemia. On the other hand, hemodilution with HbV, suspended in HSA sustained both blood pressure and renal cortical O_2 tension, and all the rats survived. These results clearly demonstrate that HbV, has sufficient O_2 transporting capability.

To observe the microcirculatory response to the infusion of Hb products, we used the intravital microscopy equipped with all the units to measure blood flow rates, vascular diameter, O_2 tension, and so on. This system was developed by Professor Intaglietta at the University of California, San Diego. We used the hamster dorsal-skin fold preparation that allows observation of blood vessels from small arteries to capillaries. We evaluated the HbV suspension as a resuscitative fluid for hemorrhagic-shocked hamsters [26]. About 50% of the blood was withdrawn, and the blood pressure was maintained at around 40 mmHg for 1 h. The hamsters either received HbV suspended in HSA (HbV/HSA), HSA alone, or shed blood (Fig. 3). Immediately after infusion, all the groups showed increases in mean arterial pressure, however, only the albumin infusion resulted in incomplete recovery. The HbV/HSA group showed the same recovery with the shed autologous blood infusion. During the shock period, all the groups showed significant hyperventilation that was evident from the significant increase in arterial O_2 partial. Simultaneously,

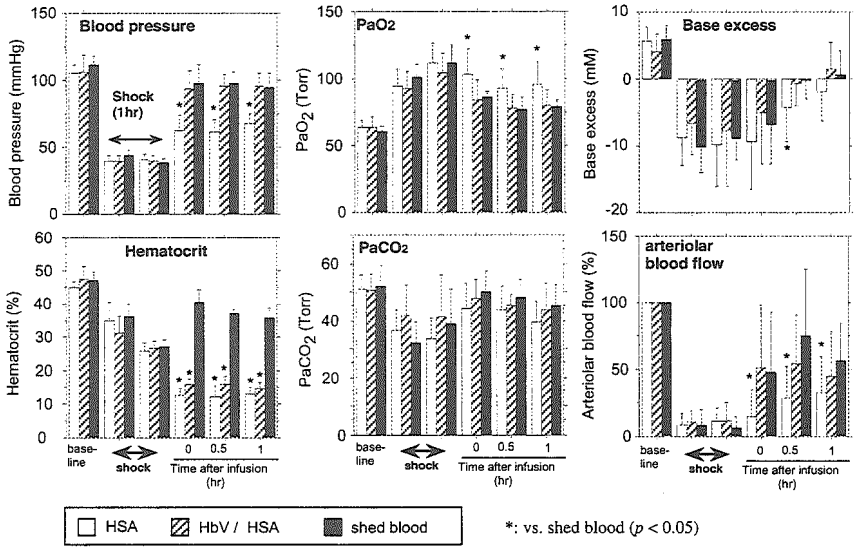


FIG. 3. Resuscitation from hemorrhagic shock with HbV suspended in HSA (HbV/HSA) in hamster dorsal skinfold model. Mean ± SD

base excess and pH decreased significantly. Immediately after resuscitation, all the groups tended to recover. However, only the HSA group showed sustained hyperventilation. Base excess for the HSA group remained at a significantly lower value one hour after resuscitation. Blood flow decreased significantly in arterioles to 11% of basal value during shock. The HbV/HSA and shed autologous blood groups immediately showed significant increases in blood flow rate after resuscitation, while the albumin group showed the lowest recovery.

In Vivo Safety of HbV

We further examined the safety profile of HbV such as cardiovascular responses, pharmacokinetics, influence on reticulo endothelial system (RES), influence on clinical measurements and daily repeated infusion [29–35].

We observed the responses to the infusion of intra-molecularly cross-linked Hb (XLHb) and HbV into conscious hamsters. XLHb (7 nm in diameter) showed a significant increase in hypertension equal to 35 mmHg, and simultaneous vasoconstriction of the resistance artery equal to 75% of the baseline levels [30] (Fig. 4). On the other hand, HbV at 250 nm, showed minimal change. The small acellular XLHb is homogeneously dispersed in the plasma, and it diffuses through the endothelium layer of the vascular wall and reaches the smooth muscle. Intra-moleculary cross-linked Hb traps nitric oxide (NO) as an endothelium-derived relaxation factor, and induces vasoconstriction, and hypertension. On the other hand, the large HbV stay in the lumen and do not induce vasoconstriction. Several mechanisms are proposed for Hb-induced vasoconstriction. These include NO-binding, excess O_2 supply, reduced shear stress, or the presence of Hb recognition site on the

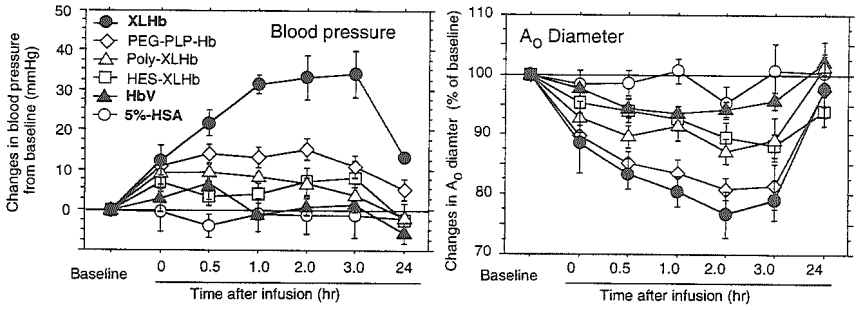


FIG. 4. Changes in mean arterial pressure and the diameters of the resistance artery in hamster dorsal skin microcirculation after the bolus infusion of Hb-based O_2 carriers. Mean ± SD

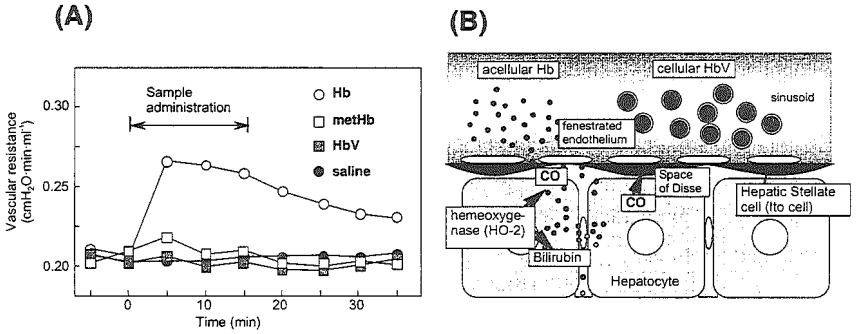


FIG. 5. A Changes in vascular resistance during perfusion of exteriorized rat liver with HbV, Hb, metHb, or saline. B Schematic representation of hepatic microcirculation: The small Hb molecule extravasate across the fenestrated endothelium to reach to the space of Disse, where heme of Hb is catabolised by hemeoxygenase-2 (HO-2) and CO is released as a vasorelaxation factor. However, the excess amount of the extravasated Hb traps CO and induces vasoconstriction and the resulting higher vascular resistance. On the other hand, the larger HbV retains in the sinusoid and there is no extravasation and vasoconstriction

endothelium. But it is clear that Hb-encapsulation shields against the side effects of acellular Hbs.

Professor Suematsu at Keio University has revealed the effects of Hb-based O_2 carriers in hepatic microcirculation [29,32] (Fig. 5). On the vascular wall of the sinusoid in hepatic microcirculation, there are many pores, called fenestration, with a diameter of about 100 nm. The small Hb molecules with a diameter of only 7 nm extravasate through the fenestrated endothelium and reach the space of Disse. On the other hand, HbV particles, which are larger than the pores, do not extravasate. Heme of extravasated Hb is excessively metabolized by hemeoxygenase-2 in hepatocyte to produce CO and bilirubin. Even though CO acts as a vasorelaxation factor in the liver, the excess amount of Hb rapidly binds CO, resulting in the vasoconstriction and an increase in vascular resistance. On the other hand, HbV (250 nm in diameter) is large enough to remain in the sinusoid, and the vascular resistance is maintained.

So, what is the optimal molecular dimension of Hb-based O_2 carriers? The upper limitation is below the capillary diameter to prevent capillary plugging, and for sterilization by membrane filters (Fig. 6). On the other hand, smaller sizes exhibit a higher rate of vascular wall permeability with side effects such as hypertension and neurological disturbances. HbV exhibits a very low level of vascular wall permeability. Therefore, the HbV appears to be appropriate from the viewpoint of hemodynamics. We have clarified the influence of HbV on the RES, because the fate of HbV is RES trapping.

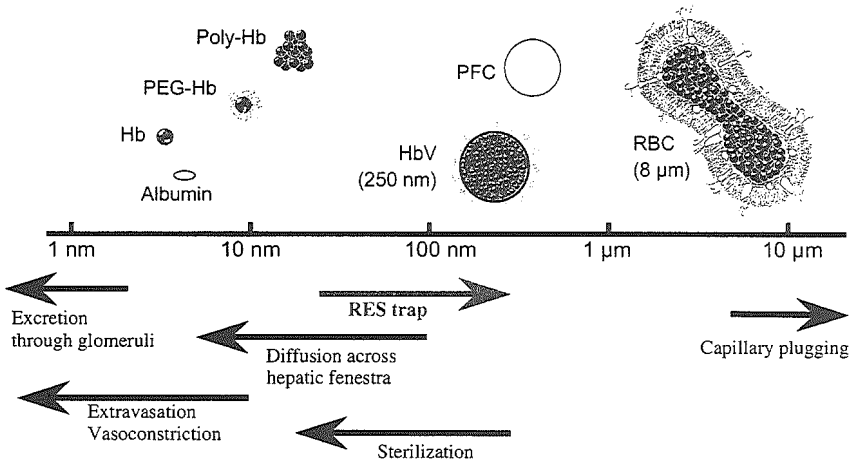
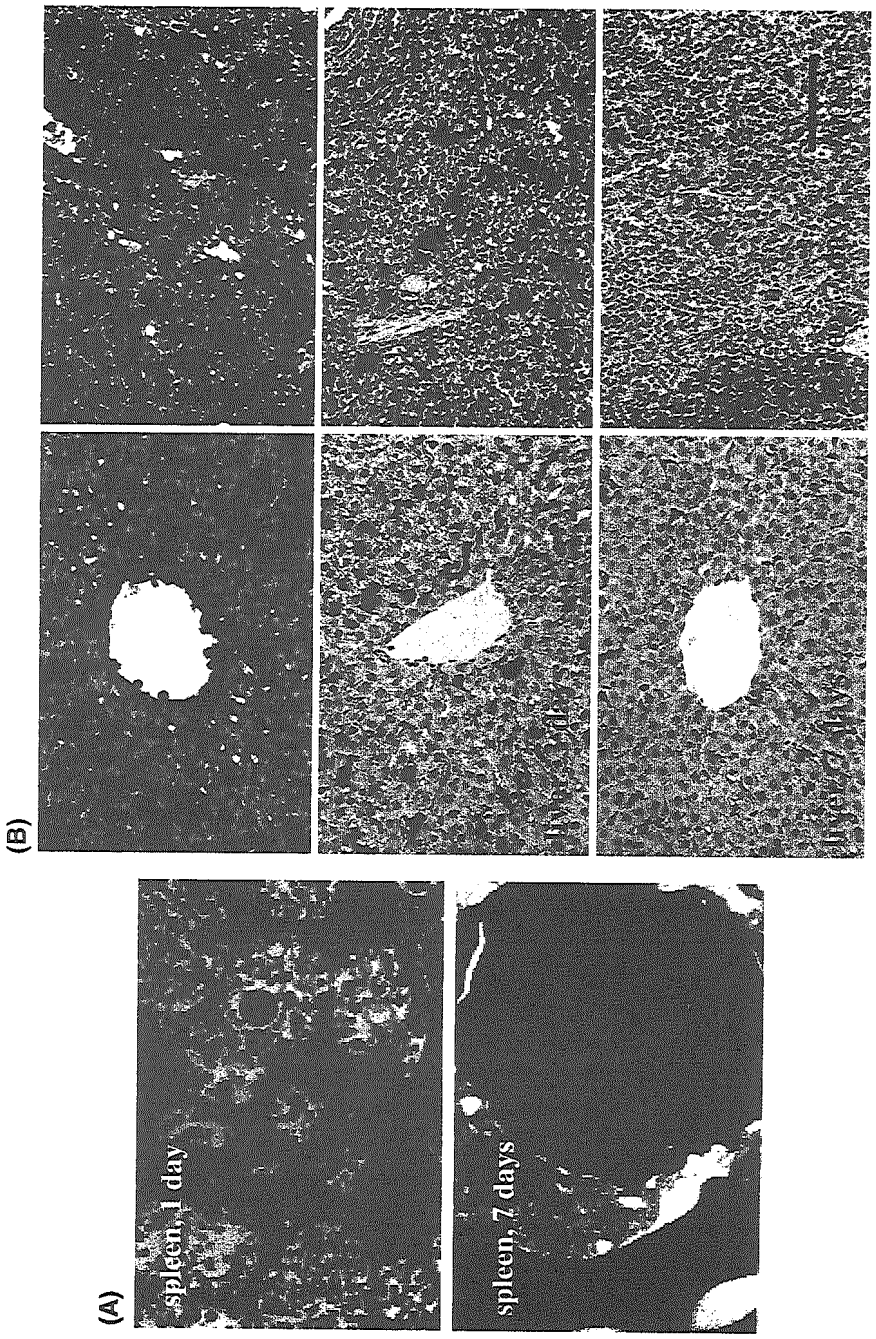


FIG. 6. Optimal diameter of Hb-based oxygen carriers from the view point of physiological response and production process

Circulation persistence was measured by monitoring the concentration of radioisotope-labeled HbV in collaboration with Dr. W.T. Phillips at the University of Texas at San Antonio. The circulation half-life is dose dependent, and when the dose rate was 14 ml/kg, the circulation half-life was 24 h. The circulation time in the case of the human body can be estimated to be twice or three times longer; or about 2 or 3 days at the same dose rate. Gamma camera images of radioisotope-labeled HbV showed the time course of biodistribution. Just after infusion, HbV remains in the blood stream so that the heart and liver that contain a lot of blood showed strong intensity. However, after it is finished playing its role in O_2 transport, a total of 35% of HbV are finally distributed mainly in the liver, spleen and bone marrow.

The time course of liver uptake was monitored with a confocal fluorescence microscope. Hb-vesicle was stained with a lipid fluorophore. The liver of an anesthetized hamster was exposed and a fluorescence-labeled-HbV was infused intravenously. Due to the motion of respiration, the picture oscillates. However, a static frame can be obtained. The individual particles of HbV cannot be recognized. When the vesicles are accumulated in phagosomes of Kupffer cells, they can be recognized with a strong fluorescence. How is HbV metabolized in macrophages? The transmission electron microscopy (TEM) of the spleen 1 day after infusion of HbV clearly demonstrated the presence of HbV particles in macrophages, where HbV particles that appear as black dots are captured by the phagosomes [34] (Fig. 7). Red blood cells and HbV contain a lot of ferric iron with a high electron density, so that they show



strong contrast in TEM. However, after 7 days, the HbV structure cannot be observed. We confirmed no abnormalities in the tissues and no irreversible damage to the organs or complete metabolism within a week. A Polyclonal anti-human Hb antibody was used as the marker of Hb in the HbV. This antibody does not recognize rat Hb. The red-colored parts indicate the presence of Hb in HbV, and almost disappear after 7 days in both the spleen and liver. This shows that HbV can be metabolized quite promptly.

One issue of the Hb-based O_2 carriers is that they have a significant influence on clinical laboratory tests. They remain in the plasma phase in hematocrit capillaries after centrifugation of blood samples, and interfere with the colorimetric and turbidimetric measurements. However, HbV can be simply removed from blood plasma either by ultracentrifugation or centrifugation in the presence of a high-molecular-weight dextran to enhance precipitation. We can obtain a very clear supernatant for accurate analyses [35]. This is one advantage of HbV in comparison with acellular Hb solutions. Accordingly we examined the influence on organ functions by serum clinical laboratory tests after the bolus infusion of HbV at a dose rate of 20 ml/kg. Albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactic dehydrogenase (LDH), which reflect the liver function, moves their values within normal range. Concentrations of bilirubin and ferric ion are maintained at a low level. The concentration of lipids transiently changed. In particular, the cholesterols increased significantly. And phospholipids slightly increased, however, they returned to the original level after 7 days. These results indicate that the membrane components of HbV, once they reappear from RES, are metabolized on the physiological pathway.

We recently tested a daily repeated infusion of HbV in Wistar rats as a safety study. The dose rate was a 10-ml/kg/day infusion for 14 days. All rats well tolerated and survived. Body weight showed a monotonous but slightly depressed increase in comparison with the saline group. However, after 2 weeks there was no significant difference with the saline control group. All the rats seemed very healthy and active. There was no piloerection. As for the hematological parameters, the numbers of white blood cells and platelets did not exhibit a significant difference from the HbV group and the saline control group. Hematocrit showed a slight reduction for the HbV group, probably due to the accumulation of the large amount of HbV in the blood. Histopathological examination one day after the final infusion of HbV showed signifi-

FIG. 7. A Transmission electron microscopy of rat spleen one day after the infusion of HbV (20 ml/kg) and after 7 days. Black dots are HbV particles captured in phagosomes in the spleen macrophages, and they disappeared at 7 days. B Staining with anti-human Hb antibody showed the presence of HbV in spleen and liver. They disappeared within 7 days. Cited from: Sakai et al (2001) Am J Pathol 159:1079–1088

cant accumulation of HbV in spleen macrophages, and liver Kupffer cells. Berlin Blue staining revealed the presence of hemosiderin indicating that the metabolism of Hb was initiated. There were no other morphological abnormalities, and the serum clinical chemistry indicated transient but reversible increases in lipid components. AST and ALT were within the normal range. From these results we are confident with the safety of HbV.

Design and Physicochemical Properties of rHSA-Heme

We have been conducting research on totally synthetic O_2 carriers, or so-called albumin-heme that does not require Hb. Human serum albumin is the most abundant plasma protein in our blood stream, but its crystal structure has not been elucidated for long time. In 1998, Dr. Stephen Curry of the Imperial College of London was the first elucidate the crystal structure of the human serum albumin complexed with seven molecules of myristic acids [36]. He found that the dynamic conformational changes of albumin take place by the binding of fatty acid.

In Japan, recombinant human serum albumin is now manufactured on a large scale by expression in the yeast Pichia pastoris, and it will appear on the market soon [37]. A large-scale plant, which can produce one million vials per year, has been already established. From the viewpoint of clinical application, O_2-carrying albumin is quite exciting and may be of extreme medical importance. With this background, we have found that synthetic heme derivative is efficiently incorporated into recombinant human serum albumin (rHSA), creating a red-colored rHSA-heme hybrid. This rHSA-heme can reversibly bind and release O_2 molecules under physiological conditions in the same manner as Hb. In other words, our rHSA-heme hybrid is a synthetic O_2-carrying hemoprotein, and we believe that its saline solution will become a new class of red blood cell substitute. We have already published these chemistry findings and technologies in international journals [38–49].

Figure 8 summarizes the structure of the albumin-heme molecule. The Maximal binding numbers of heme to one albumin are eight, and the magnitude of the binding constants ranged from 10^6 to 10^4 (M^{-1}). The isoelectric point of rHSA-heme was found to be 4.8, independent of the binding numbers of heme. This value is exactly the same as that of albumin itself. Furthermore, the viscosity and density did not change after the incorporation of heme molecules, and the obtained solution showed a long shelf life of almost two years at room temperature. The O_2-binding sites of rHSA-heme are iron-porphyrin, therefore the color of the solution changed in a similar way to Hb. Upon addition of O_2 gas through this solution, the visible absorption pattern immediately changed to that of the O_2-adduct complex. Moreover, after bubbling carbon monoxide gas, albumin-heme formed a very stable carbonyl complex.

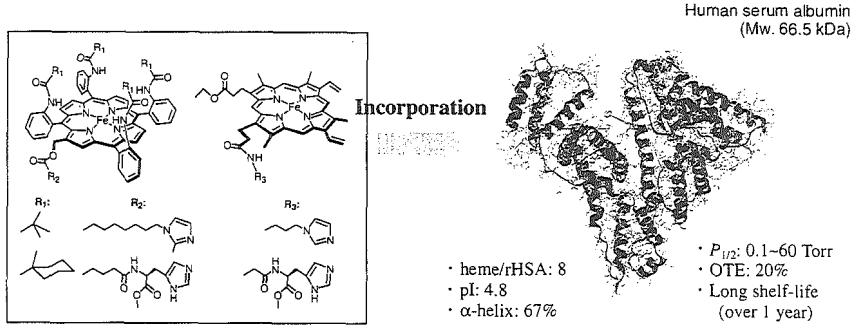


FIG. 8. Structure of the albumin-heme molecule

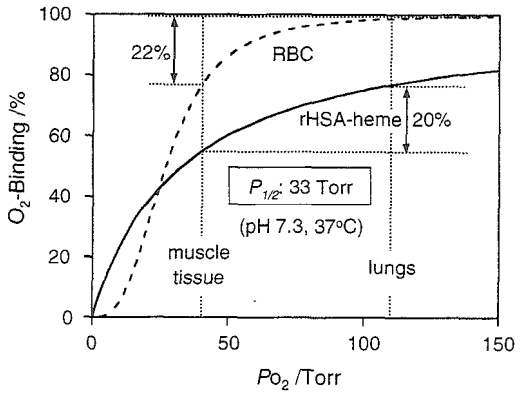


FIG. 9. O_2-binding equilibrium curve of albumin-heme

Figure 9 shows the O_2-binding equilibrium curve of rHSA-heme. The O_2-binding affinity, of rHSA-heme is always constant, independent of the number of heme, and the O_2-binding profile does not show cooperativity. However, the O_2-transporting efficiency of albumin-heme between the lungs where PaO_2 is 110 Torr and muscle tissue where PtO_2 is 40 Torr increases to 20%, which is similar to 22% efficiency of red blood cells. The O_2-binding property of albumin-heme can be controlled by changing the chemical structure of heme derivatives incorporated. More recently, we have found that a protoheme derivative is also incorporated into albumin and can bind and release O_2 as well [50].

In Vivo Safety and Efficacy of rHSA-Heme

Based on these findings, we can say that rHSA-heme can become an entirely synthetic O_2-carrier, and satisfy the initial clinical requirements for a red blood cell substitute. However, we have another problem to solve before we can use this material as an O_2-carrier in the circulatory system. This problem is NO scavenging. Of course, it can bind NO, and it may be anticipated that the injection of rHSA-heme also induce hypertensive action. We have evaluated the efficacy and safety of this rHSA-heme solution with animal experiments.

As described before, small Hb molecules extravasate through the vascular endothelium and react with NO, thus inducing vasoconstriction and acute increases in systemic blood pressure. Contrary to the expectations, the observation of the intestinal microcirculation after the infusion of rHSA-heme into an anesthetized rat revealed that the diameters of the venules and arterioles were not deformed at all [51]. Indeed, only a small change in the mean arterial pressure was observed after the administration of the rHSA-heme solution (Fig. 10). In contrast, the infusion of Hb elicited an acute increase in blood pressure. Why does albumin-heme not induce vasoconstriction or hypertension? The answer probably lies in the negatively charged molecular surface of albumin. One of the unique characteristics of serum albumin is its low permeability through the capillary pore, which is less than 1/100 that for Hb due to the electrostatic repulsion between the albumin surface and the glomerular basement membrane around the endothelial cells.

We are now evaluating the O_2-transporting ability of this albumin-heme molecule in the circulatory system with further animal experiments [52]. First, we determined the physiological responses to exchange transfusion with rHSA-heme solution into rats after 70% hemodilution and 40% hemorrhage

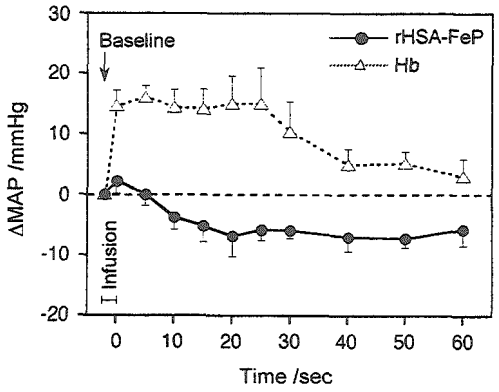


FIG. 10. Change of MAP after the administration of rHSA-heme solution in the anesthetized rats (n = 5). All data are shown as changes from the basal values (ΔMAP) just before the infusion and expressed as mean ± S.E. Basal value is 90.1 ± 3.0 mmHg

(Fig. 11). The declined mean arterial pressure and blood flow after a 70% exchange with albumin and further 40% bleeding of blood showed a significant recovery of up to 90% of the baseline values by the infusion of the rHSA-heme solution. On the other hand, all rats in the control group only injected with albumin died within 30 min. Furthermore, muscle tissue O_2-tension significantly increased. These responses indicate the in vivo O_2-delivery of the rHSA-heme solution.

More recently, we have synthesized human serum albumin dimer, which can incorporate sixteen hemes in its hydrophobic domain [53]. The human serum rHSA-heme dimer solution dissolves 1.2 times more O_2 compared to that of red blood cells and keeps its colloid osmotic pressure at the same level as the physiological value.

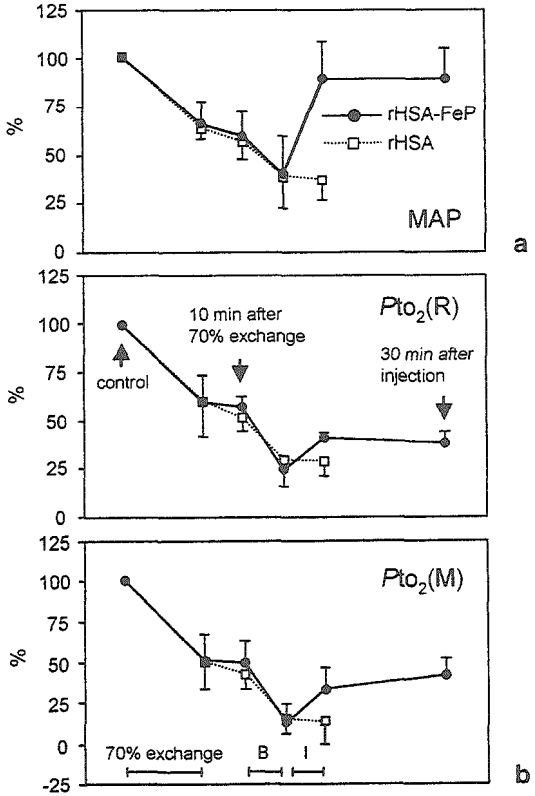


FIG. 11. Change of (a) MAP and (b) O_2-tension in renal cortex during the 70% hemodilution with 5 wt% rHSA and further 40% exchange transfusion with rHSA-heme in anesthetized rats (n = 5). All data are shown as changes from the basal values and expressed as mean ± S.E.

Potential Applications of Artificial O_2 Carriers

For almost 20 years our group at Keio University in collaboration with Dr. Tsuchida's group at Waseda University have been trying to produce artificial O_2 carriers. To date, we have produced several types of O_2 carriers and evaluated their efficacy and biocompatibility. In this chapter, we have shown what we have done to produce O_2 carriers. Below, we would like to show you the potential applications of artificial O_2 carriers, as well as a glimpse of the vast possibilities that lie ahead.

Tumor Oxygenation

Unlike vessels in normal tissues, the development of a vasculature in a tumor lacks normal course of angiogenesis and is hence, highly heterogeneous. Consequently, areas of hypoxia are quite common in tumors. In these hypoxic regions, it can be added that tumor cells acquire resistance to treatments such as chemotherapy and radiation. Our rHSA-heme was injected into the responsible artery that supplies circulation to an implanted tumor (Fig. 12) [54]. O_2 tension of the tumor rises immediately after intra-arterial infusion of albumin heme up to 2.4 times that of the baseline value. Our findings in animals indicate that tumor tissue O_2 levels can be elevated by the administration of artificial O_2 carriers due to the difference in O_2 transporting properties from red blood cells. Whether this increase in tissue O_2 can potentiate cancer treatment is currently under investigation.

Organ Preservation

One of the most important agenda in transplantation medicine is long-term organ preservation and circumvention of ischemia reperfusion injuries. We

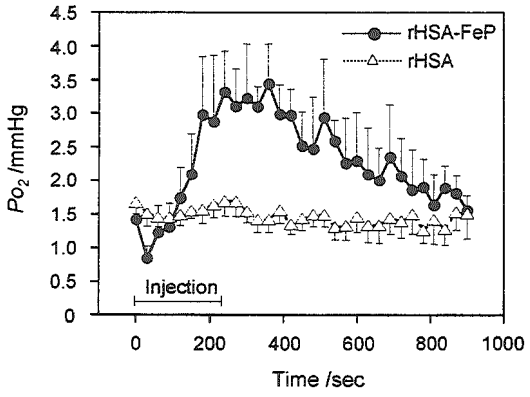


FIG. 12. Changes in the O_2 tension of the hypoxic region of the ascites hepatoma LY80 solid tumor after the administration of the O_2 saturated rHSA-heme or rHSA solutions in the anesthetized rats (n = 4 each). All data are shown as changes from the basal values (Po_2) just before the infusion and expressed as mean ± S.E.

think that artificial O_2 carriers can be applied as a perfusate for donor tissue in order to overcome these problems. In particular, its O_2 carrying capacity has the potential to significantly extend the preservation period. This will make it easier to transport organs. Also, utilizing the extra time, we may in the future be able to perform additional organ tests for better compatibility, or even perform genetic modifications during this period. We believe that through these applications, the concept of organ preservation can be expanded to organ culture, and furthermore to include the preservation of cells derived from donor tissues.

Extracorporeal Circulation

Extracorporeal circulation is quite common in cardiac surgery. Improvements are being made in the priming solutions but red cells are often still required to fill the device circuit, particularly in compromised cases and in children [55]. We believe that the use of artificial O_2 carriers in the priming solution can decrease or completely eliminate the need for a transfusion in such cases, and hence reduce the incidence of infection or GVHD.

Tissue Ischemia

Tissue ischemia can ensue from impairment of peripheral perfusion due to a variety of diseases such as arteriosclerosis obliterans, diabetes, and Burger's disease. The key event in the progression of ischemic diseases is the inability of red cells to flow through the capillaries, beyond obstruction ulceration and gangrene formation become imminent. We believe that this critical phase can be avoided or delayed by the application of artificial O_2 carriers, which can be designed to flow through these damaged capillaries or collateral circulation [27,28].

Liquid Ventilation for Acute Lung Injury

For patients who present acute lung injury or acute respiratory distress syndrome (ARDS), gas exchange in the lung exhibits severe deterioration and sometimes even the newest mechanical ventilation method fails to establish adequate oxygenation of the blood. In this type of critical case, liquid ventilation using an artificial O_2 carrier can establish optimal oxygenation of the blood and may reproduce the integrity of lung parenchyma [56]. Briefly explained, oxygenated liquid ventilation fluid is administered into the lung through trachea and O_2 molecules are transferred through diseased alveolus by diffusion and oxygenate the blood. Currently, this method is thought to be effective for patients with congenital diaphragmatic herniation. Efficacy for adult acute lung injuries is now under investigation. Perfluorochemicals are the main fluid used for clinical use, however, aqueous artificial O_2 carriers may have the potential to be used for liquid ventilation.

Epilogue

The research field of the red cell substitutes is moving forward very rapidly. Also as you have seen, the paradigm in this field is expanding from red cell substitutes to "O_2 therapeutics". The quality control and the pre-clinical test will be completed on the carriers produced at the pilot plant, after which clinical trials will proceed. We look forward to the day that our research will play an effective role in treating patients.

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Hemoglobin-Vesicles (HbV) as Artificial Oxygen Carriers

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Summary. Considering the physiological significance of the cellular structure of a red blood cell (RBC), it may be reasonable to mimic its structure for designing a hemoglobin (Hb)-based oxygen carrier. In this chapter, we have summarized the characteristics and performances of Hb-vesicles (HbV) that have been developed on the basis of molecular assembly. Collaborative in vitro and in vivo studies have revealed sufficient safety and efficacy of HbV.

Key words. Blood substitutes, Hemoglobin-vesicles, Red blood cells, Oxygen transport, Liposome

Introduction: Importance of Cellular Structure

When we design an artificial oxygen carrier based on hemoglobin (Hb) molecules, we may have to reconsider why Hb is encapsulated in RBCs in our body. Barcroft et al. (1923) insisted that the reasons for Hb encapsulation in RBCs were: (1) a decrease in the high viscosity of Hb and a high colloidal osmotic pressure; (2) prevention of the removal of Hb from blood circulation; and (3) preservation of the chemical environment in the cells such as the concentration of phosphates (2,3-DPG, ATP, NADPH, etc.) and other electrolytes [1]. Moreover, during the long history of development of Hb-based oxygen carriers, many side effects of molecular Hb have become apparent such as renal toxicity due to the dissociation of tetrameric Hb subunits to two dimers ($\alpha_2\beta_2 \rightarrow 2\alpha\beta$), which may induce renal toxicity and entrapment of gaseous messenger molecules (NO and CO) inducing vasoconstriction,

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hypertension, reduced blood flow and tissue oxygenation in the microcirculatory levels, neurological disturbances, and malfunctioning of esophageal motor function. These side effects of molecular Hb would imply the importance of cellular structure.

The pioneering work was performed by Chang (1957) [2], who started encapsulation of Hb like a RBC and prepared microcapsules (5 μm) made of nylon, collodion, etc. Toyoda (1965) [3] and Kitajima of the Kambara-Kimoto group (1971) [4] also covered Hb solution with gelatin, gum Arabic, or silicone; however, it was very difficult to regulate the particle size appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility. After Bangham and Horne reported in 1964 that phospholipids assembled to form vesicles in aqueous media [5], and that they encapsulate water-soluble materials in their inner aqueous interior, it was quite reasonable to use such vesicles for Hb encapsulation. Djordjevici and Miller (1977) prepared liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acid, etc [6]. Hunt, Kondo, Chapman, Gaber, Farmer, Beissinger, Chang, Schmidt, Farmer, Rudolph and others attempted LEH [7–16]. In the United States, Naval Research Laboratories showed the remarkable progress of LEH. Hemoglobin-vesicles (HbV), with a high efficiency production process and improved properties, have been established by Tsuchida's group, based on the technologies of molecular assembly and precise analysis of pharmacological and physiological aspects [1,17] (Fig. 1).

In this chapter we summarize the characteristics of HbV based on the science of molecular assembly and its excellent results.

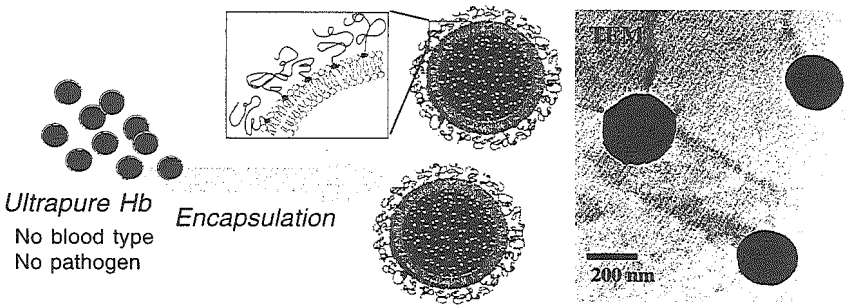

FIG. 1. Hb-vesicles (diameter, ca. 250 nm) are prepared from ultrapure Hb obtained from outdated RBC. The surface of the vesicles is modified with polyethyleneglycol that ensures the dispersion stability during storage and during circulation in the blood stream. Transmission electron micrograph (TEM) clearly demonstrates the well-regulated particle size and high Hb content within the vesicles

Preparation and Characteristics of HbV as a Molecular Assembly

Purification of Hb for the Utmost Safety

The primary advantage of using an artificial oxygen carrier should the absence of risk of infectious diseases derived from human blood. Even though strictly inspected RBCs after expiration of limitation period are used as a source of Hb, it is necessary to introduce additional procedures to inactivate and remove viruses in the process of Hb purification in order to guarantee the utmost safety from infection. In our purification process, virus inactivation was performed by pasteurization at 60°C for 12 h, which are the same conditions used for the pasteurization of human serum albumin [18,19]. This process can be introduced by utilizing the stability of carbonylhemoglobin (HbCO). The thermograms of HbCO indicated the denaturation temperature at 78°C, which is much higher than that for oxyhemoglobin (64°C) [20] (Fig. 2).

The virus inactivation efficiency was evaluated by the Hokkaido Red Cross Blood Center [21,22]. The Hb solution spiked with vesicular stomatitis virus (VSV) was treated at 60°C for 1 h under either an air or CO atmosphere. VSV was inactivated at less than 5.8 $\log_{10}$ and less than 6.0 $\log_{10}$ under the air and

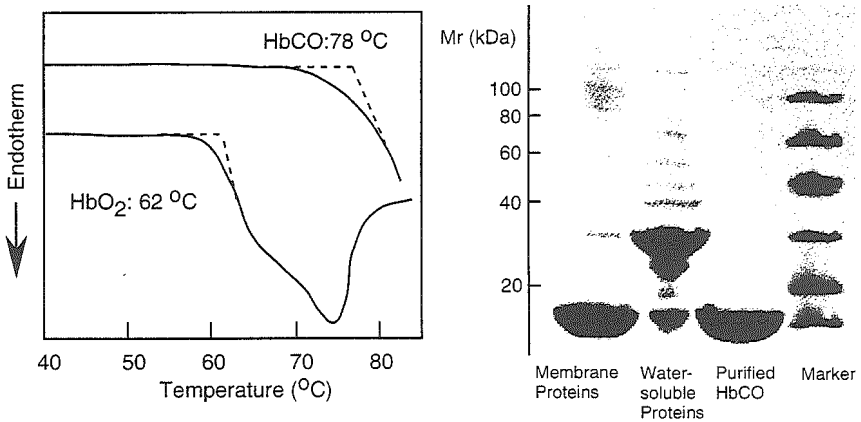

FIG. 2. **Left**, the calorimetric thermograms of HbCO solution showed the decomposition temperature at 78°C, indicating the thermal stability for pasteurization at 60°C. The amount of sample was 60 μl (6.0 g/dl). The heating speed was 1.0°C/min. **Right**, SDS-PAGE of HbCO after the heat treatment (60°C, 12 h), indicates the purity and no contamination of other proteins derived from RBC. The concentration of Hb applied to the gel was 10 mg/ml, which was 10 times higher than the maximum concentration described in the instruction manual of PhastSystem, in order to show the absence of other bands in the purified HbCO

CO atmosphere, respectively. Although the methemoglobin (metHb) rate increased after the heat treatment under the air atmosphere, no metHb formation was observed by the treatment under the CO atmosphere. Isoelectric focusing analysis revealed the denaturation of Hb after the heat treatment under the air, while the Hb band was not altered in the carbonylated condition. Some protein bands other than Hb had disappeared on sodiumdodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) after the heat treatment. During the pasteurization, all the other concomitant proteins are denatured and precipitated. As a result, we obtain ultrapure Hb solution. This high purity is essential to prevent membrane plugging during the next ultrafiltration process.

We tested ultrafiltration of HbCO solution to remove viruses with PLANOVA-35N and -15N (Bemberg Microporous Membrane: BMM; Asahi Kasei, Tokyo, Japan) [23]. The virus removal mechanism is by size exclusion through the capillary pores, and the filtration method is a depth filtration. The unit membrane which has a network structure of capillaries and voids is accumulated to form 150 layers. PLANOVA -35N and -15N have mean pore sizes of 35 nm and 15 nm, respectively. PLANOVA-35N is suitable for removing envelope-type viruses such as HIV, and HCV of which the size ranges from 40 nm to 100 nm, PLANOVA -15N can be used to remove the nonenvelope-type viruses, such as parvoviruses, of which the size is less than 40 nm. However, when the pores of the membrane filter are plugged by impurities, the PLANOVA-35N is sometimes used as a prefilter for PLANOVA-15N. The permeation flux (LMH) and the permeated ratio of HbCO solution ([Hb] = 5.6 g/dl) through PLANOVA-35N at 13°C were 36 (L/m^2/h) and almost 100 (%), respectively. Those through PLANOVA-15N at 13°C were 15 (L/m^2/h) and 95 (%), respectively. The LMH increased to 18 (L/m^2/h) at 25°C. Under the same conditions, a high removal efficiency of a bacteriophage, $\phi \times 174$, (>7.7 log) was confirmed. These results indicate that PLANOVA-15N is effective for the process of virus removal from Hb solutions. We also confirmed the effectiveness of other virus removal ultrafiltration systems such as Viresolve.

Thus, purified HbCO solution can be concentrated to above 40 g/dl very effectively using an ultrafiltration process. After regulation of the electrolyte concentrations, this is supplied for encapsulation procedure. The ligand of the resulting HbV, CO, is converted to O_2 by illuminating the liquid membrane of HbV suspension to a visible light under O_2 flow [24].

Other groups have selected methods to preserve the well-organized enzymatic systems originally present in the RBCs aiming at the prolonged stability of ferrous state of Hb [25,26]. However, this may cause insufficient virus removal or inactivation and cannot guarantee the utmost safety of the resulting artificial oxygen carrier. One advantage of HbV is that any reagent can be coencapsulated in the vesicles. It has been confirmed that coencapsulation of an appropriate amount of a reductant, such as glutathione or homocysteine, and active oxygen scavengers, such as catalase, effectively retards the metHb formation [27–31].

Effective Hb Encapsulation

The performance of HbV depends on the weight ratio of Hb to lipid ([Hb]/[Lipid]). This value is improved by lowering the number of bilayer membrane (lamellarity) of the vesicle and raising the concentration of Hb in the interior of the vesicle. We studied the optimal conditions for Hb encapsulation using an extrusion method and considering the behaviors of Hb and lipid assemblies as a kind of polymer electrolyte [32–35].

The maximum [Hb]/[Lipid] ratio that would relate to the isoelectric point (pI) of Hb can be obtained at ca. pH 7. The Hb molecule is negatively charged when pH is above 7.0, and the electrostatic repulsion between Hb and the negatively charged bilayer membrane results in lower encapsulation efficiency. However, the lower pH should enhance Hb denaturation by interaction with the lipid bilayer membrane and metHb formation at a lower pH. Therefore, the physiological pH, 7.0–7.4, would be optimal. It was also revealed that the higher ionic strength shields the repulsion between the negatively charged lipid bilayer membranes and increases the lamellarity.

The number of bilayer membranes decreases with increased microviscosity (decreased lipid mobility). Multilamellar vesicles are converted to smaller vesicles with smaller lamellarity during the extrusion procedure. When membrane fluidity is high, deformation of vesicles during extrusion occurs more easily, even for multilamellar vesicles, resulting in larger lamellarity in the final vesicles. Therefore, the use of lipids with higher phase transition temperature is preferred. However, these lipids would make extrusion more difficult, because a higher shear stress (high extrusion pressure) is required. Based on this reasoning, mixed lipids contain 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) as the main component.

Based on the precise analysis of the characterization of the physicochemical properties of the components, the encapsulation efficiency of Hb solution in a size-regulated phospholipid vesicle has been improved using an extrusion method [36,37]. Mixed lipids (DPPC, cholesterol, 1,5-*O*-dioctadecyl-*N*-succinyl-L-glutamate (DPEA), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[monomethoxy poly(ethylene glycol) (5,000)] (PEG-DSPE)] at a molar ratio of 5, 5, 1, and 0.033 were hydrated with a NaOH solution (7.6 mM) to obtain a polydispersed multilamellar vesicle dispersion (50 nm–30 μm in diameter). The polydispersed vesicles were converted to smaller vesicles having an average diameter of ca. 500 nm with a relatively narrow size distribution by freeze-thawing at a lipid concentration of 2 g/dl

and cooling rate of −140°C /min. The lyophilized powder of the freeze-thawed vesicles was rehydrated into a concentrated Hb solution (40 g/dl) and retained the average size and distribution of the original vesicles. The resulting vesicle dispersion smoothly permeated through the membrane filters during extrusion. The average permeation rate of the freeze-thawed vesicles was ca. 30 times faster than that of simple hydrated vesicles. During the extrusion process, Hb solution was encapsulated into the reconstructed vesicles effectively with a diameter of 250 ± 20 nm, and the [Hb]/[lipid] ratio reached 1.7–1.8.

Regulation of Oxygen Affinity

Oxygen affinity of purified Hb (expressed as P_{50}, O_2 tension at which Hb is half-saturated with O_2) is about 5 Torr, and Hb strongly binds O_2 and does not release O_2 at 40 Torr (partial pressure of mixed venous blood). Historically, it has been regarded that the O_2 affinity of an Hb-based O_2 carrier (HBOC) should be regulated similarly to that of RBC, namely about 25–30 Torr, using an allosteric effector, or by a direct chemical modification of the Hb molecules. Theoretically, this allows sufficient O_2 unloading during blood microcirculation, as could be evaluated by the arterio-venous difference in O_2 saturation in accordance with an O_2 equilibrium curve. It has been expected that decreasing the O_2 affinity (increasing P_{50}) results in an increase in the O_2 unloading. This expectation is supported by the result that the RBC with a high P_{50} shows an enhanced O_2 release for improved exercise capacity in a mouse model [38].

If this theory is correct, P_{50} of Hb in HbV should be equivalent to that of human RBCs, i.e., 28 Torr, or higher. Pyridoxal 5′-phosphate (PLP) is coencapsulated in HbV as an allosteric effector to regulate P_{50} [39]. The main binding site of PLP is the *N*-terminal of the α- and β-chains and β-82 lysine within the β-cleft, which is part of the binding site of natural allosteric effector, 2,3-diphosphoglyceric acid (2,3-DPG) [40]. The bound PLP retards the dissociation of the ionic linkage between β-chains of Hb during conversion of deoxy to oxyHb in the same manner as does 2,3-DPG. Thus, oxygen affinity of Hb decreases in the presence of PLP. The P_{50} of HbV can be regulated to 5–150 Torr by coencapsulating the appropriate amount of PLP or inositol hexaphosphate as an allosteric effector [41]. Equimolar PLP to Hb (PLP/Hb = 1/1 by mol) was coencapsulated, and P_{50} was regulated to 18 Torr. When the molar ratio PLP/Hb was 3/1, P_{50} was regulated to 32 Torr. The O_2 affinities of HbV can be regulated quite easily without changing other physical parameters, whereas in the case of the other modified Hb solutions their chemical structures determine their O_2 affinities, thus regulation is difficult. The appropriate O_2 affinities for O_2 carriers have not yet been completely decided; however, the easy regulation of O_2 affinity may be useful to meet the requirement of the clinical indications such as oxygenation of ischemic tissues (see section "Improved Oxygenation in Ischemic Hamster Flap Tissue by Hemodilution with HbV").

Surface Modification of HbV and Its Stability During Long-Term Storage

Since Hb autoxidizes to form metHb and loses its oxygen-binding ability during storage as well as in blood circulation, the prevention of metHb formation is required. The conventional long-term preservation methods are to store modified Hbs in a frozen state or to store them as a freeze-dried powder with some cryoprotective or lyoprotective agents such as saccharides or polyols [42,43]. Some groups have recently reported a method to preserve deoxygenated Hbs in a liquid state [44], using the well-known intrinsic characteristic of Hb that the Hb oxidation rate in a solution is dependent on the oxygen partial pressure and deoxyHb essentially is not autoxidized at ambient temperature [45,46].

In the case of HbV, not only the inside Hb, but also the cellular structure has to be physically stabilized in order to prevent intervesicular aggregation, fusion, and leakage of encapsulated Hb and other reagents. Phospholipid vesicles are molecular assemblies and generally regarded as unstable capsules which require some reinforcement. We studied the γ-ray polymerization in the bilayer membranes of phospholipids bearing dienoyl groups, and the resulting polymerized phospholipid membrane significantly stabilized HbV [47–49]. The polymerized vesicles preserved the particle diameter and function of the inside Hb even after 10 repeated freeze-thawings and freeze-dryings and rehydrations [50,51]. However, the relatively slow rate of metabolism of the polyphospholipid in the reticuloendothelial systems is considered to be a problem. Cryoprotection and lyoprotection of the HbV were performed by the addition of saccharides such as trehalose [52] or glycolipid [53]. However, preservation of the oxygen carriers in a liquid state may be more useful for infusion in emergency situations than the time-consuming procedures such as redissolving the Hb powder or thawing several hundred milliliters of the frozen Hb solution stored in a freezer.

Surface modification of phospholipid vesicles with the poly(ethylene glycol) (PEG)-conjugated lipid is a well-known method to prolong the circulation time of the vesicles in vivo for drug delivery systems [54,55]. For HbV, the surface of HbV was also modified with PEG chains to improve its dispersion state of the vesicles when mixed with blood components [56]. The PEG-modified HbV has shown an improved blood circulation and tissue oxygenation due to the absence of HbV aggregate formation and viscosity ele-

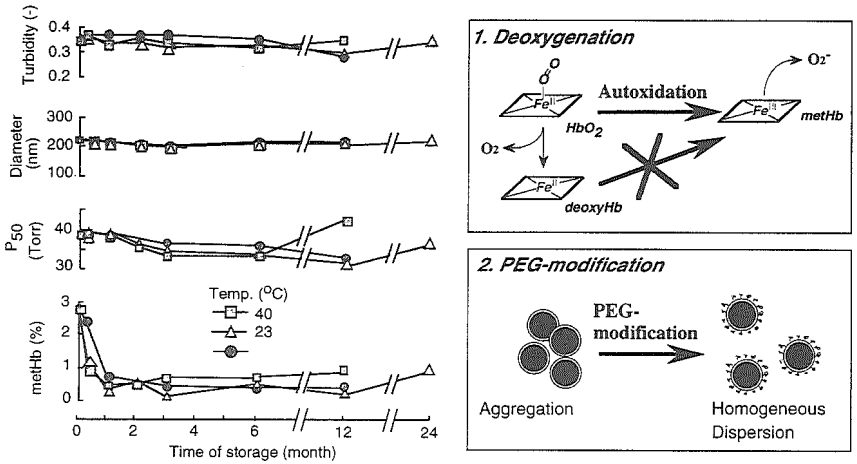

FIG. 3. Stability of HbV during storage for up to 24 months. Deoxygenation is important to prevent autoxidation to metHb, and PEG-modification suppresses aggregation and fusion of HbV to stabilize the dispersion condition. Both techniques are essential for the long-term storage of HbV. A reductant coencapsulated in HbV not only consumed a *trace amount of oxygen* but also gradually reduced metHb

vation [57,58] and prolonged circulation persistence in vivo [59]. However, little attention has been paid to the ability of PEG modification for the long-term preservation of vesicles or liposomes in the liquid state [60,61].

We studied the possibility of the long-term preservation of HbV by the combination of two technologies, i.e., surface modification of HbV with PEG chains and deoxygenation during storage for 2 years [62] (Fig. 3). The samples stored at 4° and 23°C showed a stable dispersion state for 2 years, although the sample stored at 40°C showed the precipitation and decomposition of vesicular components, a decrease in pH, and 4% leakage of total Hb after 1 year. The PEG chains on the vesicular surface stabilize the dispersion state and prevent the aggregation and fusion due to their steric hindrance. The original metHb content (ca. 3%) before the preservation gradually decreased to less than 1% in all the samples after 1 month due to the presence of homocysteine inside the vesicles which consumed the residual oxygen and gradually reduced the trace amount of metHb. The rate of metHb formation was strongly dependent on the partial pressure of oxygen, and no increase in metHb formation was observed due to the intrinsic stability of the deoxygenated Hb. These results indicate the possibility that HbV suspension can be stored at room temperature for at least 2 years.

Generally, phospholipid vesicles are regarded as unstable capsules; however, the establishment of this pivotal technology will enhance the application of PEG-modified vesicles in other fields. The long-term preservation of oxygen carriers overcomes the limitation of the blood transfusion system and will be of benefit to clinical medicine.

Interaction of Lipopolysaccharide (LPS) with HbV and Quantitative Measurement of LPS

The production process of HbV has to be guaranteed with a good manufacturing practice (GMP) standard as a biological product regarding the strict regulation of impurity and viral and bacterial contamination. It is required to strictly monitor the content of the lipopolysaccharide (LPS), known as an endotoxin, a component of the outer membrane of gram-negative bacteria possessing a large variety of biological influences on numerous mammalian cells and tissues. Endotoxin is an extremely potent toxin with a lethal dose (LD_{50}) of 3 mg/kg and 1 mg/kg in rats and dogs, respectively [63,64]. The U.S. Food and Drug Administration (FDA) has established a guideline on human maximum endotoxin dose permissible for parenteral products (5 EU/kg) [65] that may include Hb-based oxygen carriers. This limit is based on the endotoxin activity (Endotoxin Unit: EU; 1 EU = 100 pg), and can be measured via the *Limulus* amebocyte lysate (LAL) assay, in which LAL clots and forms a gel in the presence of LPS [66]. In general, the LAL method has advantages over rabbit pyrogen testing, because the LAL method requires a smaller sample, and the assays can easily be repeated [67]. Since the volume of oxygen carriers to be infused for shock resuscitation or acute hemodilution is estimated to be less than 20 ml/kg, the specific endotoxin limits per millimeter should be 0.25 EU/ml (= 5/20), similar to that for water for injection (0.25 EU/ml).

Bacterial LPS is an amphiphilic macromolecule; therefore, it hydrophobically interacts with protein and biomembranes [68]. Hb strongly interacts with LPS showing synergistic toxicity [69,70]. The constituent of endotoxin that causes LAL gelation is a glycophospholipid-designated lipid-A [71]. Lipid-A possesses several fatty acid constituents that are readily inserted into the bilayer membrane of the phospholipid vesicles. The inclusion of lipid-A in the phospholipid vesicles markedly reduces several functions of lipid-A such as its LAL gelation activity [72]. Using isothermal titration calorimetry (ITC), we quantitatively clarified for the first time that LPS from the *Salmonella minnesota* wild type (smooth form) was inserted into the phospholipid vesicles with an enthalpy change (ΔH) of −80 kcal/mol and the maximum incorporation of 7.6 mol% on the outer surface of the vesicles [73]. To our knowledge, the ΔH value of PEG_{5000}-DSPE (MW of PEG = 5 kDa) for the same phospholipid vesicles is only −13 kcal/mol. This comparison indicates that LPS inserted into the bilayer membrane is thermodynamically more stabi-

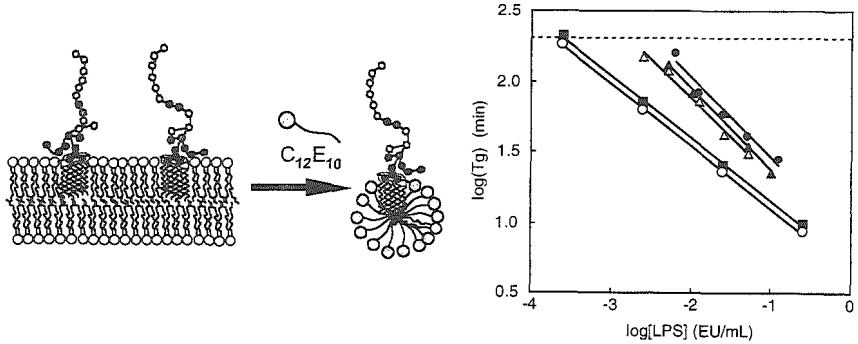

FIG. 4. (*Left*) Solubilization of LPS-contaminated vesicles with a detergent to form mixed micelles. (*Right*) Calibration curves for the quantitative measurement of LPS in the presence of $C_{12}E_{10}$ at various concentrations (vol%); 2.0 (●), 0.8 (▲), 0.4 (△), 0.1 (■), 0 (○). [LPS] is the final concentration in the test tube for turbidimetry. Dilution factors should be multiplied to obtain [LPS] in the HbV suspension at [Hb] = 10 g/dl. For example, [LPS] should be multiplied with 400 at $[C_{12}E_{10}]$ = 0.1 wt%. The *broken line* indicates the gelation time limit for Toxinometer (200 min)

lized than PEG_{5000}-DSPE. The large difference in ΔH is probably due not only to the hydrophobic interactions by the eight alkyl chains of LPS but also to the hydrogen bonding of the amide bonds into the interface of the hydrophobic and hydrophilic regions that contribute to the interaction of LPS with the phospholipid bilayer membrane. As for the interaction between Hb and LPS, the ITC analysis was recently reported by Jurgens et al. [74], who clarified that 3–5 LPS molecules bind to one Hb molecule. As a consequence, the researchers who study HbV or other phospholipid vesicles for delivering other functional molecules encountered a problem in measuring the LPS content for the quality control of these materials [75–77].

Considering this background information, we tested the solubilization of HbV with deca(oxyethylene) dodecyl ether ($C_{12}E_{10}$) to release the LPS entrapped in the vesicles as a pretreatment for the succeeding LAL assay of the kinetic-turbidimetric gel clotting analysis using Toxinometer (Wako Pure Chem. Ind. Ltd., Tokyo, Japan) (detecting wavelength, 660 nm) [73] (Fig. 4). The $C_{12}E_{10}$ surfactant interferes with the gel clotting in a concentration-dependent manner, and the optimal condition was determined in terms of minimizing the dilution factor and $C_{12}E_{10}$ concentration. We clarified the condition that allowed the measurement of LPS higher than 0.1 EU/ml in the HbV suspension. This modified LAL assay using $C_{12}E_{10}$ and the Toxinometer is routinely used in our production system of HbV. Significant attention is paid to the quality control of HbV for preclinical studies, and all the HbV prepared under sterile conditions showed an LPS content less than 0.1 EU/ml at [Hb] = 10 g/dl.

Moreover, the utilization of the histidine-immobilized agarose gel (Pyrosep; Wako Pure Chem. Ind. Ltd., Tokyo, Japan) effectively concentrated the trace amount of LPS from the $C_{12}E_{10}$-solubilized HbV solution and washed out $C_{12}E_{10}$ as an inhibitory element [78]. The LAL assay with the LPS-adsorbed gel resulted in the detection limit of 0.0025 EU/ml. The pretreatment with $C_{12}E_{10}$ would be applicable not only to HbV but also to other drug delivery systems using phospholipid vesicles encapsulating or incorporating functional molecules.

Interaction with Active Oxygen Species

It has been pointed out that that heme-mediated reactions of chemically modified Hbs such as ligand coordinations and redox reactions could cause organ dysfunction and/or tissue damage. Especially, redox reactions may affect the physiological protection against reactive oxygen species [79]. The oxidation of oxyHb by H_2O_2 is known to generate ferrylHb and metHb accompanied by heme degradation and the release of free iron. Furthermore, during the autoxidation of oxyHb to metHb, reactive oxygen species such as superoxide, hydrogen peroxide, and the hydroxyl radical are generated to damage not only the remaining oxyHb but also living cells and organs. Especially, ferrylHb is known to be a potent oxidant which catalyzes the peroxidation of lipids comprising the biomembrane and other biomaterials [80,81]. In normal human plasma, the concentration of H_2O_2 is 4–5 μM [82] and elevates to 100–600 μM under inflammatory [83] or ischemia/reperfusion conditions [84]. In fact, ferrylHb can be found both in the RBCs [85] and in the endothelial cells model after hypoxia reoxygenation [86,87]. Several *in vitro* studies suggest that free radicals or degradation products catalyzed by ferrylHb could damage the endothelial cells in the presence of acellular-type Hb modifications. Hb-mediated cytotoxicity via ferrylHb is one of the important safety issues of HBOCs [88].

On the other hand, in the cellular-type HbV, reactive oxygen species generated within the HbV during metHb formation were completely consumed by Hb. Although such a reaction leads to Hb oxidation, no reactive oxygen species have been detected outside the vesicles. However, reaction of Hb inside the vesicle with exogenous H_2O_2 is one of the important safety issues to be clarified and compared with a free Hb solution. We confirmed that during the reaction of the Hb solution with H_2O_2, metHb and ferrylHb are produced, and H_2O_2 is decomposed by the catalase-like reaction of Hb [89]. The aggregation of discolored Hb products due to heme degradation is accompanied by the release of iron (ferric ion). On the other hand, the concentrated Hb within the vesicles reacts with H_2O_2 that permeated across the bilayer membrane, and the same products as the Hb solution were formed inside the vesicles. However, there is no turbidity change, no particle diameter change of the HbV,

and no peroxidation of lipids comprising the vesicles after the reaction with H_2O_2. Furthermore, no free iron is detected outside the vesicle, though ferric ion is released from the denatured Hb inside the vesicle, indicating the barrier effect of the bilayer membrane against the permeation of ferric ion. When vesicles composed of egg yoke lecithin (EYL) as unsaturated lipids are added to the mixture of Hb and H_2O_2, the lipid peroxidation is caused by ferrylHb and hydroxyl radical generated from reaction of the ferric iron with H_2O_2. However, no lipid peroxidation is observed in the case of the HbV dispersion because the saturated lipid membrane of the HbV should prevent the interaction of the ferrylHb or ferric iron with the EYL. These results indicate the high safety of the Hb vesicles which enclose the reactive Hb products in the reaction with H_2O_2.

Influence of HbV on Clinical Laboratory Tests and Countermeasures

One of the remaining issues of the chemically modified acellular Hbs is the interference during clinical laboratory tests by the presence of Hbs in the serum. This topic has been extensively discussed in the field of clinical chemistry and laboratory medicine [90–94]. Even though clinical laboratory assays of blood serum components play an important role in the diagnosis and the care of many peri- or postoperative and traumatic patients, both hemolysis and lipemia are well known to cause interference in many colorimetric and spectrophotometric methods in routine automated assays. Accordingly, the presence of HBOCs interferes with the measurements due to the strong optical absorbances attributed to the Hb species (400–600 nm in wavelength). An appropriate pretreatment or calculation to subtract the deviation should be required to obtain accurate concentrations of the analytes. HbV particles (diameter, 250 nm), which possess both the Hb absorption and light scattering, show strong interference in various measurements [95]. It is important to clarify the interference of the HbV suspension in clinical laboratory tests performed on serum and to establish a pretreatment method to avoid such interference (Fig. 5).

The HbV suspension, acellular Hb solution ([Hb] = 10 g/dl) or saline, was mixed with a pooled human serum at various ratios up to 50 vol% ([Hb] = 5 g/dl), and the magnitude of the interference effect of HbV and Hb on 30 analytes was studied. The mixture of the HbV suspension and serum was ultracentrifuged (50,000 g, 20 min) to remove the HbV particles as precipitate, and the supernatant was analyzed and compared with the saline control group. The HbV particles were also removed by centrifugation (2,700 g, 30 min) in the presence of dextran (Mw 200 kDa). The HbV suspension showed considerable interference effects in most analytes. The majority of these

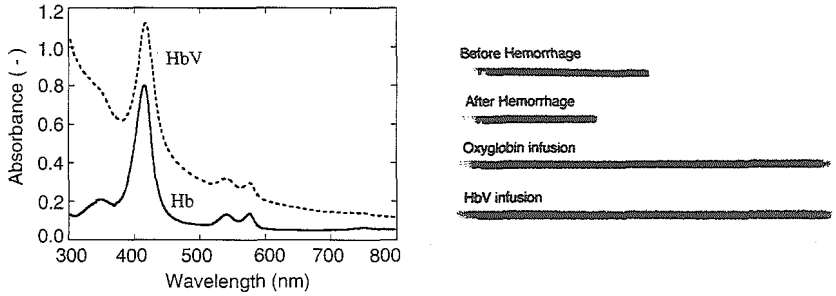

FIG. 5. **Left**, absorption spectrum of Hb and HbV at [Hb] = 0.01 g/dl in a 10 mm cuvette measured using a UV/vis spectrophotometer. The significant turbidity is confirmed especially at shorter wavelength for an HbV suspension. **Right**, glass capillaries for the hematocrit measurements during the resuscitation of hemorrhagic shocked hamsters. The centrifuged glass capillary containing blood after the infusion of glutaraldehyde-polymerized bovine Hb showed a red colored supernatant due to the presence of Hb components. In the case of HbV infusion, HbV does not precipitate at the normal centrifugation condition (10,000 g, 5 min), therefore, the supernatant is red and turbid

effects were more serious than those of the acellular Hb solution. These findings are thought to be due to the light absorption of Hb in HbV and/or the light scattering generated in the suspension that interferes with the colorimetric and turbidimetric measurements. The components of HbV may also interfere with the chemical reactions of the studied assays. However, removal of the HbV from the supernatant diminished the interference in most of the assays. This pretreatment of plasma allows accurate measurements of total protein, albumin, globulin, AST, ALT, LDH, ALP, γ-GTP, bilirubin, creatinine, urea nitrogen, uric acid, amylase, lipase, creatinine phosphokinase, total cholesterol, free cholesterol, β-lipoprotein, HDL-cholesterol, total lipid, free fatty acid, phospholipid, and electrolytes (Na, K, Cl, Ca, inorganic phosphate, Mg, Fe, and Cu). Neutral fat was not measured accurately due to the sedimentation by ultracentrifugation.

This pretreatment may be applicable to the removal of perfluorocarbon particles as another type of O_2 carrier, because it is reported that the particles form aggregation in the presence of dextran [96]. We currently utilize the pretreatment of ultracentrifugation for the safety study of HbV in animal tests. Addition of Dex may be an alternative way to facilitate the precipitation of HbV at a lower centrifugal force that is more convenient for a clinical situation, because not all clinical laboratories are equipped with an ultracentrifuge. In this research, we studied the major analytes only. Further research is necessary to clarify the interference for the other measurements of analytes including hydrophobic or amphiphilic drugs or biological components that may interact with the hydrophobic bilayer membrane of HbV.

Efficacy of HbV as Oxygen Carriers in Vivo

The advantages of the HBOCs are the absence of blood-type antigens and infectious viruses, and stability for long-term storage that overwhelm the RBC transfusion. The shorter half lives of HBOCs in the blood stream (2–3 days) limit their use but they are applicable for a shorter period of use such as: (1) a resuscitative fluid for hemorrhagic shock in an emergency situation for a temporary time or bridging until the packed RBCs are available, (2) a fluid for preoperative hemodilution or perioperative O_2 supply fluid for a hemorrhage in an elective surgery to avoid or delay allogeneic transfusion, (3) a priming solution for the circuit of an extracorporeal membrane oxygenator (ECMO), and (4) other potential indications, e.g., so-called O_2 therapeutics to oxygenate ischemic tissues.

One particle of HbV (diameter, ca. 250 nm) contains about 30,000 Hb molecules. Since HbV acts as a particle in the blood, not as a solute, the colloid osmotic pressure of the HbV suspension is nearly zero. It requires the addition of a plasma expander for a large substitution of blood to maintain blood volume. The candidates of plasma expanders are human serum albumin (HSA), hydroxyethyl starch, dextran, or gelatin depending on the clinical setting, cost, countries and clinicians. The absence of any infectious disease from humans is the greatest advantage of recombinant human serum albumin (rHSA) and it will soon be approved as an alternative for clinical use in Japan. Moreover, there should be no immunological and hematological abnormalities that are often seen in the use of dextran and hydroxyethyl starch. Aiming at application of HbV suspended in a plasma expander to the above indications, HbV was tested for resuscitation from hemorrhagic shock [97–100] and extreme hemodilution [57,58,101–105] in the Waseda-Keio group and with Prof. Intaglietta at the University of California, San Diego. Moreover, HbV was tested for oxygenation of an ischemic skin flap by Dr. Erni et al. at Inselspital University Hospital, Bern [106,107], and this implies the further application of HbV for other ischemic diseases such as myocardial and brain infarction and stroke. Some of the published results are summarized in this section.

Resuscitation from Hemorrhagic Shock with HbV Suspended in Recombinant Human Serum Albumin [100]

Objective. The ability of the suspension of HbV to restore the systemic condition after hemorrhagic shock was evaluated in anesthetized Wistar rats for 6 h after resuscitation.

Methods. The HbV was suspended in a 5 g/dl recombinant human serum albumin solution (HbV/rHSA) at an Hb concentration of 8.6 g/dl. Forty male

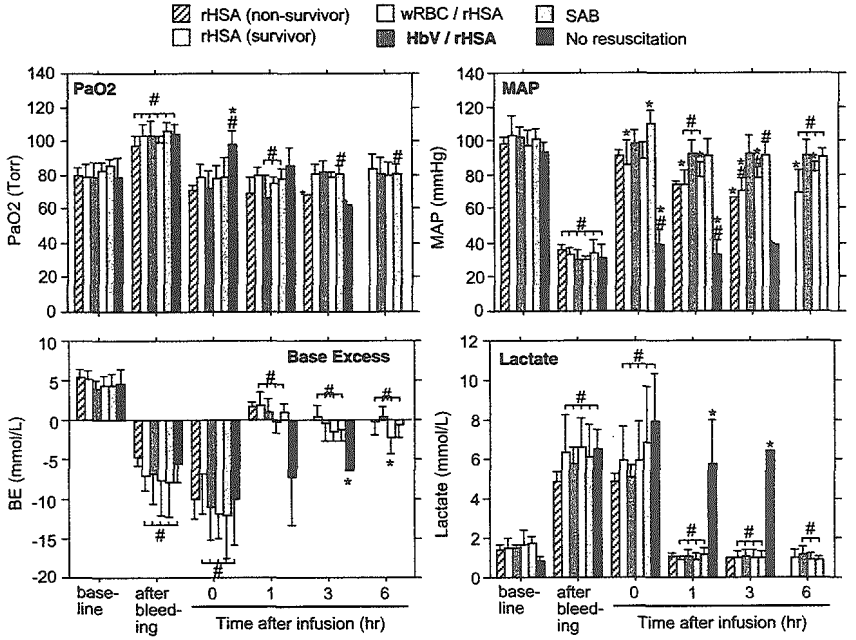

FIG. 6. Changes in mean arterial pressure (MAP) and blood gas parameters during hemorrhagic shock and resuscitation with infusion of HbV suspended in recombinant human serum albumin (HbV/rHSA), shed autologous blood (SAB), washed red blood cells suspended in recombinant human serum albumin (wRBC/rHSA), and recombinant human serum albumin (rHSA) alone. The sham group did not receive a resuscitative fluid after the hemorrhage, and died within 3 h. The number of surviving rats was 3 at 1 h. In the rHSA group, 2 of the 8 rats died between 1 and 6 h. Accordingly, the rHSA group was divided into the rHSA(survivor) group and the rHSA(non-survivor) groups until they died. Therefore, the number of rats (n) for the rHSA(survivor) and rHSA(non-survivor) groups were 6 and 2, respectively. # significantly different from baseline ($p < 0.05$), * significantly different versus the HbV/rHSA group ($p < 0.05$)

Wistar 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 (MAP) monitoring.

Measurements and Main Results. Shock was induced by 50% blood withdrawal. The rats showed hypotension (MAP = 32 ± 10 mmHg) and significant metabolic acidosis and hyperventilation (Fig. 6). After 15 min, 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.

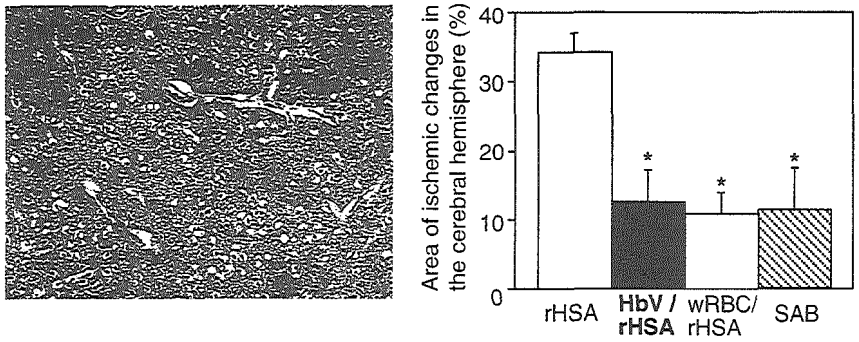

FIG. 7. Resuscitation from hemorrhagic shock with HbV suspended in rHSA in comparison with rHSA, wRBC/rHSA. The cerebral hemisphere on the right side of the rHSA group showed significant ischemic changes, a pyknotic change of the nuclei and an edematous change (34 ± 3% of the total section area) as shown in the left picture, relating to the ligation of the right carotid artery. However, the other groups that were resuscitated with O_2-carrying fluids showed minimal changes (* $p < 0.001$ vs. rHSA; HbV/rHSA, 13 ± 5%; SAB, 11 ± 6%; wRBC/rHSA, 11 ± 3%). The non-resuscitated rats did not show such ischemic changes

The HbV/rHSA group restored MAP to 93 ± 8 mmHg at 1 h, similar to the SAB group (92 ± 9 mmHg), which was significantly higher compared with the rHSA (74 ± 9 mmHg) and wRBC/rHSA (79 ± 8 mmHg) 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 h. After 6 h, 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 (Fig. 7). These results indicate that HbV suspended in recombinant human serum albumin provides restoration from hemorrhagic shock that is comparable with that obtained using shed autologous blood.

90% Exchange Transfusion with HbV Suspended in Human Serum Albumin [58,103,104]

Objective. The effect of surface modification of HbV with poly(ethylene glycol) (Mw. 5 kDa) on hemodynamics and O_2 transport was studied by 90% exchange transfusion with the PEG-modified HbV and unmodified HbV suspended in 5% HSA in anesthetized Wistar rats.

Methods. Male Wistar rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Catheters (PE-20) were introduced into the right jugular vein for infusion (1 ml/min) and the right common carotid

artery for blood withdrawal (1 ml/min) and MAP measurements. MAP and heart rate were monitored through the arterial catheter. Arterial blood samples for gas analyses were also obtained from the arterial catheter. Abdominal aortic blood flow was measured by an ultrasonic pulsed Doppler flow meter as an indicator of cardiac output. The O_2 tension of blood withdrawn from the right atrium was measured as an indicator of mixed venous O_2 tension. These values were employed to calculate O_2 delivery and consumption. Renal cortical and skeletal muscle tissue O_2 tensions were monitored as indicators of tissue perfusion. Unmodified HbV/HSA, HSA alone, and washed rat RBC suspended in 5% HSA containing 10 g/dl of Hb (ratRBC/HSA) were employed as controls.

Measurements and Main Results. Both the PEG-modified HbV/HSA and unmodified HbV/HSA groups showed sustained MAP and blood gas parameters which were comparable with ratRBC/HSA group. Only the HSA group showed the significant decline in these parameters and resulting death within 30 min after completion of exchange. The blood flow in the abdominal aorta increased 1.5 times, and the total peripheral resistance decreased in the PEG-modified HbV/HSA-administered group in comparison with the unmodified HbV/HSA group. As for the blood gas parameters, the base excess and pH remained at higher levels in the PEG-modified HbV/HSA group, and the O_2 tension in mixed venous blood for the PEG-modified HbV/HSA group tended to be maintained at a higher level than that for the unmodified HbV/HSA group. Owing to the physicochemical properties, the PEG modification of HbV reduced the viscosity by the suppression of aggregation and resulted in prompt blood circulation in vivo.

Subcutaneous Microvascular Responses to 80% Exchange Transfusion with PEG-modified and Unmodified HbV [57]

Objective. The function of PEG-modified and unmodified HbV as a blood replacement was tested in the subcutaneous microvasculature of conscious hamsters during severe hemodilution in which 80% of the RBC mass (70 ml/kg) was substituted with suspensions of the vesicles in 5% HSA solution (Fig. 8).

Methods. Conscious male Syrian golden hamsters (60–70 g) with dorsal skinfold preparation were used. Blood withdrawal and sample infusions were simultaneously performed at a rate of 0.3 ml/min. At 30%, 60%, and 80% blood exchange levels, MAP, heart rate, blood gases, and microvascular parameters were measured.

Measurements and Main Results. Both materials yielded normal MAP, heart rate, and blood gas parameters at all levels of exchange, which could not be

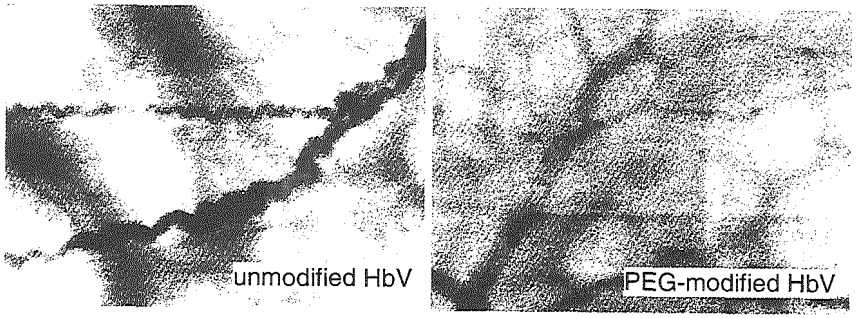

FIG. 8. Micrographs of microvasculature after 80% exchange transfusion with unmodified HbV or PEG-modified HbV suspended in 5% HSA. A high contrast was obtained by illumination with a wavelength range of around 420 nm, being absorbed at the Soret band of Hb molecules in HbV and RBC. Left, the aggregated vesicles block and chains in collecting venules were observed. Right, the microvasculature of postcapillaries is blackened owing to the homogeneous dispersion of PEG-modified HbV particles in the plasma phase. This is effective for better blood flow in the microcirculation

achieved with HSA 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. PEG reduced vesicular aggregation and viscosity, improving microvascular perfusion relative to the unmodified type. However, the microvascular perfusion with PEG-modified HbV/HSA was lower than the blood perfused one.

Improved Oxygenation in Ischemic Hamster Flap Tissue by Hemodilution with HbV [107]

Objective. The aim of this study was to test the influence of oxygen affinity of HbVs and level of blood exchange on the oxygenation in collateralized, ischemic, and hypoxic hamster flap tissue during normovolemic hemodilution.

Methods. Microhemodynamics were investigated with intravital microscopy. Tissue oxygen tension was measured with Clark-type microprobes. HbVs with a P_{50} of 15 Torr (HbV_{15}) and 30 Torr (HbV_{30}) were suspended in 6% Dextran 70 (Dx70). The Hb concentration of the solutions was 7.5 g/dl. A stepwise replacement of 15%, 30%, and 50% of total blood volume was performed, which resulted in a gradual decrease in total Hb concentration.

Measurements and Main Results. In the ischemic tissue, hemodilution led to an increase in microvascular blood flow to maximally 141%–166% of baseline in all groups (median; $P < 0.01$ vs. baseline, not significant between groups). Tissue oxygen tension was transiently raised to 121 ± 17% after the 30% blood exchange with Dx70 ($P < 0.05$), whereas it was increased after each step of hemodilution with HbV15-Dx70 and HbV30-Dx70, reaching 217 ± 67% ($P < 0.01$) and 164 ± 33% ($P < 0.01$ vs. baseline and other groups), respectively, after the 50% blood exchange. From these results it can be concluded that despite a decrease in total Hb concentration, the oxygenation in the ischemic, hypoxic tissue could be improved with increasing blood exchange with HbV solutions. Furthermore, better oxygenation was obtained with the left-shifted HbVs.

Safety of HbV (In Vitro and In Vivo Tests)

Rheological Property and Oxygen Releasing Behavior

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. It has been suggested that the higher viscosity and the resulting higher perfusion pressure would be beneficial to increase the shear stress on the vascular wall for vasorelaxation and to homogeneously transmit the pressure to microvascular networks and thus to supply blood to whole capillaries [108]. PEG-modified HbV suspended in 5% HSA solution was mixed with human blood and the viscosity was measured. The viscosity was similar to that of blood, and the mixtures at various mixing ratios showed a viscosity of 3–4 cP. RBC is the main component to determine blood viscosity and the results indicate no significant interaction between HbV and RBC [39]. To observe the flow pattern of the mixture of HbV and RBC, they were mixed in various volume ratios at [Hb] = 10 g/dl in isotonic saline containing 5% HSA, and the suspension was perfused at the centerline flow velocity of 1 mm/s through an O_2 permeable fluorinated ethylenepropylene copolymer tube (inner diameter, 28 μm) exposed to a deoxygenated environment [109]. The mixtures of acellular Hb solution and RBC were also tested. Since HbV was homogeneously dispersed in the HSA solution, increasing the volume of the HbV suspension resulted in a thicker marginal RBC-free layer (Fig. 9).

In the same experimental model, measurement of the O_2 release from the narrow tube was performed using a scanning-grating spectrophotometer with a photon count detector, and the rate of O_2 release was determined based on the visible absorption spectrum in the *Q* band of Hb [109]. Irrespective of the mixing ratio, the rate of O_2 release from the HbV-RBC mixtures was

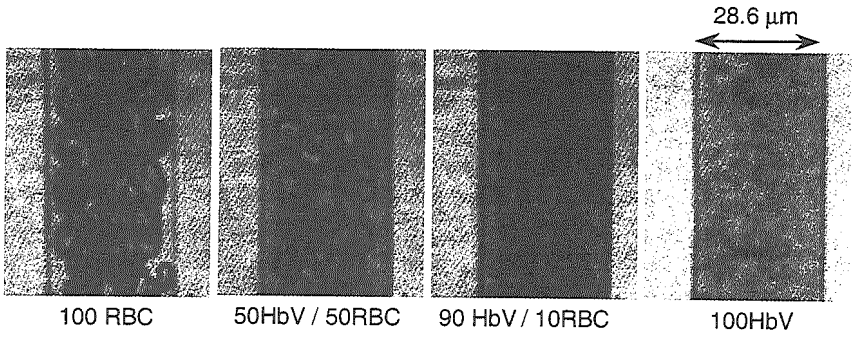

FIG. 9. Flow patterns of the mixture of HbV and RBC suspended in HSA in a narrow tube. HbV particles were homogeneously dispersed in a suspension medium. They tended to distribute in the marginal zone of the flow. The thickness of the RBC-free layer increased with the increasing amount of HbV. The RBC-free phase becomes darker and more semi-transparent, indicating the presence of HbV. Diameter of the tube = 28 μm; [Hb] = 10 g/dl; centerline flow velocity = 1 mm/s

similar with that from RBC alone. 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 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 [110]. However, this concept is controversial regarding the recent finding that an excess O_2 supply would cause autoregulatory vasoconstriction and microcirculatory disorders [111–113]. We confirmed that HbV does not induce vasoconstriction and hypertension, due to not only the reduced inactivation of nitric oxide as an endothelium-derived vasorelaxation factor, but also possibly the moderate O_2 releasing rate similar to RBC as confirmed in this study.

Effects on Hematological Functions

The biocompatibility of HbV is important to clinical use. Transient thrombocytopenia was one of the most significant hematological effects observed after infusion of liposome-encapsulated Hbs in rodents [114]. Exchange transfusion with unmodified HbV (containing DPPG as a lipid component) in anesthetized rats also resulted in a slightly decreased platelet count, although the change was insignificant [104]. These effects were also observed for administration of negatively charged liposomes [115,116]. The transient reduction in platelet counts caused by liposomes was also associated with

sequestration of platelets in the lung and liver. Platelet activation is necessary to prevent bleeding in vivo; however, nonphysiological activation leads to initiation and modulation of inflammatory responses because platelets contain an array of potent proinflammatory substance. RANTES (Regulated upon activation, normal T-cell expressed and presumably secreted), one of the C-C chemokines, is a useful marker for platelet activation as it is stored in α-granules of platelets and was shown to be released after stimulation. Accordingly, the biocompatibility of HbV was examined by estimating their effects on agonist-induced platelet aggregation response and RANTES release from platelets in vitro [117]. This study on biocompatibility was performed in collaboration with Dr. H. Ikeda at the Hokkaido Red Cross Blood Center (Sapporo), and his colleagues.

The effect of low concentration of HbV (Hb: 5.8 mg/dl) on platelet function was assessed by examining an agonist-induced aggregation response, and that of relatively high concentrations of HbV (Hb: 0.29, 1 and 2 g/dl) by measuring the release of RANTES from platelets, which is regarded as a marker of platelet activation. The pre-incubation of platelets with HbV at 5.8 mg/dl of Hb did not affect platelet aggregation induced by collagen, thrombin, and ristocetin. The pretreatment of platelet-rich plasma (PRP) with HbV at concentration up to 2 g/dl of Hb had no aberrant effects on the collagen-induced RANTES release. Furthermore, the collagen-induced release of RANTES from PRP was not affected by longer incubation with HbV at 2 g/dl of Hb. The basal levels of RANTES from PRP were unchanged in the presence of HbV. These results suggest that HbV, at the concentrations studied, have no aberrant effects on platelet functions in the presence of plasma.

The effect of HbV on the coagulation time (PT, APTT) was tested with human plasma. HbV was mixed with human plasma at the ratios of 20%, 40% and 60% v/v. The prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured automatically. The results were compared with saline and phosphate buffered saline. The PT value increased from 10 s to 15 s with increasing the mixing ratio; however, there was no significant difference between the groups. The APTT value increased from about 40 s to about 50 s with an increase of the mixing ratio; however, there were no significant differences between the groups. The delayed coagulation is due to the dilution of the blood components, and there is no significant effect on the blood coagulation system.

Polymorphonuclear neutrophils (PMNs) are essential cells in the host defense against a variety of infectious agents. Circulating PMNs require activation to migrate to inflammatory sites and then effectively kill pathogens. Previously in the field of drug delivery systems, sterically stabilized liposomes with PEG have been reported to reduce the chemotactic activity of human PMNs in response to zymosan and the bacterially derived peptide, *N*-formyl-

methionyl-leucyl-phenylalanine (fMLP) [118]. Therefore, the effects of the PEG-modified HbV on human PMNs in vitro were studied, focusing on the functional responses to fMLP as an agonist [119]. The pretreatment of PMNs with HbV up to a concentration of 56 mg/dl Hb did not affect the fMLP-triggered chemotactic activity. In parallel to these results, the fMLP-induced upregulation of CD11b (Mac-1) levels on HbV-pretreated PMNs was comparable to that of untreated cells. Furthermore, the pretreatment of PMNs with HbV even at 580 mg/dl Hb did not affect the gelatinase B [Matrix methalloproteinase-9 (MMP-9)] release, suggesting that the fMLP-induced release of secondary and tertiary granules was normal. In addition, the fMLP-triggered superoxide production of PMNs was unchanged by the pretreatment of HbV at 580 mg/dl Hb. Thus, these results suggest that HbV, at the concentrations studied, have no aberrant effects on the fMLP-triggered functions of human PMNs.

Hypertension and Vasoconstriction in Relation 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 [120]. However, vasoconstriction reduces blood flow, lowering functional capillary density, and therefore affecting tissue perfusion and oxygenation [113,121]. Nitric oxide (NO) scavenging by Hb due to intrinsic high affinity of NO to Hb is the mechanism presumed to cause vasoconstriction and hypertension [122,123]. This theory was validated indirectly using exteriorized rabbit aortic rings in organ baths, where constriction was observed following the addition of acellular Hb solutions as well as an NO synthase inhibitor [124,125]. Different modifications of the Hb molecule cause hypertension that is qualitatively and quantitatively different, and red blood cells (RBCs) and cellular HbV (liposome-encapsulated Hb) do not cause either vasoconstriction or hypertension [99,100,105]. Most evidence for the pressor response is obtained from measurements of systemic pressure, and direct evidence about the mechanism involved is scarce. In previous studies in conscious hamsters fitted with a dorsal skinfold, we found that small arteries of 130–160 μm diameter, termed resistance vessels, exhibit the greatest reactivity in hemorrhagic shock [126], playing a significant role in the regulation of blood flow. Constriction of these resistance vessels in this model was also directly correlated to the pressure response following administration of NO synthase inhibitor [127].

In collaboration with Prof. Intaglietta, 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 [128]. Conscious Syrian golden hamsters with dorsal skinfold preparation were used. After the top load infusion of Hb products (7 ml/kg) into arterial catheters inserted into the jugular vein, mean arterial pressure and heart rate were monitored through the 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), hydroxyethyl-starch-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 h after infusion. There was a simultaneous decrease in the diameter of the resistance vessels (79 ± 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 HSA was used as a 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 Hb molecules.

The liver is a major organ that detoxifies excess amounts of heme by the action of heme oxygenase (HO). HO decomposes protoheme IX to generate biliberdin-IXα and CO. Under normal conditions, the 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 [129]. 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. Dr. 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% [130]. 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 HO-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 vasoconstriction and