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Chapter 44

Hemoglobin Vesicles as a Molecular Assembly: Characteristics of Preparation Process and Performances as Artificial Oxygen Carriers

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IMPORTANCE OF CELLULAR STRUCTURE

Physicochemical analysis has revealed that the cellular structure of RBCs may not be effective for the facilitated O_2 releasing and binding of Hb molecules in comparison with a homogeneous Hb solution (Vandegriff and Olson, 1984; Page *et al.*, 1998; Sakai *et al.*, 2003a); however, nature has selected this cellular structure during its evolution. Historically, Barcroft *et al.* insisted that the reasons for the Hb encapsulation in red blood cells were: (1) a decrease in the high viscosity of Hb and a high colloidal osmotic pressure; (2) prevention of the removal of hemoglobin from the blood circulation; and (3) preservation of the chemical environment in the cells such as the concentration of phosphates (2,3-DPG, ATP, etc.) and other electrolytes (Tsuchida *et al.*, 1995). Moreover, during the long history of the development of Hb-based O_2 carriers (HBOCs) many side effects of molecular Hb have become apparent, such as the dissociation of tetrameric Hb subunits into two dimers ($\alpha_2\beta_2 \rightarrow 2\alpha\beta$) that may induce renal toxicity, and entrapment of gaseous messenger molecules (NO and CO) inducing vasoconstriction, hypertension, reduced blood flow and tissue oxygenation at microcirculatory levels (Goda *et al.*, 1998; Sakai *et al.*, 2000a), neurological disturbances, and the malfunctioning of the esophageal motor function (Murray *et al.*, 1995). These side effects of molecular Hb would imply the importance of the cellular structure.

The pioneering work of the Hb encapsulation was performed by Chang (1991) using a polymer membrane. After Bangham and Horne (1964) had reported that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior, it was quite reasonable to use such vesicles for the Hb encapsulation. Djordjevici and Miller (1977) prepared a liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acid etc. The Naval Research Laboratory showed the remarkable progress of LEH (Rudolph

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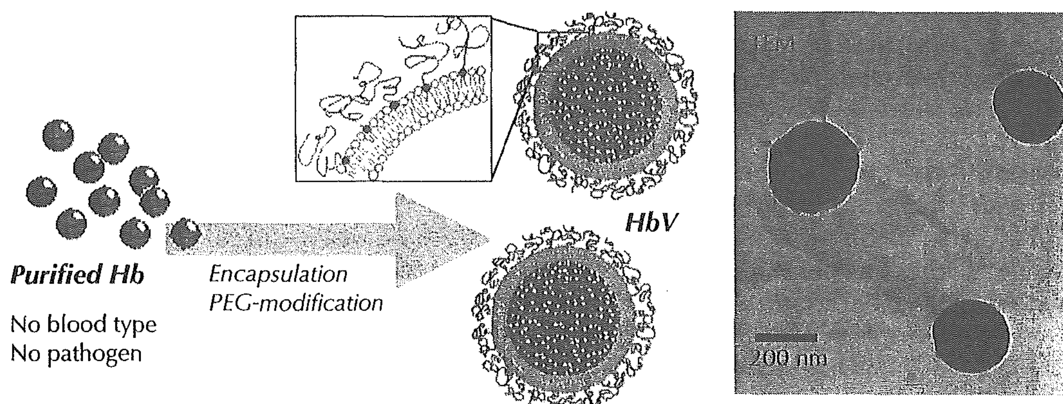

Figure 44.1 Hb vesicles (HbV, diameter *ca.* 250 nm) are prepared from ultra-pure Hb obtained from outdated RBC. One particle contains about 30 000 Hb molecules. The surface of one HbV is modified with about 6000 polymer chains of PEG that ensure the dispersion stability of HbV during storage and during circulation in the bloodstream. The transmission electron micrograph (TEM) clearly demonstrates the well-regulated particle size and high Hb content within the vesicles. See color plate 22.

et al., 1991). What we call Hb vesicles (HbV) with a high-efficiency production process and improved properties have been established by Tsuchida's group based on the nano-technologies of molecular assembly and precise analyses of the pharmacological and physiological aspects (Tsuchida, 1998; Figure 44.1). The *in vivo* studies of HbV have revealed O_2 transporting efficiency comparable to that of RBCs (Izumi *et al.*, 1996, 1997; Kobayashi *et al.*, 1997; Sakai *et al.*, 2004a; Yoshizu *et al.*, 2004), safety in terms of blood compatibility (Ito *et al.*, 2001; Wakamoto *et al.*, 2001), the importance of the particle size and the cellular structure of HbV (Goda *et al.*, 1998; Sakai *et al.*, 2000a), and prompt degradation in the reticuloendothelial system (Sakai *et al.*, 2001, 2004b, 2004c, 2004d), all of which make us confident about advancing to the further development of HbV. The joint collaborative partnership of academia (Waseda and Keio Universities), a biotechnology venture company (Oxygenix, Inc., Tokyo) and a corporation (Nipro Co., Osaka) is aiming for clinical trials of HbV within a few years.

In this chapter we scientifically summarize the characteristics of the preparation process of HbV based on the sciences of molecular assembly to induce their excellent performances. It should be emphasized that the components of HbV, lipids and Hb assemble to form a functional nanoparticle through secondary binding forces (hydrophobic interaction, Coulombic force, hydrogen bond, van der Waals force).

PREPARATION OF HEMOGLOBIN VESICLES

Virus inactivation and removal during hemoglobin purification

The primary advantage of artificial O_2 carriers should be no fear of infectious disease derived from human blood. In Japan, the donated blood is strictly inspected by the nucleic acid amplification test (NAT). However, it is necessary also to introduce procedures to inactivate and remove viruses during the process of Hb purification from outdated RBC in order to guarantee the utmost safety from infection, based on the unforgettable tragedy of HIV transmission due to the distribution of non-pasteurized plasma-derived products. In our purification process, virus inactivation was performed by pasteurization at 60°C for 10 hours – the same conditions for the pasteurization of human serum albumin (Sakai *et al.*, 1993; Fukutomi *et al.*, 2002). This process can be introduced by utilizing the stability of carbonylhemoglobin (HbCO). The thermograms of HbCO indicated a denaturation temperature of 78°C, which is much higher than that for oxyhemoglobin (64°C) (Sakai *et al.*, 2002a).

The virus inactivation efficiency was evaluated by the Hokkaido Red Cross Blood Center (Abe *et al.*, 2001; Huang *et al.*, 2002). The Hb solution spiked with vesicular stomatitis virus (VSV) was treated at 60°C for 1 hour under a CO atmosphere. VSV was inactivated at $> 6.0 \log_{10}$ without metHb

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-meth oxy 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 (= I5/20), 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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Albumin-Heme: A Synthetic Heme-Based Oxygen Carrier

Chapter 46

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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

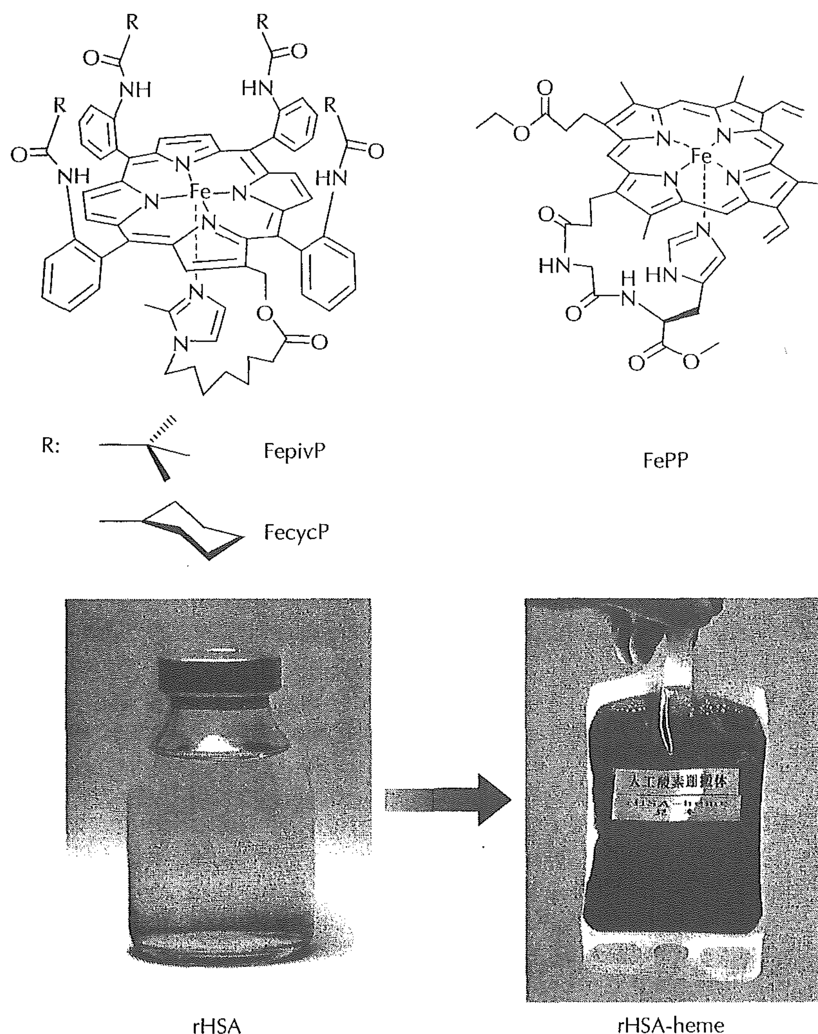

Figure 46.1 Super-structured heme derivatives for the albumin-heme hybrids and the red-colored rHSA-heme solution ([rHSA] = 5 g/dl). See color plate 23.

(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 $P50$ 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

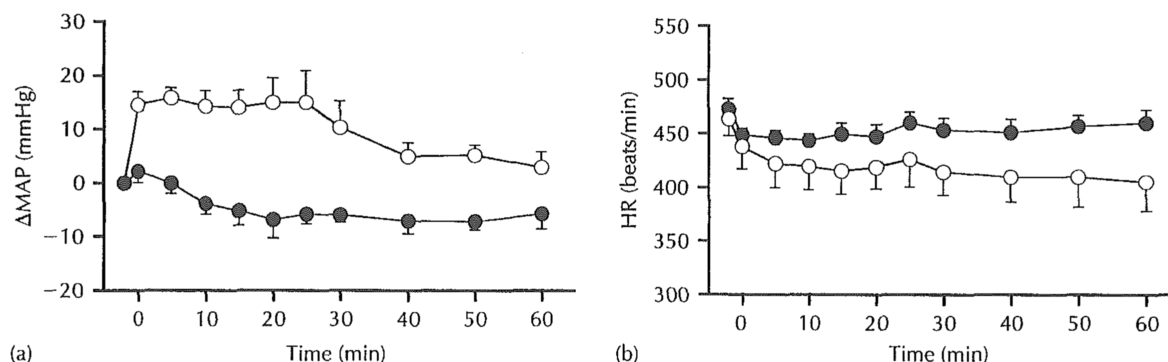

Figure 46.2 Changes of (a) MAP and (b) HR in anesthetized rats before and after infusion of rHSA-heme solution (n = 5) (●, rHSA-heme group; ○, Hb group). MAP is represented as change from the basal value (ΔMAP) just before the infusion with mean ± SEM (n = 5) (basal value is 90.1 ± 3.0 mmHg). HR was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

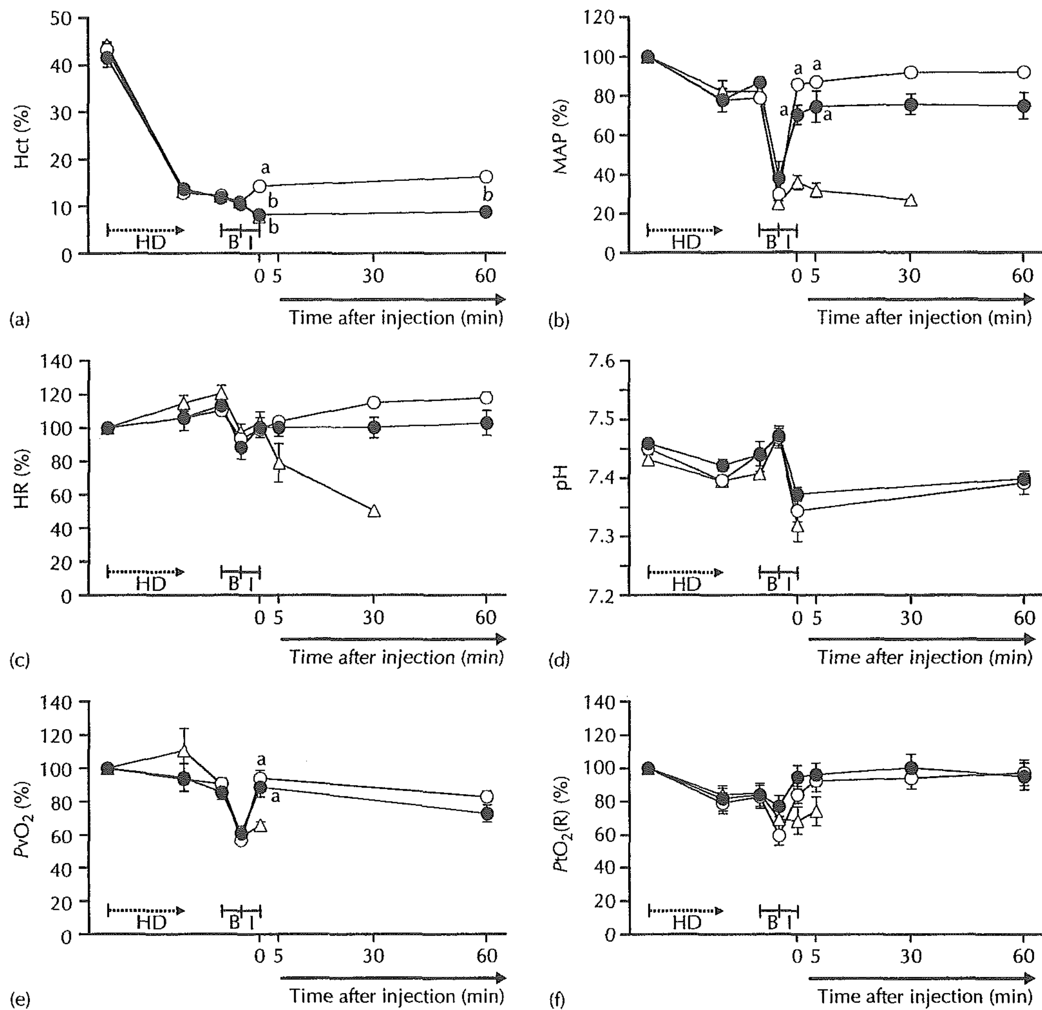

Figure 46.3 Time courses of (a) Hct, (b) MAP, (c) HR, (d) pH, (e) PvO_2 and (f) PtO_2(R) in anesthetized rats after 70 per cent hemodilution with rHSA and 30 per cent exchange transfusion with rHSA-heme solution (n = 6) (●, rHSA-heme group; ○, whole blood group; △, rHSA group). MAP, HR, PvO_2 and PtO_2(R) 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.

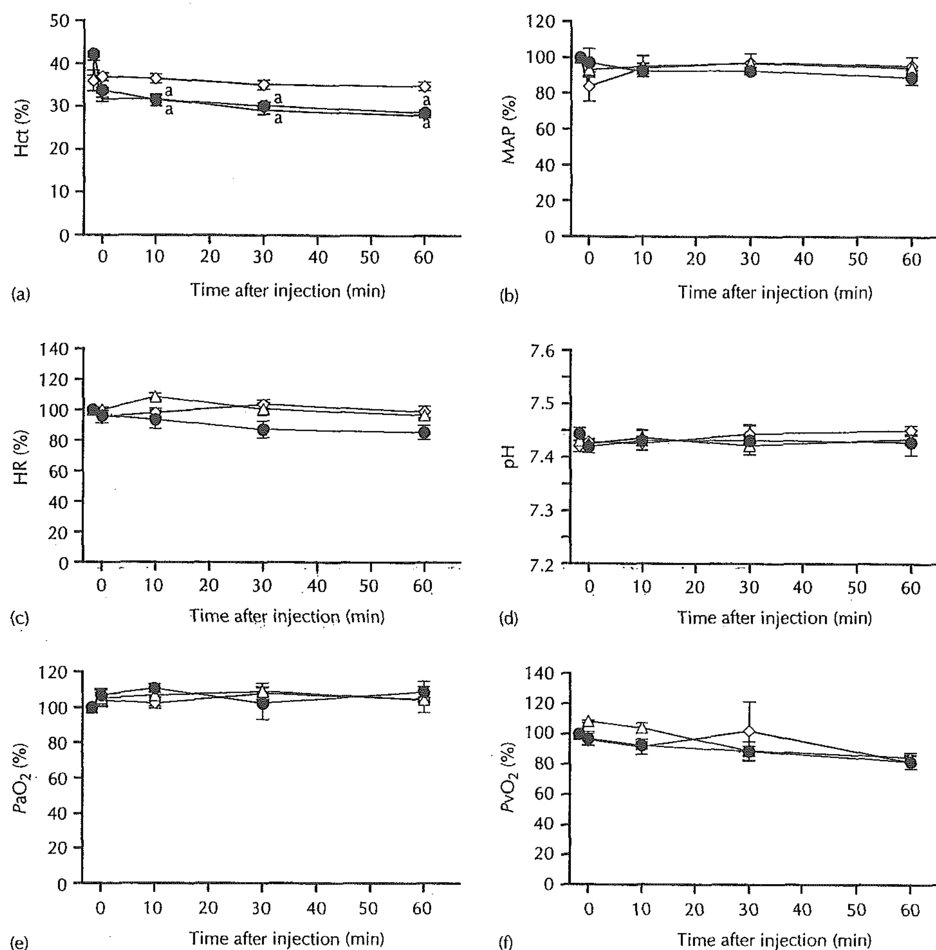

Figure 46.4 Time courses of (a) Hct, (b) MAP, (c) HR, (d) pH, (e) PaO_2 and (f) PvO_2 in anesthetized rats after 20 per cent exchange transfusion with rHSA-heme or rHSA solution (n = 6) (◇, control group (only surgery treatments without infusion); △, rHSA group; ■, rHSA-heme group). MAP, HR, PaO_2 and 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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CHAPTER 160

Blood Substitutes' Efficacy Microvascular and Rheological Determinants

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The development of a blood substitute, also called "artificial" blood, or more exactly an oxygen-carrying plasma expander (OCPE), is still a major goal of transfusion medicine, driven by blood shortages, problems associated with the transmission of disease by available blood, and the complex logistics of acquiring, analyzing, storing, distributing, and delivering the needed blood. To date blood is unequaled (or appears to be) in its capacity to restore circulatory volume; however, it is often remarked that if blood were proposed today as an oxygen-carrying volume restoration fluid, it would not be approved by regulatory agencies.

Blood is needed in the presence of blood losses; however, the initial anemia is usually inconsequential to the organism's survival, whereas the associated hypovolemia is tolerated only within a narrow margin. As a result, a blood substitute should ideally target both events, possibly sequentially. Therefore, the development of an effective blood substitute is also in part related to availability and detailed understanding of an effective plasma expander, which is the term used to describe a volume restoration fluid used prior to reaching the transfusion trigger, or the point in volume restitution at which the introduction of an oxygen carrier, blood, is determined to be essential.

Blood exerts its principal functions in the microcirculation, the locale of exchange of the materials that it transports. Thus any attempt to introduce a blood surrogate must ensure efficacy at this level. In contrast, most efforts aimed at developing artificial blood were made in the absence of detailed information and analysis of how oxygen is managed at the level of the microcirculation. The cornerstones in the development of artificial blood up to the present are that this fluid should restore most of the oxygen-carrying capacity of the shed blood, that it is beneficial for the resulting mixture of remaining blood and resuscitation fluid (e.g., OCPE) to have a viscosity lower than that of natural blood, and that the material should have very low oxygen affinity so that oxygen would be readily released when blood arrives to the microcirculation.

These principles have guided the development of products that are now undergoing clinical trials. It is apparent that the initial impetus for the development of artificial blood was based on the restoration of systemic functions after acute blood losses with little or no emphasis on understanding the behavior of the resuscitation fluids in the microcirculation, which is the organ system where blood performs its functions. This was in part due to the imperfect understanding of how oxygen is managed in the microcirculation in both normal and pathophysiological conditions, the role and regulation of shear stress–dependant mediators produced by the endothelium, and the lack of techniques for measuring the key transport parameters that determine efficacy in maintaining microvascular function upon the introduction of a blood surrogate.

Oxygen-carrying capacity and oncotic pressure were prescribed to be similar to that of blood, while the experience with hemodilution suggested that improvements in transport would be obtained by lowering blood viscosity to values significantly below those of whole blood. An additional

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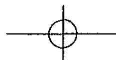

presumed beneficial modification was the use of oxygen carriers based on modified hemoglobins with high p50s, presumed to facilitate oxygen unloading and tissue oxygenation. As acellular modified molecular hemoglobin became the oxygen carrier of choice, it was found this material was vasoactive, causing hypertension, which is deleterious in resuscitation. Vasoactivity was attributed to hemoglobin scavenging NO, leading to vasoconstriction, which gave rise to a significant effort aimed at modifying the hemoglobin molecule so that its affinity for NO was reduced.

At present an optimal OCPE is still perceived to have the following properties: oxygen-carrying capacity equivalent to 10 to 14 g/dL hemoglobin, p50 greater than 30 mmHg, viscosity 1 cP, oncotic pressure approximately 25 mmHg, and low NO binding. However, studies in the microcirculation show that a fluid configured according these concepts yields problematic outcomes in terms of resuscitation from anemic hypovolemia. Furthermore, since the most favored source of hemoglobin is human, even if it were possible to obtain a one to one conversion from blood to blood substitute, the problem of blood shortages is not solved. In an effort to circumvent the human hemoglobin source there have been various attempts to obtain hemoglobin by recombinant technology. Biopure Inc. (Boston, MA) has progressed to Phase III clinical trials with a molecular hemoglobin-based fluid derived from bovine blood. However, recombinant technology has not progressed to the development of an efficacious product to date, and the bovine-based product was not developed on the basis of microcirculatory data.

The Design of an Efficacious Oxygen-Carrying Plasma Expander

To date there are virtually no rivals to hemoglobin as a transporter of oxygen from the lung to the tissue because of its ability to bind a large amount of oxygen through a chemical reaction. The discovery that fluorocarbons could dissolve a comparatively large amount of oxygen, albeit at high oxygen partial pressures, suggested using this vehicle as the oxygen transporter. However, the use of fluorocarbon-based blood replacement fluids has not materialized, in part because of the lack of definitive experimental studies on the physiology related to altered blood physical properties and changes in the distribution of oxygen partial pressure in the circulation.

Various modifications of hemoglobin have optimized its performance and mostly eliminated the vasoactivity of this molecular species. Human hemoglobin remains the most favored source because of the well-defined methodology for obtaining blood from donors, which in advanced medical systems is virtually free of parasitic, bacterial, or viral contamination. The present formulations of bovine hemoglobin appear to be vasoactive and in the long term could present unknown risks of introducing diseases that may have extraordinarily long incubation times.

If the perceived and now frequently reported blood shortage is the driving force behind the development of hemoglobin-based oxygen-carrying blood substitutes, then the use of human hemoglobin is problematic since the processing technology and formulation would require that a unit of original blood yield at least an equivalent unit of "artificial blood," a zero-sum result that does not relieve shortages. A realistic process should produce several units of equivalent hemoglobin-based oxygen-carrying blood substitute from a unit of natural blood.

The present goal in devising a human hemoglobin-based blood substitute is to circumvent the inherent toxicity of the hemoglobin molecule and to be as efficacious as an equivalent unit of natural blood but at a lower hemoglobin concentration than blood, thus introducing a multiplying factor between the original source of human hemoglobin and the final product. Advances in microvascular technology allow us to critically analyze each of the "cornerstone" precepts that have guided the development of blood substitutes to date, namely the viscosity of the material, the affinity for oxygen, the effective concentration, and the resulting colloidal osmotic pressure when the material is present in the circulation. In the following these parameters will be analyzed from a microcirculatory perspective.

The Role of Viscosity in Oxygen Transport

Blood viscosity depends on red blood cell concentration (hematocrit) and on plasma viscosity. The manipulation of these two viscosities is the basis of the clinical practice of hemodilution. Accordingly, the restitution of blood losses with conventional plasma expanders can be effectively and safely accomplished up to a 50 percent loss of the red blood cell mass, using fluids with plasma-like viscosity. The decrease in viscosity due to hemodilution causes a compensatory increase in cardiac output due to the lowered flow resistance, thus maintaining oxygen delivery.

A specific decrease in oxygen-carrying capacity is one of the parameters that defines the so-called transfusion trigger. Microcirculatory experimental studies do not support the contention that lack of oxygen-carrying capacity is the actual determining factor in the decision of transfusing blood. In the awake hamster window chamber model, neither oxygen-carrying capacity nor tissue oxygenation is in jeopardy with red blood cell losses of two-thirds of the original mass (Figure 1).

A factor that is significantly changed upon reaching the transfusion trigger is blood viscosity, which is approximately half of normal, because of the loss of red blood cells. Thus, additional losses of red blood cells will further reduce blood viscosity, which is strongly dependent on hematocrit. The reduction of blood viscosity is initially compensated by increased cardiac output. However, cardiac output seldom doubles, and blood viscosity has the potential of decreasing