formation and denaturation. Some protein bands other than Hb disappeared on SDS-PAGE and IEF after the heat treatment. During pasteurization, all the other concomitant proteins are denatured and precipitated. As a result, we obtain an ultra-pure Hb solution. This high purity is essential for preventing membrane plugging during the subsequent ultrafiltration process to remove virus. The FDA requires two orthogonal steps of not only virus inactivation but also virus removal.

We tested the ultrafiltration of the HbCO solution to remove viruses with PLANOVA™-35N and -15N (P35N, P15N, Bemberg Microporous Membrane: BMM; Asahi Kasei Co.) (Naito *et al.*, 2002). The virus removal mechanism is by size exclusion through the capillary pores, and a depth filtration. The unit membrane, which has a network structure of capillaries and voids, is accumulated to form 150 layers. P35N and P15N have mean pore sizes of 35 nm and 15 nm, respectively. P35N is suitable for removing envelope-type viruses of which the size ranges from 40 nm to 100 nm, such as HIV, HCV etc., and P15N can be used to remove the non-envelope-type viruses with size of less than 40 nm, such as parvoviruses. The permeation flux and the permeated ratio of the HbCO solution ([Hb] = 5.6 g/dl) through P35N at 13°C were 36 (l/m^2 per hour) and almost 100 (per cent), respectively. Those through P15N were 15 (l/m^2 per hour) and 95 (per cent), respectively. Under the same conditions, a high removal efficiency of a bacteriophage, $\phi \times 174$, (>7.7 log) was confirmed. These results indicate that P15N is effective for the process of virus removal from Hb solution. We also confirmed the effectiveness of other virus removal ultrafiltration systems such as Viresolve (Millipore Co.).

The obtained purified HbCO solution can be very effectively concentrated to above 40 g/dl using an ultrafiltration process. After regulation of the electrolyte concentrations, this is supplied for the encapsulation procedure. The ligand of the resulting HbV, CO, is converted to O_2 by illuminating the liquid membrane of the HbV suspension with a visible light under flowing O_2 (Chung *et al.*, 1995).

Other groups have selected the way to preserve the well-organized but relatively unstable enzymatic systems originally present in RBCs, aiming at the prolonged stability of the ferrous state of Hb (Ogata *et al.*, 1997). The enzymatic system can partly be preserved with the compensation of insufficient virus removal or inactivation, but this cannot guarantee the utmost safety of the resulting HBOCs. One advantage of HbV is that any reagent can be co-encapsulated in the vesicles. It has been confirmed that co-encapsulation of the appropriate amount of a reductant (such as glutathione or homocysteine) and active oxygen scavengers (such as catalase) effectively retards the metHb formation (Takeoka *et al.*, 1997, 2002; Sakai *et al.*, 2000a; 2004d; Teramura *et al.*, 2003). However, our recent idea is that the metHb formation may not be a serious problem in the emergency situation because HbV will be infused to bridge to the blood transfusion in a clinical setting.

Encapsulation of concentrated Hb in HbV

The performance of Hb vesicles depends on the weight ratio of Hb to lipid ([Hb]/[lipids]), that is, the ability to carry more Hb with fewer vehicles made of lipids. This value is improved by lowering the number of bilayer membranes (lamellarity) of the vesicle and raising the concentration of Hb in the interior of the vesicle. We studied the optimal conditions for the Hb encapsulation using the extrusion method, and considered the behaviors of the Hb and lipid assemblies as a kind of polymer electrolyte (Takeoka *et al.*, 1993, 1994a, 1994b, 1996).

The maximum ([Hb]/[lipids]) ratio can be obtained at ~pH 7, which relates to the isoelectric point (pI) of Hb. The Hb molecule is negatively charged when the 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 enhances the Hb denaturation by too much interaction with the lipid bilayer membrane and metHb formation at a lower pH. Therefore, the physiological pH, 7.0–7.4, is 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 increasing the microviscosity (decreased lipid mobility). Multilamellar vesicles are converted to smaller vesicles with a smaller lamellarity during the extrusion procedure. When the membrane fluidity is high, deformation of the vesicles during extrusion occurs more easily even for multilamellar vesicles, resulting in larger lamellarity in the final vesicles. Therefore, the use of lipids with a higher phase transition temperature is preferred. However, these lipids make extrusion more difficult, because a higher shear rate (high

extrusion pressure) is required. Based on this reasoning, mixed lipids contain dipalmitoylphosphatidylcholine (DPPC) as the main component.

Based on the precise analysis of the characterization of the physicochemical properties of the components, the encapsulation efficiency of the Hb solution in a size-regulated phospholipid vesicle has been improved using an extrusion method (Sakai *et al.*, 1996; Sou *et al.*, 2003a). Mixed lipids (DPPC, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[mono-methoxy poly(ethylene glycol)$_{5000}$]) at a molar ratio of 5:5:1:0.033 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 ~500 nm and with a relatively narrow size distribution by freeze-thawing at a lipid concentration of 2 g/dl and a 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 size and size 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 ~30 times faster than that of the simple hydrated vesicles. During the extrusion process, the Hb solution was effectively encapsulated into the reconstructed vesicles with a diameter of 250 ± 20 nm, and the Hb/lipid ratio reached 1.7–1.8. This improvement of the Hb encapsulation procedure is a breakthrough for the scalability for commercialization.

REGULATION OF OXYGEN AFFINITY

The O_2 affinity of purified Hb (expressed as *P*50, the O_2 tension at which Hb is half-saturated with O_2) is about 14 mmHg, and Hb strongly binds O_2 and does not release O_2 at 40 mmHg (the partial pressure of mixed venous blood). Historically, it has been considered that the O_2 affinity should be regulated to a level similar to that of RBC, namely about 25–30 mmHg, 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 can be evaluated by the arteriovenous difference in the levels of O_2 saturation in accordance with an O_2 equilibrium curve. It has been supposed that decreasing the O_2 affinity (increasing *P*50) will result in an increase in the O_2 unloading, which is supported by the result that RBC with a high *P*50 shows an enhanced O_2 release for improved exercise capacity in a mouse model (Shirasawa *et al.*, 2003).

If this theory is correct, the *P*50 of Hb in HbV should be equivalent to that of human red blood cells, i.e., 28 mmHg, or higher. Pyridoxal 5′-phosphate (PLP) is co-encapsulated in HbV as an allosteric effector to regulate *P*50 (Sakai *et al.*, 2000b). 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). The bound PLP retards the dissociation of the ionic linkage between the β-chains of Hb during conversion of deoxy to oxyHb in the same manner as does 2,3-DPG. Thus the O_2 affinity of Hb decreases in the presence of PLP. The *P*50 of HbV can be regulated to 5–150 mmHg by co-encapsulating the appropriate amount of PLP or inositol hexaphosphate as an allosteric effector (Wang *et al.*, 1992). Equimolar PLP to Hb (PLP/Hb = 1/1 by mol) was co-encapsulated, and *P*50 was regulated to 18 mmHg. When the molar ratio PLP/Hb was 3/1, *P*50 was regulated to 32 mmHg. The O_2 affinities of HbV can be regulated quite easily without changing the other physical parameters, whereas in the case of the other modified Hb solutions their chemical structures determine their O_2 affinities and thus regulation is difficult. The appropriate O_2 affinities for O_2 carriers have not been yet completely decided; however, the easy regulation of the O_2 affinity may be useful in meeting the requirement for clinical indications such as oxygenation of ischemic tissues (Contaldo *et al.*, 2003).

STORAGE STABILITY

Since Hb autoxidizes to form metHb and loses its O_2-binding ability during storage as well as during blood circulation, the prevention of metHb formation is required. Some groups have reported a method of preserving the deoxygenated Hb in the liquid state (Kerwin *et al.*, 1999), using the well-known intrinsic characteristic of Hb that its oxidation rate in a solution is dependent on the O_2 partial pressure and deoxyHb is essentially not autoxidized at ambient temperature (Levy *et al.*, 1988). For the 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 the encapsulated Hb.

The surface modification of phospholipid vesicles with poly(ethylene glycol) (PEG)-conjugated lipid is a well-known method of prolonging the circulation time of the vesicles *in vivo* for drug delivery systems (Klibanov *et al.*, 1990). For HbV, the surface was also modified with PEG chains to improve the dispersion state of the vesicles when mixed with blood components (Yoshioka, 1991). The PEG-modified HbV has shown an improved blood circulation and tissue oxygenation due to the absence of HbV aggregate formation and viscosity elevation (Sakai *et al.*, 1997, 1998) and prolonged circulation persistence *in vivo* (Sou *et al.*, 2003b). However, little attention has been paid to the ability of the PEG modification for the long-term preservation of vesicles or liposomes in the liquid state. We studied the possibility of the long-term preservation of Hb vesicles by the combination of two technologies – surface modification of HbV with PEG chains, and deoxygenation during storage for 2 years (Sakai *et al.*, 2000c). The samples stored at 4° and 23°C showed a stable dispersion state for 2 years, though the sample stored at 40°C underwent precipitation and decomposition of the vesicular components, a decrease in pH, and 4 per cent leakage of the total Hb after 1 year. The PEG chains on the vesicular surface stabilize the dispersion state and prevent aggregation and fusion due to their steric hindrance. The original metHb content (~3 per cent) before the preservation gradually decreased to less than 1 per cent in all the samples after 1 month due to the presence of homocysteine inside the vesicles, which consumed the residual O_2 (thiol groups in homocysteines reacted with oxygen to generate disulfide and active oxygen species) and gradually reduced the trace amount of metHb. The rate of metHb formation was strongly dependent on the O_2 partial pressure, and no increase in the metHb formation was observed due to the intrinsic stability of the deoxygenated Hb. These results clearly indicate the possibility that the 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 O_2 carriers overcomes the limitation of the blood transfusion system and will contribute to benefiting clinical medicine.

ENDOTOXIN

The production process of HbV has to be guaranteed by a good manufacturing practice (GMP) standard as a biological product regarding the strict regulation of impurities and viral and bacterial contamination. Monitoring 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, is strictly required. The US FDA has established a guideline for the human maximal endotoxin dose permissible for parenteral products (5 EU/kg) that may include HBOCs. 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 (Levin and Bang, 1964). Since the volume of O_2 carriers to be infused for shock resuscitation or acute hemodilution is estimated to be less than 20 ml/kg, the specific endotoxin limits per ml should be 0.25 EU/ml (= 15/20), similar to that for water for injection (0.25 EU/ml).

Bacterial LPS is a gigantic amphiphilic macromolecule, therefore it interacts hydrophobically with protein and biomembranes. Hb strongly interacts with LPS, showing synergistic toxicity. The constituent of endotoxin that causes LAL gelation is a glycophospholipid – designated lipid-A. 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 (Richardson *et al.*, 1983). As a consequence, the researchers who have studied HbV or other phospholipid vesicles for delivering other functional molecules have encountered a problem in measuring the LPS content for the quality control of these materials (Cliff *et al.*, 1995; Harmon *et al.*, 1997).

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 subsequent LAL assay of the kinetic-turbidimetric gel clotting analysis using a Toxinometer® (Sakai *et al.*, 2004e). 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 HbV production system. 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 0.2 EU/ml at [Hb] = 10 g/dl. Moreover, the utilization of the histidine-immobilized agarose gel (Pyrosep®) 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. 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.

HEMOGLOBIN VESICLES AS OXYGEN CARRIERS *IN VIVO*

The advantages of HbV and other HBOCs are the absence of blood-type antigens and infectious viruses, and stability for long-term storage that outdoes the RBC transfusion. The shorter half-lives of the HBOCs in the bloodstream (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 during 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 extacorporeal membrane oxygenator (ECMO) (Yamazaki *et al.*, 2004); and (4) other potential indications, e.g. so-called O_2 therapeutics to oxygenate ischemic tissues.

One particle of HbV (diameter ~250 nm) contains about 30 000 Hb molecules. Since HbV acts as a particle in the blood and not as a solute, the colloid osmotic pressure of the HbV suspension is nearly zero. It requires addition of a plasma expander for a large substitution of blood to maintain blood volume. The candidates for plasma expanders are HSA, hydroxyethyl starch, dextran or gelatin, depending on the clinical setting, cost, country concerned and clinicians. Recombinant human serum albumin (rHSA) is the alternative. 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 immunological and hematological abnormalities, which are often seen when using dextran and hydroxyethyl starch. Aimed at the application of HbV suspended in a plasma expander for the above indications, HbV was tested in resuscitation from hemorrhagic shock (Sakai *et al.*, 2002b, 2004a; Yoshizu *et al.*, 2004) and extreme hemodilution (Izumi *et al.*, 1997; Kobayashi *et al.*, 1997; Sakai *et al.*, 1997, 1998, 1999) in collaboration with Waseda–Keio and Professor Marcos Intaglietta at UCSD. Moreover, HbV with a high O_2 affinity (low *P*50) suspended in HES or dextran was tested for oxygenation of an ischemic skin flap by Erni *et al.* at the Inselspital University Hospital, Berne (Contaldo *et al.*, 2003; Erni *et al.*, 2003) and the results imply the further application of HbV for other ischemic diseases such as myocardial and brain infarction, and stroke.

SUMMARY

Based on the above establishment of the HbV production system and the potential clinical applications of HbV, significant efforts have been made in the joint collaboration partnership of Waseda–Keio–Oxygenix–Nipro to produce HbV with a facility of GMP standard, and to start preclinical and, finally, clinical trials. Since the combination of recombinant Hb (rHb)-vesicles suspended in recombinant albumin (rHSA) would be the ideal 'artificial red blood cells', this project has recently initiated the next generation HbV (Kai *et al.*, 2004).

EDITOR'S SUMMARY

Liposome encapsulated hemoglobin is a long-sought goal in Japan, where the product is called hemoglobin vesicles (HbV), which distinguishes this product from the one developed primarily in the US, whose designation is LEH. HbV is the result of a long series of studies in which the size of the vesicles, including the number of lipid layers, the surface composition and materials co-encapsulated have been optimized.

HbV is produced by an extrusion process that has commercial potential, although at this time the product has not yet been produced in

quantities sufficient for clinical trials. Sterilization of the hemoglobin, prior to encapsulation, is performed using heat, and antioxidants are co-encapsulated to retard hemoglobin oxidation. Oxygen affinity is regulated to any desired *P*50 by co-encapsulation of allosteric effectors, and this group has contributed important studies on the effect of different *P*50 on oxygen delivery to tissues by HbV. The product is claimed to be stable when stored for up to 2 years.

A commercial effort has been launched in Japan, and it is hoped that HbV could be in human clinical trials within the next few years.

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Chapter 46

Albumin-Heme: A Synthetic Heme-Based Oxygen Carrier

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INTRODUCTION

The risk of transmission of viral illness by transfused blood has become extremely low and the transfusion of donor blood is currently a routine procedure. However, this level of safety has been achieved at great cost, and hepatitis virus or unknown pathogens cannot be completely excluded by the NAT system. Furthermore, the transfusion of donor blood requires cross-matching and compatibility tests to avoid a hemolytic reaction in the recipient, and the purified red blood cells (RBC) need to be stored in the refrigerator at 4°C. These requirements limit the availability of blood in a disaster or emergency. Against this background, several types of hemoglobin (Hb)-based oxygen carriers (HBOCs) have been studied as potential RBC substitutes or O_2 therapeutic reagents (Chang, 1997; Tsuchida, 1998; Winslow, 1999; Squires, 2002; Greenburg and Kim, 2004). Unfortunately, these materials do not fulfill all the requirements of blood replacement compositions. The first concern is the source of human Hb, which is limited by the availability of outdated human blood. Animal blood raises concerns about the transmission of animal pathogens, as Hb products potentially carry risks due to the biological origin of the raw materials. The second problem of the HBOCs (i.e., modified Hb) is the high colloid osmotic pressure (Keipert and Chang, 1988) and its vasoconstriction effect (Schultz *et al.*, 1993; Abassi *et al.*, 1997; Moisan *et al.*, 1998). About 50 per cent of the products in advanced clinical trials still increase blood pressure and decrease cardiac output (Squires, 2002). The precise mechanism of this hypertension is controversial, but many researchers suspect that the Hb molecules penetrate the vascular endothelium and capture the endothelial-derived relaxing factor (EDRF), namely NO. Others believe that the excessive delivery of oxygen to arteriolar vascular walls induces autoregulatory vasoconstriction (Guyton *et al.*, 1964; Tsai *et al.*, 1995; Rohlfs *et al.*, 1998; Winslow, 2000).

RATIONALE FOR ALBUMIN-HEME

In our circulatory system, free hemin (iron(III) complex of protoporphyrin IX dissociated from metHb) is captured by hemopexin, which is a unique protein having an extremely high binding constant of hemin ($K > 10^{12} M^{-1}$) (Tolosano and Altruda, 2002). Crystal structure analysis of the hemopexin–hemin complex has revealed that the hemin is tightly bound by double histidine

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coordinations to the central ferric ion and multiple hydrogen bondings with the amino acid residue (Paoli *et al.*, 1999). Nevertheless, the concentration of hemopexin in the plasma is rather low (<17 μM) and human serum albumin (HSA) may provide a reserve binding capacity of hemin in various conditions, for instance trauma, inflammation, hemolysis etc. In fact, HSA binds hemin with a relatively high affinity ($K > 10^8 M^{-1}$) (Adams and Berman, 1980). If HSA can transport O_2 like Hb, it would be of extreme medical importance not only as a blood replacement composition but also as an O_2 therapeutic reagent.

We have found that a series of super-structured heme derivatives with a covalently linked proximal-base were incorporated into HSA, and the obtained red-colored albumin–heme hybrids (Figure 46.1) can reversibly bind and release O_2 under physiological conditions in the same manner as Hb and myoglobin (Mb) (Komatsu *et al.*, 1999, 2000, 2001a, 2002; Tsuchida *et al.*, 1999; Nakagawa *et al.*, 2004). Since recombinant HSA (rHSA) is manufactured on a large scale by yeast expression, the rHSA–heme hybrid has become entirely synthetic hemoprotein and absolutely free of infectious pathogens. Our recent animal experiments demonstrated that rHSA-heme actually works as an oxygen-carrying plasma protein in the bloodstream (Tsuchida *et al.*, 2000; Komatsu *et al.*, 2004). Although the NO-binding affinity of rHSA-heme is higher than that of Hb (Komatsu *et al.*, 2001b), it does not induce an unfavorable vasopressor effect at all

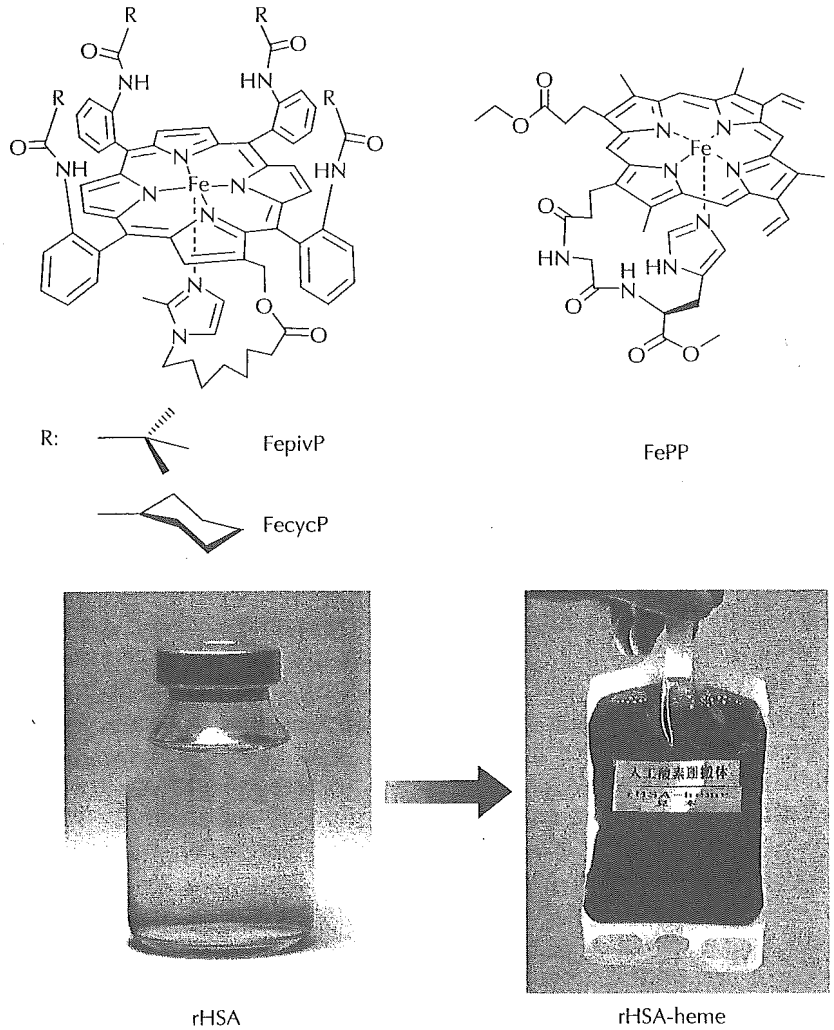

Figure 46.1 Super-structured heme derivatives for the albumin-heme hybrids and the red-colored rHSA-heme solution ([rHSA] = 5 g/dl). See color plate 23.

(Tuschida *et al.*, 2003). We suspect that the electrostatic repulsion between the albumin surface and glomerular basement membrane around the endothelial cell retards the rapid leakage of the rHSA-heme molecule and quick scavenging of NO. The albumin-heme is now recognized to be a promising material for a new class of RBC substitutes. In this chapter, we describe the O_2-transporting efficacy and preclinical safety of this synthetic heme-based O_2-carrier.

OXYGEN BINDING AND PHYSICOCHEMICAL CHARACTERISTICS

From the 30 super-structured heme compounds that were all synthesized by the authors, we found that oxygenated rHSA-FecycP showed a high stability against the autoxidation; its half-time against the ferric form *in vitro* (9 h at 37°C) was close to that of the native Mb (Komatsu *et al.*, 2002). We selected rHSA-FecycP with a similar *P*50 value (34 mmHg at 37°C) to that of RBCs as the most suitable material for an artificial O_2-carrier. The physicochemical characteristics and shelf-life of the rHSA-heme solution ([rHSA], 5 g/dl; heme/rHSA, 4 (mol/mol); isoelectric point, 4.8; COP, 18 mmHg; viscosity, 1.1–1.2 cPs; shelf-life greater than 2 years) had already been reported elsewhere (Komatsu *et al.*, 1999, 2002; Tsuchida *et al.*, 2002).

BLOOD COMPATIBILITY *IN VITRO*

The viscosity of the rHSA-heme solution (1.2 cPs at a high shear rate of 230 s^{-1}) was much lower than that of whole blood (4.0 cPs) and exhibited Newtonian type shear rate dependence just like rHSA itself. After mixing the rHSA-heme solution into whole blood at 10–44 per cent of the volume, the heme concentration in the plasma phase remained constant for 6 hours at 37°C, and no significant time dependence was observed in the numbers of RBCs, white blood cells and platelets (Huang *et al.*, 2003). The microscopic observations clearly showed that the shapes of the RBC had not been deformed during the measurement period. These results suggest that the rHSA-heme has no effect on the morphology of the blood cell components *in vitro*. With respect to the blood coagulation parameters (prothrombin time and activated partial thromboplastin time), the coexistence of rHSA-heme has only a negligibly small influence. Moreover, it was also shown that the rHSA-heme solution has no influence on the complement factors (CH50, SC5b-9) and platelet activation. Although more functional assay is necessary to establish firmly the biocompatibility of rHSA-heme with whole blood, we can conclude that it has a good compatibility with blood cells.

IN VIVO EFFECTS

Blood pressure effects

The administration of extracellular HBOCs often elicits an acute increase in blood pressure by vasoconstriction. At the beginning of this study, our concern was that the small rHSA-heme 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-heme strongly binds NO; the NO-binding affinity ($P50^{NO} = 1.8 \times 10^{-8}$ mmHg) is nine-fold higher than that of the Hb, and is high enough to react with 1 μM NO in the wall of the vasculator (Komatsu *et al.*, 2001b). 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-heme solution in anesthetized rats (Tsuchida *et al.*, 2003). Contrary to our expectations, only a negligibly small change in the mean arterial pressure (MAP) was observed after the administration of the rHSA-heme solution (5 g/dl, 300 mg/kg; Figure 46.2a). If anything, the difference from the baseline (ΔMAP) slowly decreased to −6.8 ± 3.4 mmHg within 20 minutes 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 set-up. In contrast, the administration of extracellular Hb solution elicited an acute increase in blood pressure (ΔMAP 16 ± 1.9 mmHg), which followed a graduated decrease throughout the 60-minute period of observation (Tsuchida *et al.*, 2003). Why does rHSA-heme not induce the hypertension? 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

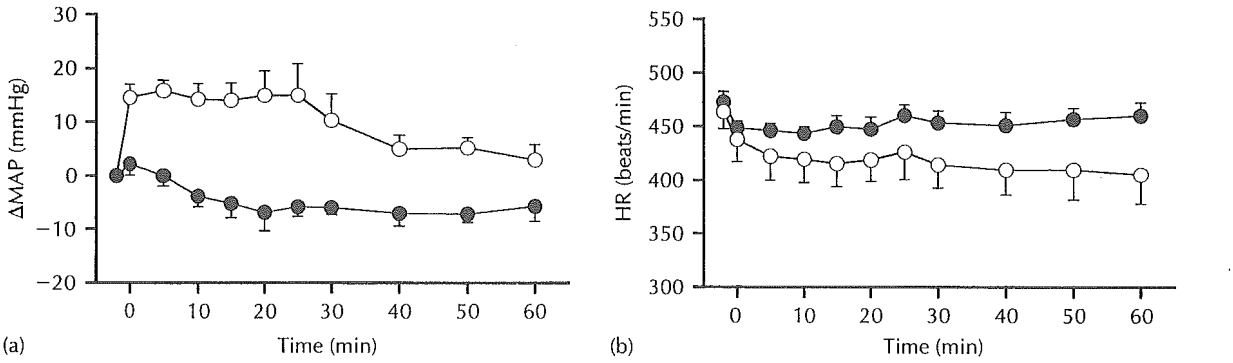

Figure 46.2 Changes of (a) MAP and (b) HR in anesthetized rats before and after infusion of rHSA-heme solution (n = 5) (●, rHSA-heme group; ○, Hb group). MAP is represented as change from the basal value (ΔMAP) just before the infusion with mean ± SEM (n = 5) (basal value is 90.1 ± 3.0 mmHg). HR was shown as mean ± SEM (n = 5) (Tsuchida, 2003).

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. In the blood vessels, rHSA-heme presumably circulates for a longer time compared to Hb without extravasation. The heart rate (HR) responses after the rHSA-heme injection were also negligibly small (Figure 46.2b). Visualization of the intestinal microcirculatory changes clearly showed that the widths of the venule and arteriole are fairly constant (Tsuchida *et al.*, 2003).

Hemodilution

The physiological responses to a 30 per cent exchange transfusion with rHSA-heme solution after 70 per cent hemodilution with 5 g/dl rHSA were investigated using anesthetized rats (Komatsu *et al.*, 2004). First, the isovolemic 70 per cent hemodilution was carried out using 5-g/dl rHSA solution. The blood withdrawal via the common carotid artery (2 ml) and the rHSA infusion from the femoral vein (2 ml) (each 1 ml/min) were repeated for nine cycles until Hct was reduced to 13.6 per cent (32 per cent of the initial Hct value of 42.6 per cent). After 10 minutes, a 30 per cent volume of the circulatory blood was withdrawn, producing a severe hemorrhagic shock state. The same volume of the samples was then intravenously injected. As negative or positive control groups, the rats were infused with the 5-g/dl rHSA solution (rHSA group) or the shed rat blood ([heme] = 5.3 mM, whole blood group). The circulation parameters, blood parameters, renal cortical PO_2 (PtO_2(R)) and muscle tissue PO_2 (PtO_2(M)) were carefully monitored for 60 minutes after the injection.

Following administration of the 5-g/dl rHSA solution, the MAP, HR, respiration rate, PtO_2(R), PtO_2(M), arterial blood O_2 pressure (PaO_2), venous blood O_2 pressure (PvO_2), and arterial blood CO_2-pressure ($PaCO_2$) did not recover, leading to death within 32 minutes (Figure 46.3). By contrast, the infusion of whole blood improved these values, except for PtO_2(M), to their initial levels. In the rHSA-heme group, the animals survived over 60 minutes after the infusion, and the HR, respiration rate, PtO_2(R) and PvO_2 showed similar recoveries to those as observed in the whole blood group (Komatsu *et al.*, 2004). MAP, PtO_2(M), PaO_2, pH and PCO_2 also significantly recovered. We are certain that the albumin-heme solution has the potential to resuscitate hemorrhagic shock, stabilize the blood circulation, and transport oxygen throughout the body.

PRECLINICAL SAFETY

In order to evaluate the preclinical safety of this synthetic O_2 carrier, we performed a 20 per cent exchange transfusion with rHSA-heme into anesthetized rats and measured the time courses of the circulation parameters (MAP, HR, respiration rate) and blood parameters (PaO_2, PvO_2, pH, blood cell numbers) for 6 hours, which is adequate time to identify acute toxicity (Huang *et al.*, 2004a). After stabilization of the animals' condition, the

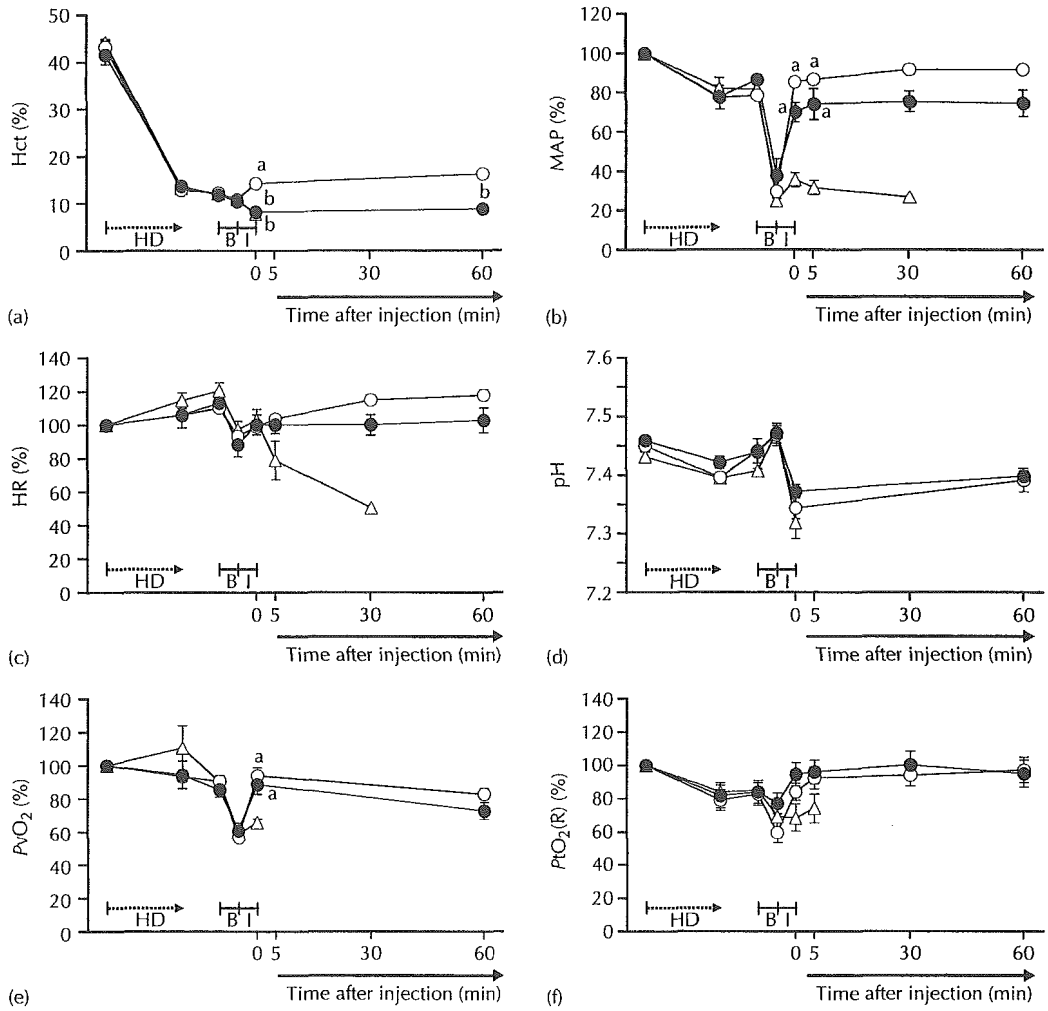

Figure 46.3 Time courses of (a) Hct, (b) MAP, (c) HR, (d) pH, (e) PvO_2 and (f) $PtO_2(R)$ in anesthetized rats after 70 per cent hemodilution with rHSA and 30 per cent exchange transfusion with rHSA-heme solution (n = 6) (●, rHSA-heme group; ○, whole blood group; △, rHSA group). MAP, HR, PvO_2 and $PtO_2(R)$ are represented as percentage ratios of the basal values with mean ± SEM. Hct, HR and pH were shown as mean ± SEM. HD, hemodilution; B, bleeding; I, sample injection. $^aP < 0.05$ versus rHSA group. $^bP < 0.05$ versus whole blood group (Komatsu, 2004).

20 per cent exchange transfusion was performed by 1 ml blood withdrawal via the common carotid artery and 1 ml rHSA-heme infusion from the femoral vein (each 1 ml/min) with four repeating cycles.

The appearance of all the animals showed absolutely no change for 6 hours after the exchange transfusion. The physiological responses of the blood circulation, gas equilibria and blood cell numbers in the rHSA-heme group were almost the same as those of the control group (surgery treatments without infusion) and rHSA groups (Figure 46.4; Huang *et al.*, 2004a). MAP and HR did remain constant after the injection of the rHSA-heme, suggesting again that the albumin-based O_2 carrier does not induce the vasoconstriction. It is also noteworthy that autoxidation of the ferrous rHSA-heme to the ferric state was retarded in the bloodstream; the half-time of the oxygenated rHSA-heme *in vivo* was approximately four-fold longer than that *in vitro* (Tsuchida *et al.*, 2000). It was found that autoxidated rHSA-hemin was certainly reduced in the whole blood suspension. A physiological concentration of ascorbic acid continuously provided by RBC probably re-reduces the ferric hemin, leading to the apparent long lifetime of the oxygenated species.

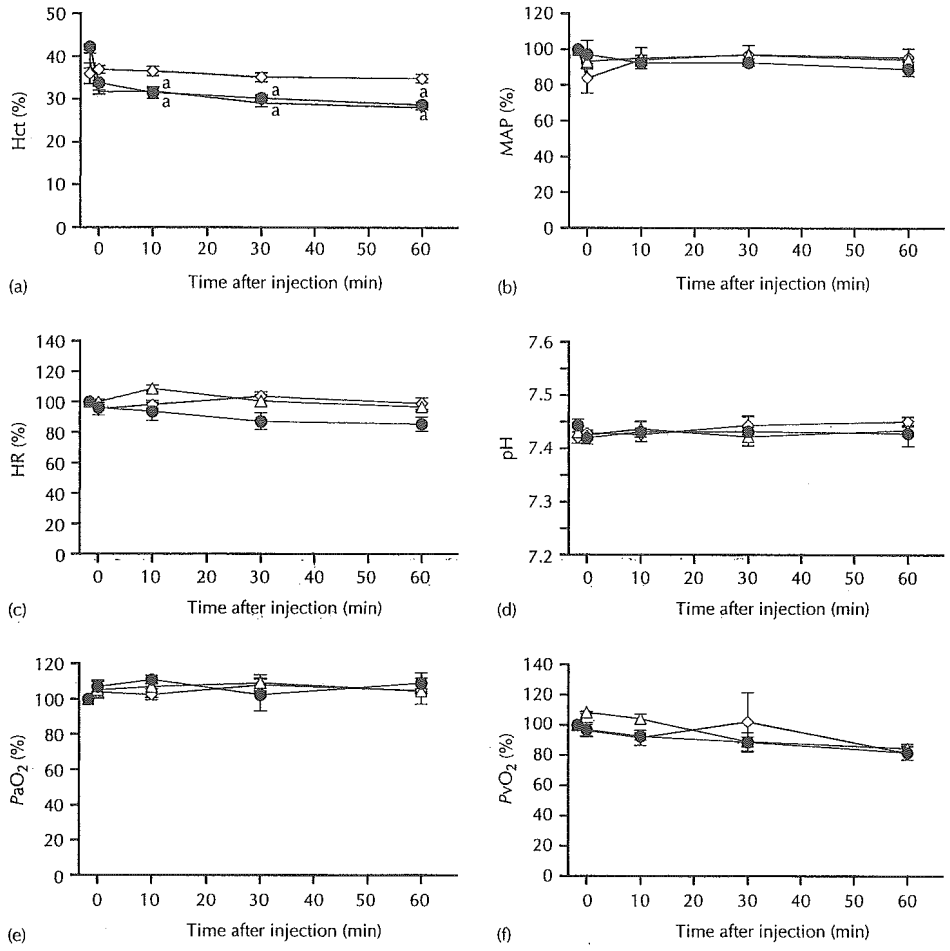

Figure 46.4 Time courses of (a) Hct, (b) MAP, (c) HR, (d) pH, (e) PaO_2 and (f) PvO_2 in anesthetized rats after 20 per cent exchange transfusion with rHSA-heme or rHSA solution (n = 6) (◇, control group (only surgery treatments without infusion); △, rHSA group; ■, rHSA-heme group). MAP, HR, PaO_2 and PvO_2 are represented as percentage ratios of the basal values with mean ± SEM. Hct, HR and pH are shown as mean ± SEM. (Huang, 2004a).

Furthermore, 20 per cent exchange transfusions with rHSA-heme into anesthetized rats were followed by blood biochemical tests of the withdrawn plasma and histopathology observations of the vital organs for 7 days (Huang *et al.*, 2004b).

In the albumin-heme group, a total of 30 analytes showed almost the same values, by blood biochemical tests, as those observed in the reference rHSA group, implying that there was no significant toxicity caused by the exchange transfusion with rHSA-heme (Huang *et al.*, 2004b). Histopathology observations implied that the administration of rHSA-heme did not produce any negative side effects on the vital organs. All these results showed the preclinical safety of the rHSA-heme solution.

FUTURE RESEARCH

As described in this chapter, results have shown O_2-transporting efficacy and initial clinical safety of the rHSA-heme solution that allow us to undergo further advanced preclinical testing of this synthetic O_2 carrying plasma protein. Exchange transfusion with rHSA-heme into beagles is now under investigation.

Furthermore, rHSA-heme as a monomolecular O_2 carrier was tested for its ability to increase O_2 tension in the hypoxia of the solid tumor rat model. By the direct administration of the rHSA-heme solution (10 ml/kg) into the ascites hepatoma LY80 tumor on the femur, the O_2 tension of the hypoxic region immediately increased

to 3.45 ± 1.43 mmHg, which corresponds to a 2.4-fold increase compared to that of the baseline value (Kobayashi *et al.*, 2003). These high O_2 levels continued for 300 s after the infusion. While more research is required to consider how rHSA-heme behaves in the tumor blood vessel and is related to the increase in the O_2 partial pressure, the present results obviously indicate that rHSA-heme led to increased O_2 release in the hypoxic region in the solid tumor. Experiments regarding combined treatment with the rHSA-heme administration and radiation therapy are currently underway.

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EDITOR'S SUMMARY

Albumin-heme is a unique product – an oxygen transporter that is totally synthetic. By this approach, human serum albumin is produced in a recombinant yeast system, and then synthetic heme is coordinated to its surface. Up to 8 heme groups per molecule have been incorporated so far.

Albumin-heme has been prepared to have a *P*50 similar to that of red blood cells, but the oxygen binding is not cooperative. It avidly binds NO, but is not hypertensive in preliminary animal tests. It appears to be as stable with regard to oxidation as native human hemoglobin. A number of early preclinical tests have been performed, including biocompatibility and effects on coagulation, and no significant toxic effects have been noted.

While it is still early in the development of albumin-heme as a therapeutic agent for use in humans, and the cost of production is likely to be high, it is an intriguing product that could find use in specialized applications such as oxygenating tumors to increase the effects of radio- or chemotherapy.

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Effects of Hemoglobin Vesicles on Resting and Agonist-Stimulated Human Platelets In Vitro

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Abstract: Hemoglobin vesicles (HbV) are artificial oxygen carriers that encapsulate a concentrated hemoglobin (Hb) solution with a phospholipid bilayer membrane. The oxygen transporting ability of HbV in vivo has been demonstrated by the transfusion of HbV into hemorrhagic shock rodent models. However, the compatibility of HbV with human blood cells must be evaluated. Preincubation of platelets with concentrations of 20% or 40% HbV had no effect on the binding of PAC-1, a monoclonal antibody that detects activation-dependent conformational changes in $\alpha_{IIb}\beta_3$ on platelets, or the surface expression of CD62P in whole blood. ADP-induced increases in PAC-1 binding were significantly enhanced by exposing the platelets to concentrations of either 20% or 40% HbV, whereas the ADP-induced increases in CD62P expression were not affected by HbV treatment at either concentration. Preincubation of platelet-rich plasma (PRP) with HbV minimally reduced the spontaneous release of TXB_2 and RANTES, but did not significantly affect the formation of TXB_2 or the release of RANTES and β-TG in platelets stimulated with ADP. Similarly, preincubation of PRP with HbV minimally reduced the spontaneous release of RANTES but did not significantly affect the formation of TXB_2 or the release of RANTES and β-TG in platelets stimulated with collagen, although collagen-induced serotonin release tended to decrease with HbV pretreatment. These data suggest that the exposure of human platelets to high concentrations of HbV (up to 40%) in vitro did not cause platelet activation and did not adversely affect the formation and secretion of prothrombotic substances or proinflammatory substances triggered by platelet agonists, although one of the earliest events in ADP-induced platelet activation was slightly potentiated by HbV pretreatment at the doses tested. Taken together,

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these results imply that HbV, at concentrations of up to 40%, do not have any aberrant interactions with either unstimulated or agonist-induced platelets.

INTRODUCTION

Vigorous efforts have been made to develop hemoglobin (Hb)-based oxygen carriers (HBOCs) for use as red blood cell substitutes [1], and some of these carriers are now in the final stages of clinical trials [2–4]. HBOCs offer several potential benefits for red blood cell transfusion applications, including the absence of blood-type antigens and infectious viruses and the ability to be stably stored for long time periods [5]. HBOCs can be categorized into two types: acellular modified Hb molecules and cellular liposome-encapsulated Hb, or Hb vesicles (HbV) [6]. Acellular modified Hb molecules are composed of intramolecularly cross-linked Hb, recombinant cross-linked Hb, polymerized Hb, or intramolecularly polymer-conjugated Hb. An acellular polymerized bovine Hb has already been used in clinical practice in South Africa.

Cellular HbV have a phospholipid vesicle structure and contain concentrated Hb molecules, similar to actual red blood cells [7–11]. Although HbV have not been clinically tested, the oxygen transporting abilities of HbV have been shown to be sufficient using a 40% exchange transfusion with HbV suspended in saline [8] and a 90% exchange transfusion with HbV in the presence of albumin as a plasma expander in rats [7]. Surface modification of HbV with poly(ethyleneglycol)-phospatidylethanolamine reduced the viscosity by suppressing inter-vesicular aggregation, allowing prompt blood circulation in vivo [9]. A sufficient O_2 transporting ability, comparable with that of blood, was also established in another model [11], and the prompt metabolism of HbV in the reticulo-endothelial system has been demonstrated [10].

The biocompatibility of HbV is an important factor for the clinical use of these materials. The administration of HbV could lead to interactions with blood cells, including platelets. Circulating platelets bind to the subendothelial matrix of injured vessels and subsequently become activated, resulting in the release or the expression of components in their intracellular granules and the formation of metabolic products. These products include prothrombotic substances (e.g., adenine nucleotides, thromboxane A_2 [TXA_2], serotonin, and CD62P) [12] and an array of potent proinflammatory chemokines (e.g., RANTES, MIP-1) [13]. Prothrombotic substances function as agonists for the recruitment of additional platelets into the evolving thrombus. Chemokines released from the activated platelets trigger the recruitment of leukocytes into the evolving thrombus and play a large role in the initiation and perpetuation of inflammatory responses. Platelet activation is apparently necessary to prevent bleeding in vivo; however, nonphysiological activation leads to pathological thrombosis and the

modulation of inflammatory responses. With this in mind, the biocompatibility of HbV and human platelets was evaluated by examining the effect of HbV on CD62P expression and the binding of activation–dependent $\alpha_{IIb}\beta_3$ antibody PAC-1 to platelets in the presence or absence of agonists in vitro; these two markers are the most frequently used markers of platelet activation. We also studied the effects of HbV on the secretion of other substances (i.e., serotonin, RANTES, and β-thromboglobulin [β-TG]) and the formation of thromboxane B_2 (TXB_2), a metabolite of TXA_2.

MATERIALS AND METHODS

HbV

HbV suspended in phosphate buffered saline were prepared as previously described [14]. The encapsulated carbonylhemoglobin contained pyridoxal 5′-phosphate (PLP) at a molar ratio of [Hb]/[PLP] = 1/2.5 as an allosteric effector and 5 mM of DL-homocysteine. The lipid bilayer was composed of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine, cholesterol, 1,5-dipalmitoyl-L-glutamate-*N*-succinic acid, and polyethyleneglycol-1, 2-distearyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[poly (ethylene glycol) (5,000)] at a molar ratio of 5:5:1:0.033. The Hb concentration of the HbV dispersion was adjusted to 10 g/dl. The HbV particle size was nearly 240 ± 60 nm in diameter.

Determination of CD62P and PAC-1 Expression by Flow Cytometry

The expression of CD62P and PAC-1 on platelets was measured as described previously, with slight modifications [15, 16]. Citrated whole blood was obtained from unselected healthy subjects. Whole blood (520 μl) was incubated with 480 μl of HbV or empty liposomes (at concentrations of 0%, 20%, or 40%) at 37°C for 60 minutes. After incubation, the reaction mixture was diluted to 1/5.4 with Hepes-Tyrode's buffer (KCl, 2 mM; NaCl, 127 mM; NaH_2PO_4, 0.5 mM; glucose, 5.6 mM; $NaHCO_3$, 12 mM; HEPES, 5 mM; 0.35% BSA; pH 7.3). Eighteen microliters of the diluted reaction mixture was added to 18 μl of a cocktail of FITC-conjugated PAC-1, PE-conjugated anti-CD62P and PerCP-conjugated anti-CD42a. FITC-conjugated anti-mouse IgM, PE-conjugated anti-mouse IgG, and PerCP-conjugated anti-mouse IgG were used as negative controls. All antibodies were purchased from BD bioscience-Pharmingen, San Jose, CA. The reaction mixture was then incubated with 4 μl of ADP (final concentration of 0, 0.05, 0.1, 0.5, 5, or 10 μM) for 20 minutes at room temperature in the dark. After incubation, the platelet suspension was fixed with 500 μl of paraformaldehyde (final concentration, 1%) and washed once with PBS. Finally, the platelets were resuspended in

500 μL of PBS. The samples were analyzed by flow cytometry (LSR, Becton-Dickinson, San Jose, CA). Fluorescence data from 10,000 platelet events were collected in logarithmic mode. The platelet population was identified by the number of CD42a-positive events.

Assay of Mediator Release

The platelet mediator release assay was carried out as described by Santos et al. [17], with slight modifications. Platelet-rich plasma (PRP) was obtained from citrated venous blood of unselected healthy subjects by centrifugation (140 g, 15 minutes, 22°C), and 600 μl of PRP (final concentration, 1.7×10^8/ml) was incubated with 400 μl of HbV (0%, 20%, or 40%) at 37°C for 60 minutes. After incubation, the mixture was divided into two 480 μl aliquots. For the collagen-induced platelet release reaction, the mixture was activated with 20 μl of collagen (final concentration, 1 μg/ml) (NYCOMED ARZNEIMITTEL BMBH, Germany) or buffer at 37°C for 5 minutes. For the ADP-induced platelet release reaction, the mixture was activated with 20 μl of ADP (final concentration, 2 μM) (SIGMA) or PBS at room temperature for 20 minutes. After incubation, the tube was centrifuged at 10,000 g for 1 minute. The cell-free supernatant was then transferred to another tube and centrifuged at 10,000 g for 30 minutes. The cell-free supernatant was stored at −20°C until the measurement of platelet release. Commercially available enzyme-linked immunosorbent assays (ELISAs) were used to measure the levels of RANTES (R&D Systems, Minneapolis, MN), serotonin (ICN Biomedicals Inc., Costa Mesa, CA) and TXB_2 (Cayman Chemical Company, Ann Arbor, MI) in duplicate experiments, according to the manufacturers' recommendations. Enzyme immunoassays were used to measure the levels of β-TG (Asserachrom β-TG, Roche Diagnostics, Tokyo, Japan).

Statistical Analysis

A two-way repeated measures ANOVA with Bonferroni correction was used for multiple comparisons of mediator levels and surface marker levels among different concentrations of HbV. A p value <0.05 was considered to indicate a significant difference.

RESULTS

Effect of HbV on the Binding of PAC-1 and the Expression of CD62P on Resting and ADP-stimulated Platelets In Vitro in Whole Blood

First, the effect of HbV on the binding of PAC-1 to platelets and the surface expression of CD62P on platelets with or without ADP stimulation

was examined in a whole blood environment in vitro. Without ADP stimulation, PAC-1 binding to platelets was discernible. Preincubation of whole blood with 20% or 40% HbV alone did not cause a significant difference in PAC-1 binding to the platelets. Stimulation of platelets with varying concentrations of ADP caused a gradual increase in the percentage of PAC-1 positive cells (Fig. 1A). Preincubation of whole blood with 20% or 40% HbV resulted in a slight, but significant, enhancement in the percentage of PAC-1 positive cells, compared to the results of comparable experiments without HbV, at ADP concentrations ranging from 0.05 μM to 5 μM (Fig. 1A).

Unstimulated platelets showed only a slight expression of CD62P, regardless of HbV treatment (Fig. 1B). The treatment of platelets with varying concentrations of ADP also led to gradual increases in the percentage of CD62P-positive cells, but preincubation of whole blood with 20% or 40% HbV did not affect the ADP-induced increase in the percentage of CD62P-positive cells (Fig. 1B).

Effect of HbV on Secretion of Platelet-derived Mediators in Resting and ADP-stimulated Platelets In Vitro

Next, the effect of HbV on the release of mediators from platelets stimulated with or without a submaximal dose of ADP, a weak platelet

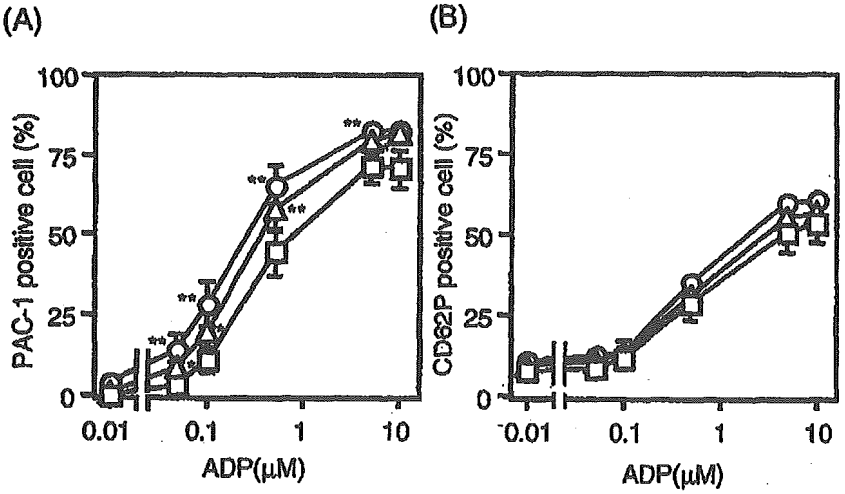

Figure 1. Effect of HbV on platelet surface activation markers. (A) PAC-1 binding to platelets and (B) CD62P expression on platelets. Whole blood was incubated with HbV at concentrations of 0% (square), 20% (triangle), or 40% (circle). Whole blood was then stimulated with or without various concentrations of ADP, as described in the Materials and Methods section. Values are the means ± SE of 4 experiments. *$p < 0.05$, **$p < 0.01$, compared with control (0% HbV).