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FIGURE LEGENDS

FIGURE 1: Hb-vesicles (HbV; diameter, ca. 250 nm) are prepared from ultra-pure Hb obtained from outdated RBC.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 6,000 polymer chains of poly(ethyleneglycol) that ensure the dispersion stability of HbV during storage and during circulation in the blood stream. The transmission electron micrograph (TEM) clearly demonstrates the well-regulated particle size and high Hb content within the vesicles.

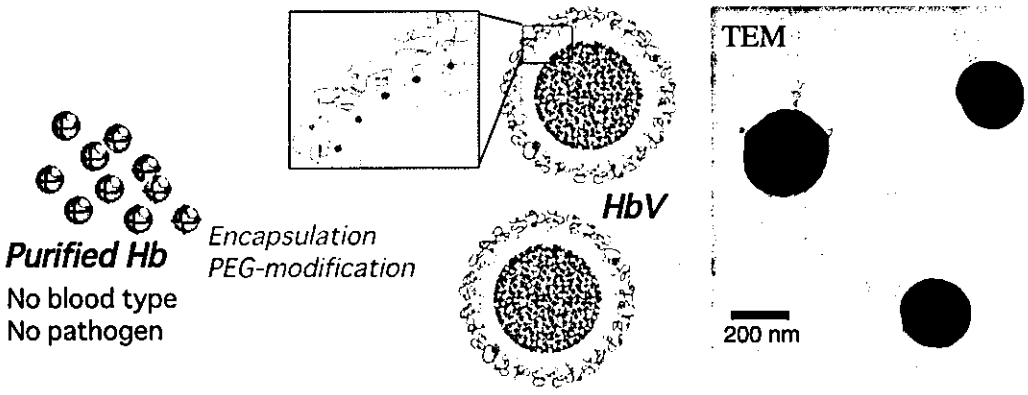

Figure 1
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Albumin-Heme: A Synthetic Heme-Based Oxygen Carrier

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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, (i) the transfusion of donor blood requires cross-matching and compatibility tests to avoid a hemolytic reaction in the recipient, and (ii) the purified red blood cells (RBC) should be stored in the refrigerator at 4°C. These requirements limit the availability of blood in a disaster or emergency. Under this background, several types of hemoglobin (Hb)-based O_2-carriers have been studied as a RBC substitute or O_2 therapeutic reagent (Chang, 1997; Greenburg, 2004; Squires, 2002; Tsuchida, 1998, Winslow, 1999). 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 The first concern is the source of human Hb, which is limited by the availability of outdated human blood.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 will raise the anxiety of the transmission of animal pathogens. The Hb products potentially carry risks due to the biological origin of the raw materials. The second problem of the Hb-based O_2-carriers (i.e. modified Hb) are the high colloid osmotic pressure (Keipert, 1988) and its vasoconstriction effect (Abassi, 1997; Moisan, 1998; Schultz,1993). About 50% 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, 1964; Rohlfs, 1998; Tsai, 1995; Winslow, 2000).

On the other hand, 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, 2002). Crystal structure analysis of the hemopexin-hemin complex revealed that the hemin is tightly bound by double histidine coordinations to the central ferric ion and multiple hydrogen-bondings with the amino acid residue (Paoli, 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, 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 1) can reversibly bind and release O_2 under physiological conditions in the same manner as Hb and myoglobin (Mb) (Komatsu, 1999, 2000, 2001a, 2002; Nakagawa, 2004; Tsuchida, 1999). 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 blood stream (Komatsu 2004; Tsuchida, 2000). Although the NO-binding affinity of rHSA-heme is higher that that of Hb (Komatsu, 2001b), it does not induce unfavorable vasopressor effect at all (Tuschida, 2003). We suspect that the electrostatic repulsion between the albumin surface and glomelular basement membrane around the endotherial cell retards the rapid leakage of the rHSA-heme molecule and quick scavenging of NO. The albumin-heme is now recognized to be one of the promising materials as a new class of RBC substitute. In this chapter, we describe the O_2-transporting efficacy and preclinical safety of this synthetic heme-based O_2-carrier.

Figure 1

O_2-Binding property and physicochemical characteristics

From the thirty super-structured heme compounds, which were all synthesized by the authors, we found that the oxygenated rHSA-FecycP showed a high stability against the autooxidation; the half-lifetime against the ferric form in vitro (9 hrs at 37°C) was close to that of the native Mb (Komatsu, 2002). We have selected rHSA-FecycP with a similar p_{50} value (34 Torr at 37 °C) to RBC 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 cP, shelf-life: over 2 years) were already reported elsewhere (Komatsu, 1999, 2002; Tsuchida, 2002)

Blood compatibility *in vitro*

The viscosity of the rHSA-heme solution (1.2 cP at a high shear rate of 230 s^{-1}) was much lower than that of whole blood (4.0 cP) and exhibited Newtonian type shear rate dependence just like rHSA itself. After the mixing of the rHSA-heme solution into whole blood at 10~44 % of the volume, the heme concentration in the plasma phase remained constant for 6 hrs at 37 °C, and no significant time dependence was observed in the numbers of RBC, white blood cells, and platelets (PLT) (Huang, 2003). The microscopic observations clearly showed that the shapes of the RBC have not been deformed during the measurement period. These results suggested 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 had only a negligibly small influence. Moreover, it was also shown that the rHSA-heme solution has no influence to

the complement factors (CH50, SC5b-9) and the PLT activation. Although more functional assay is necessary to firming establish the biocompatibility of rHSA-heme with whole blood, we can conclude that it has a good compatibility with blood cells.

Change of blood pressure after the administration

The administration of extracellular Hb-based O_2-carriers 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 circulations, and contributes 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 ($p_{50}^{NO} = 1.8 \times 10^{-8}$ Torr) is 9-fold higher compared to the Hb's and enough to react 1 μM NO in the wall of the vasculator (Komatsu, 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, 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 2(a)]. If anything, the difference from the baseline (ΔMAP) slowly decreased to -6.8 ± 3.4 mmHg within 20 min and remained constant during the monitoring period. The response is completely the same as observed following infusion with an equivalent volume of rHSA (5 g/dL) in this experimental setup. In contrast, the administration of extracellular Hb solution elicited an acute increase in blood pressure (ΔMAP: 16 ± 1.9 mmHg), followed a graduated decrease throughout the 60 min period of observation (Tsuchida, 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 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 2(b)]. Visualization of the intestinal microcirculatory changes clearly showed that the widths of the venule and arteriole are fairly constant (Tsuchida, 2003).

Figure 2

Exchange transfusion into acute anemia rat model after 70% hemodilution

The physiological responses to a 30% exchange transfusion with rHSA-heme solution after 70% hemodilution with 5 g/dL rHSA were investigated using anesthetized rats (Komatsu, 2004). First, the isovolemic 70% 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% (32% of the initial Hct value: 42.6%). After 10 min, a 30% volume of the circulatory blood was withdrawn, producing 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 min after the injection.

By 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-pressuren ($paCO_2$) did not recover, leading to death within 32 min (Figure 3). In contrast, the infusion of the whole blood improved these values to their initial levels except for ptO_2(M). In the rHSA-heme group, the animals survived

over 60 min after the infusion, and the HR, respiration rate, ptO_2(R), and pvO_2 showed similar recoveries as observed in the whole blood group (Komatsu, 2004). MAP, ptO_2(M), paO_2, pH, and pCO_2 also significantly returned. We are certain that the albumin-heme solution has the potential to resuscitate the hemorrhagic shock, stabilize the blood circulation, and transport oxygen throughout the body.

Figure 3

Preclinical safety

In order to evaluate the preclinical safety of this synthetic O_2-carrier, we performed a 20% 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 hrs, which is adequate time to know an acute toxicity (Huang, 2004a). After stabilization of the animal condition, the 20% 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 the all animals showed absolutely no change for 6 hrs 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 (only surgery treatments without infusion) and rHSA groups (Figure 4) (Huang, 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 the autooxidation of the ferrous rHSA-heme to ferric state was retarded in the blood stream; the half-lifetime of the oxygenated rHSA-heme in vivo was ca. 4-fold longer than that in vitro (Tsuchida, 2000). It has been found that autooxidated rHSA-hemin was certainly reduced in the whole blood

suspension. A physiological concentration of ascorbic acid continuously provided by RBC probably rereduces the ferric hemin, leading to the apparent long lifetime of the oxygenated species.

Figure 4

Furthermore, 20% 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, 2004b).

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

Future researches

As described in this chapter, the results showed the O_2-transporting efficacy and initial clinical safety of the rHSA-heme solution, which allows 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 Torr, which corresponds to a 2.4-fold increase compared to that of the baseline value (Kobayashi, 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 an increased O_2-release in the hypoxic region in the solid tumor. Experiments of a combined treatment with the rHSA-heme administration and radiation therapy are currently underway.

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Figure Legends

Figure 1 Super-structured heme derivatives for the albumin-heme hybrids and the red-colored rHSA-heme solution ([rHSA]= 5 g/dL).

Figure 2 Changes of (a) MAP and (b) HR in anesthetized rats before and after infusion of rHSA-heme solution (n=5) (●; rHSA-heme group and ○; Hb group). MAP is represented as change from the basal value (ΔMAP) just before the infusion with mean ± S.E.M. (n=5) (basal value is 90.1 ± 3.0 mmHg). HR was shown as mean ± S.E.M. (n=5). (Ref. Tsuchida, 2003)

Figure 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% hemodilution with rHSA and 30% 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 percent ratios of the basal values with mean ± S.E.M.. Hct, HR and pH were shown as mean ± S.E.M.. HD: hemodilution, B: bleeding, I: sample injection. $^{a}p<0.05$ vs. rHSA group. $^{b}p<0.05$ vs. whole blood group. (Ref. Komatsu, 2004)

Figure 4 Time courses of (a) Hct, (b) MAP, (c) HR, (d) pH, (e) paO_2 and (f) pvO_2 in anesthetized rats after 20% 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 percent ratios of the basal values with mean ± S.E.M.. Hct, HR and pH were shown as mean ± S.E.M.. (Ref. Huang, 2004a)

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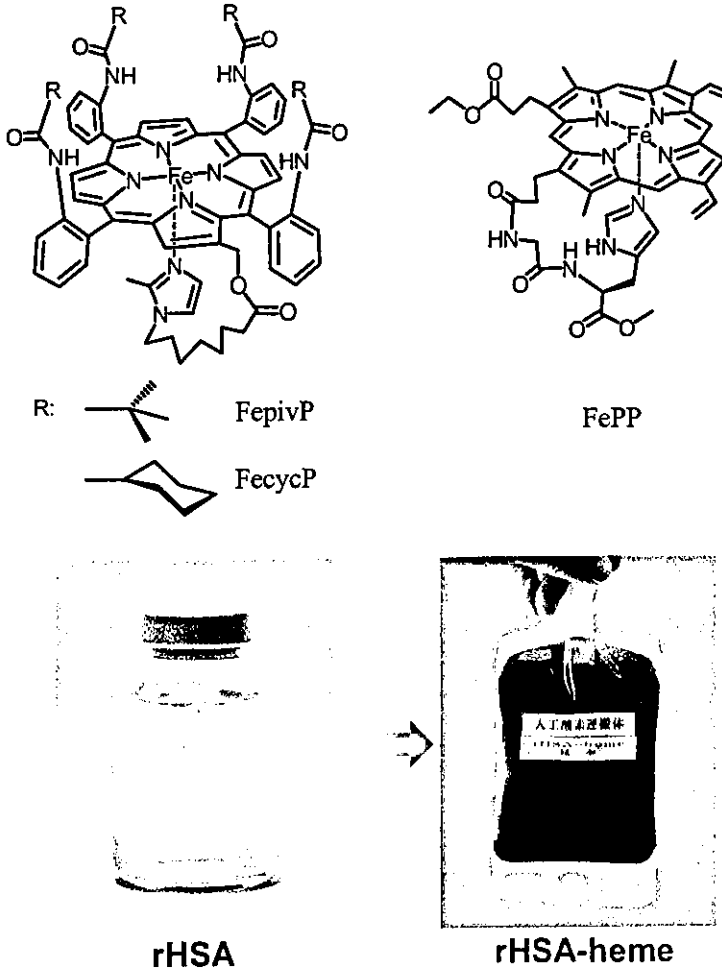
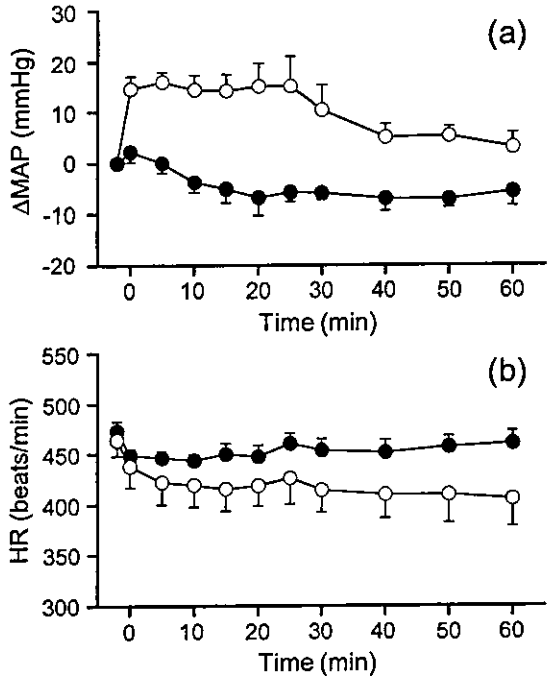


Figure 1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-20
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20
30
40
50
60
Time (min)
(b)
HR (beats/min)
550
500
450
400
350
300
0
10
20
30
40
50
60
Time (min)

Figure 2