るを得ない場合も多い。

arterial switchを行う場合は肺動脈弁が形態的機能的に正常であることが絶対条件であり，術前の血行動態によってはこれらがマスクされていることがあることに留意する。また冠動脈の走行異常も時々みられ，特に単一冠動脈で冠動脈の移植が困難になるケースも存在する。

3．1＋1/2心室修復(one-and-one-half ventricular repair)

この方法は前項のanatomical repairのうち房室関係の修復をより単純化することにより，それに伴う遠隔期合併症の発生を予防するねらいで最近，開発された方法である。特にmesocardiaやdextrocardiaに伴ってcavo-apical juxtapositionとなっているために，MustardやSenning術式を選択しにくい場合に有効な方法である。具体的にはその術式にhemi-Mustard法と呼ばれる心房内baffleを僧帽弁と肺静脈開口部の間にあて下大静脈血を三尖弁口にre-routingし，上大静脈血は両方向Glenn shuntをおいて肺動脈と吻合する(図6-58)。anatomical repairの欠点を補う可能性のある方法として遠隔期のmorbidityが少ないことが期待されるが，反面，両方向Glenn shuntによる肺動静脈瘻など別の合併症発生が危惧される。

4．modified Fontan

機能的単心室の諸疾患と同様，全体としての心室機能や肺血管抵抗値が高くないことを条件に行われる。両心室の容量の不均衡(機能的単心室)，多発性VSD，左側房室弁のstraddlingがあり心室の分割が不可能または重要な術後morbidityを呈すると思われる症例に対してこの術式を選択する。この疾患では，静脈の解剖学的異常が見られることは少ないのでtotal cavopulmonary connection術式の中でも心外導管を用いた方法を標準術式とする施設が増えつつある。

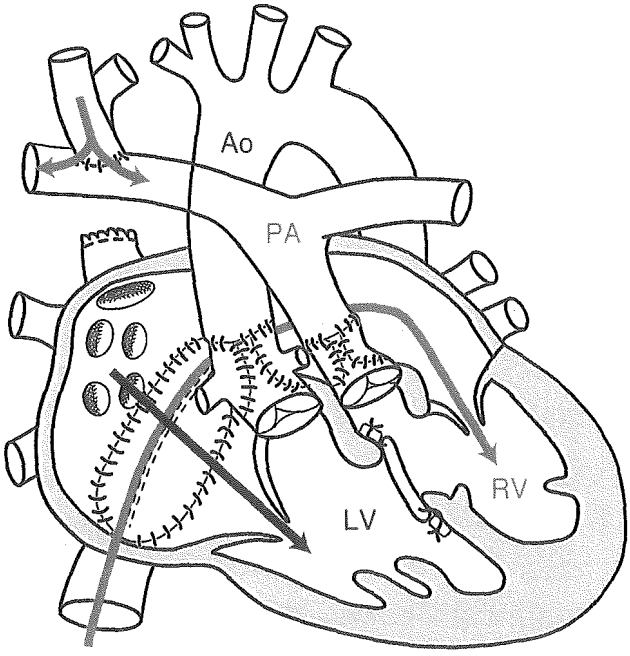

図6-58　1+1/2心室修復(arterial switch＋hemi-Mustard＋両方向Glenn shunt)の模式図

B. まとめ

本疾患の外科的治療は歴史的にも多くの変遷がありかつ現時点でも変遷している。つまり，統一された術式選択基準が存在しない。したがって，これらのオプションの長所短所を考慮に入れた上で各症例の解剖学的機能的特徴や各施設における経験に応じて外科的治療戦略を立てればよい。

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Circulation Kinetics and Organ Distribution of Hb-Vesicles Developed as a Red Blood Cell Substitute

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ABSTRACT

Phospholipid vesicles encapsulating concentrated human hemoglobin (Hb-vesicles, HbV), also known as liposomes, have a membrane structure similar to that of red blood cells (RBCs). These vesicles circulate in the bloodstream as an oxygen carrier, and their circulatory half-life times ($t_{1/2}$) and biodistribution are fundamental characteristics required for representation of their efficacy and safety as a RBC substitute. Herein, we report the pharmacokinetics of HbV and empty vesicles (EV) that do not contain Hb, in rats and rabbits to evaluate the potential of HbV as a RBC substitute. The samples were labeled with technetium-99m and then intravenously infused into animals at 14 ml/kg to measure the kinetics of HbV elimination from blood and distribution to the organs. The $t_{1/2}$ values were 34.8 and 62.6 h for HbV and 29.3 and 57.3 h for EV in rats and rabbits, respectively. At 48 h after infusion, the liver, bone marrow, and spleen of both rats and rabbits had significant concentrations of HbV and EV, and the percentages of the infused dose in these three organs were closely correlated to the circulatory half-life times in elimination phase ($t_{1/2\beta}$). Furthermore, the milligrams of HbV per gram of tissue correlated well between rats and rabbits, suggesting that the balance between organ weight and body weight is a fundamental factor determining the pharmacokinetics of HbV. This factor could be used to estimate the biodistribution and the circulation time of HbV in humans, which is estimated to be equal to that in rabbit.

Hemoglobin (Hb) isolated and purified from red blood cells (RBCs) has been tested as a principal component of RBC substitutes for carrying oxygen. However, the plasma retention time of isolated Hb is particularly short (half-life of ~0.5–1.5 h) because of the dissociation of the Hb tetramer into the dimeric form, which is subsequently filtered by the kidney, and it is known that this dimeric form is nephrotoxic (Savitsky et al., 1978). The potential of phospholipid vesicles as effective carriers of proteins and other bioactive materials has previously been proposed, since the cellular structure of such vesicles can protect the entrapped material from degradation and improve the biodistribution of proteins and other bioactive materials (Gregoriadis and Neerunjun, 1974; Papahadjopoulos et al., 1991). Phospholipid vesicles encapsulating concentrated Hb (HbV) have been proposed as a promising candidate RBC substitute, because encapsulation of Hb within a lipid membrane decreases potential side effects and toxicity of Hb, thereby making vesicles more RBC-like (Djordjevich and Miller, 1980; Gaber and Farmer, 1984; Tsuchida, 1998). The study of the safety and efficacy of HbV formulations by our research group has led to the development of an HbV formulation as a promising candidate for introduction into clinical trials (Tsuchida, 1998; Sakai et al., 2000b, 2001, 2004b; Takeoka et al., 2002).

Determination of the circulation time (half-life) of vesicles has been an important research focus, especially in RBC substitute development, because prolonged oxygen delivery is a required property for an artificial oxygen carrier. There are many reports describing the pharmacokinetics of vesicles, especially in mice and rats; however, it is difficult to apply these published data to the quantitative simulation of a clinical application. This is because of the lack of understanding of the species dependence of relevant mechanisms and correlative factors related to the clearance kinetics of vesicles. Some reports suggest that the circulatory half-life of vesicles injected in small doses into small animals such as

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ABBREVIATIONS: RBC, red blood cell; HbV, hemoglobin vesicle(s); EV, empty vesicle(s); ^{99m}Tc, technetium-99m; PEG, polyethylene glycol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPEA, 1,5-dihexadecyl-L-glutamate-*N*-succinic acid; PEG-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[monomethoxy poly(ethylene glycol) (5000)]; PLP, pyridoxal-5′ phosphate; HMPAO, hexamethylpropyleneamine oxime; %ID, percentage of infused dose; MPS, mononuclear phagocyte system.

mice or rats empirically corresponds to half-lives that are 2 or 3 times longer in humans (Gabizon et al., 2003). In addition, the infusion dose of HbV as a RBC substitute, in terms of lipid content, is nearly a hundred times larger compared with other therapeutic uses of vesicles, even though HbV encapsulate a highly concentrated form of Hb (35–40 g/dl). Furthermore, there are many other factors such as the lipid formulation (Allen et al., 1989), vesicle size (Awasthi et al., 2003), and surface modification (Klibanov et al., 1990) that influence the circulation time and distribution of the infused vesicles. There are no clinical data available for using large infusion doses of vesicles such as those required for a RBC substitute. Therefore, we focused this research on determining the correlation factors between data from different species to simulate the pharmacokinetics of HbV. In addition, empty vesicles (EV) that do not contain Hb were studied as a reference to clarify the specific influence of encapsulated Hb on the circulation properties of the vesicles.

Scintigraphic imaging is a particularly powerful tool that can be used to develop and evaluate the formulation of vesicles (Goins and Phillips 2001). Using imaging, Phillips et al. have reported on the pharmacokinetics of liposome-encapsulated Hb radiolabeled with technetium-99m (^{99m}Tc) (Rudolph et al., 1991; Phillips et al., 1992, 1999) and achieved a formulation with long circulation times. These liposomes had a small size (<200 nm), neutral surface, and PEG modification (10 mol%), and were regarded as long-circulating vesicles (so-called stealth liposomes) ($t_{1/2}$ was 65 h after 25% intravenous top-load in rabbits) (Phillips et al., 1999). However, this particular liposome formulation had a low efficiency of Hb encapsulation, because the requisites for stealth liposomes, such as small size, neutral surface, and dense PEG modification were a disadvantage for efficient Hb encapsulation (Perkins et al., 1993; Nicholas et al., 2000). As mentioned above, the infused dose of RBC substitutes will be extremely high, so high encapsulation efficiency of Hb is essential for a successful oxygen-carrying RBC substitute. We have developed HbV with a lipid formulation and encapsulation conditions that have improved the encapsulation efficiency (Takeoka et al., 1996; Sou et al., 2003), and the present HbV formulation has an oxygen-carrying capacity equal to RBCs because of this higher encapsulation efficiency (1.7–2.0 g of Hb per gram of lipids). This article is the first report on the detailed pharmacokinetics of this HbV formulation using scintigraphic imaging of ^{99m}Tc-HbV for monitoring the circulation properties and biodistribution. Factors that would permit estimation of human pharmacokinetics of large quantities of vesicles are discussed.

Materials and Methods

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, and 1,5-dihexadecyl-L-glutamate-*N*-succinic acid (DPEA) were purchased from Nippon Fine Chemical Co., Ltd. (Osaka, Japan); 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[monomethoxy poly(ethylene glycol) (5000)] (PEG-DSPE) was purchased from NOF Co. (Tokyo, Japan). DPPC, cholesterol, DPEA, and PEG-DSPE were dissolved in alcohol at a molar ratio of 5, 5, 1, and 0.033, respectively, atomized, and evaporated using a spray dryer (Cracks) to prepare a lipid powder, at Nippon Fine Chemical Co., Ltd. The mixed lipid powder was hydrated with NaOH solution, submitted to three cycles of freeze-thawing, and the resultant dispersion was then lyophilized at Kanto Chemical Co. (Tokyo, Japan). The Hb solution was obtained from outdated donated blood (Japanese Red Cross) according to the purification method described previously (Sakai et al., 2002). The Hb solution (oxyhemoglobin) was converted to carbonylHb by purging the solution with 100% carbon monoxide until testing proved conversion (99% < HbCO). The final concentration of Hb was adjusted to 40 g/dl. Homocysteine, pyridoxal-5′ phosphate (PLP), and glutathione were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of HbV. HbV were prepared according to a method described previously (Takeoka et al., 1996; Tsuchida, 1998; Sakai et al., 2001; Sou et al., 2003). All HbV preparation work was performed under sterile conditions. The purified carbonylHb solution (40 g/dl) containing 5 mM homocysteine and pyridoxal-5′ phosphate [PLP/Hb ratio of 2.5 (mol/mol)] was mixed with the lyophilized powder containing the mixed lipids (DPPC, cholesterol, DPEA, and PEG-DSPE). After controlling the size of the HbV with an extrusion method (final pore size of the filter, 0.22 μm, Fuji microfilter; Fuji Photo Film Co., Tokyo, Japan), the unencapsulated Hb was removed by three ultracentrifugation steps (10^5g, 30 min each). CarbonylHb was converted to OxyHb by exposure to visible light in an atmosphere of O_2. HbV were suspended in a physiological salt solution and filtered through sterilized filters (pore size, 0.45-μm Dismic; Toyo Roshi, Tokyo, Japan) and deoxygenated by bubbling with N_2 before storage (Sakai et al., 2000a). The control EV encapsulating glutathione (30 mM) was prepared using the same extrusion method.

Characterization of HbV and EV. The characteristics of HbV and EV are summarized in Table 1. The concentrations of Hb and phospholipid were determined by a cyanomethemoglobin method (Hemoglobin Test Wako; Wako Pure Chemicals, Tokyo, Japan) and the cholineoxidase method (Phospholipid C Test Wako; Wako Pure Chemicals), respectively. The encapsulation efficiency of Hb was represented as a w/w ratio of [Hb]/[lipid]. Methemoglobin and carbonylHb content were determined by spectrophotometry (Van Assendelft, 1970). The diameters of the resulting HbV (247 ± 44 nm) and EV (259 ± 32 nm) were determined using a submicron particle analyzer (N4SD; Beckman Coulter, Fullerton, CA). Endotoxin contamination was determined to be below 0.2 EU/ml by the *Limulus* assay test (Sakai et al., 2004a).

^{99m}Tc-Labeling of HbV and EV. Radiolabeling of HbV was performed according to a method described previously (Phillips et al., 1992). A saline solution of sodium [^{99m}Tc]pertechnetate (5 ml, 75 mCi) (Nycomed Amersham, San Antonio, TX) was injected into a vial containing lyophilized hexamethylpropyleneamine oxime (HMPAO, 0.5 mg, $SnCl_2$, 7.6 μg) (Ceretec; Amersham Biosciences Inc., Piscataway, NJ). The mixed solution was incubated for 5 min at room temperature. The ^{99m}Tc-HMPAO solution (1 ml) was then added to the HbV suspension ([Hb]; 10 g/dl, 1 ml), and the resulting mixed solution was incubated for 1 h. After removing free ^{99m}Tc-HMPAO by gel filtration (Sephadex-G25 column), total radioactivity was measured in a dose calibrator (Mark 5 model; Radex, Houston, TX) and the labeling efficiency (E) was calculated as the percentage of post-radioactivity in ^{99m}Tc-HbV to preradioactivity. The ^{99m}Tc-HbV suspension was mixed with unlabeled HbV suspension and the resultant HbV suspension ([Hb], 9.5 g/dl; [lipid], 4.75 g/dl) was used for the experiment. The ^{99m}Tc-EV were also prepared with same method and the lipid concentration was adjusted to the same lipid concentration as that of HbV suspension tested ([lipid], 4.75 g/dl). The ^{99m}Tc-labeled HbV and EV dispersion (0.5 ml) was mixed with rat

TABLE 1
Characteristics of ^{99m}Tc-HbV and ^{99m}Tc-EV suspensions

Parameter	^{99m}Tc-HbV	^{99m}Tc-EV
[Hb][a] (g/dl)	9.5	0
[Lipids] (g/dl)	4.75	4.75
Particle diameter (nm)	247 ± 44	259 ± 32
Endotoxin level (EU/ml)	< 0.2	< 0.2

[a] Methemoglobin, <1%; carbonylHb, <2%.

plasma (1.5 ml) from a donor rat and incubated at 37°C to check the labeling stability. A 100-μl aliquot of incubated sample at 48 h after mixing was passed through a Bio Gel A-15m (200–400 mesh) spin column. The sample was eluted by sequential addition of 100 μl of Dulbecco's phosphate-buffered saline (pH 7.3) under the centrifugal force of 1000 rpm for 1 min. Each fraction was collected separately and counted in a scintillation well counter (Canberra multichannel analyzer; Canberra Industries, Meriden, CT). Another 100-μl aliquot of incubation sample was used as a standard. The sum total of activity eluted with HbV or EV fractions was compared with total radioactivity in the standard.

Animal Experiments. Animal experiments were performed under the National Institutes of Health Animal Use and Care guidelines and approved by the University of Texas Health Science Center at San Antonio Institutional Animal Care Committee. Male Sprague-Dawley rats (200–274 g) were anesthetized with 3% isoflurane (VedCo, St. Joseph, MO) in 100% oxygen gas. Rats were then placed in the supine position under a Picker (Cleveland, OH) large-field-of-view gamma camera using a low-energy, all-purpose collimator and interfaced with a Pinnacle imaging computer (Medasys, Ann Arbor, MI). Image acquisition was begun as HbV or EV were infused into the tail vein at 1 ml/min. Each rat received a total dose of 0.17 to 0.37 mCi of ^{99m}Tc activity, Hb: 1.33 g/kg b.wt.; lipids: 0.67 g/kg b.wt. as an equivalent of 14 ml/kg for the HbV group (n = 5) and 0.48 to 0.55 mCi of ^{99m}Tc activity, lipids: 0.67 g/kg as 14 ml/kg for the EV group (n = 5). The infused dose (in volume) was estimated to be 25% of blood volume where the total blood volume was assumed to be 5.6% of body weight (Frank, 1976). The rabbit experiment was performed in the same manner. Male New Zealand White rabbits (2.2–2.9 kg) were anesthetized with an intramuscular injection of ketamine/xylazine (both from Phoenix Scientific, St. Joseph, MO) mixture (50 and 10 mg/kg body weight, respectively). One ear of a rabbit was catheterized with a venous line, and the other ear was catheterized with an arterial line. HbV or EV was infused in the venous line at 1 ml/min under the same gamma camera, and the blood samples were drawn from the arterial line. Each rabbit received a total dose of 3.7 to 4.5 mCi of ^{99m}Tc activity, Hb: 1.36 g/kg b.wt.; lipids: 0.68 g/kg b.wt. as 14.25 ml/kg for the HbV group (n = 5) and 3.5 to 4.9 mCi, lipids: 0.68 g/kg as 14.25 ml/kg for the EV group (n = 4). The infused dose (in volume) was estimated to be 25% of blood volume where the total blood volume was assumed to be 5.7% of body weight (Kozma et al., 1974).

Image Analysis. One-minute dynamic 64 × 64 pixel scintigraphic images were acquired over a continuous period of 0.5 and 2 h for rats and rabbits after the infusion of HbV or EV, respectively. Static images were also acquired at 3, 6, 12, 24, 36, and 48 h postinfusion. The image analysis was performed using a nuclear medicine analysis workstation (Pinnacle computer; Medasys). The regions of interest were drawn over the whole body, liver, and spleen in images. The counts of radioactivity were decay-corrected at each time and converted to a percentage of the whole body counts. Corrections were made for the blood pool contribution of the liver and spleen of the rat (17 and 6%, respectively, of the total blood volume). For rabbit, the liver was corrected by 25.4% of the total blood volume, and the spleen was individually corrected by 1.047 ± 0.076% for HbV and 1.592 ± 0.049% of the total blood volume for EV as percentage of infused dose (%ID) just after infusion, respectively.

Blood Persistence and Biodistribution. Blood was collected from the tail vein of the rat or arterial line of the rabbit (50 or 100 μl) at various times postinfusion. The radioactivity of blood samples was quantified in a scintillation well counter (Canberra multichannel analyzer; Canberra Industries) at the same time. The counts at each time were converted to the percentage of the counts of sample collected immediately after infusion. The elimination rate constants (k) were calculated by the least-squares method and half-life time ($t_{1/2}$) was calculated from eq. 1.

$$t_{1/2} = \frac{0.693}{k} \quad (1)$$

The animals were rapidly sacrificed at 48 h, and the tissue samples were collected, weighed, and counted for radioactivity in a scintillation well counter (Canberra multichannel analyzer; Canberra Industries) to calculate the biodistribution. To calculate the %ID per organ, total blood volume, muscle, and skin mass were estimated as 5.6, 40, and 13% of total body weight for rat (Frank, 1976; Petty, 1982), and 5.7, 45, and 10% of total body weight for rabbit (Kozma et al., 1974; Kaplan and Timmons, 1979), respectively. The bone was estimated as 10% of total body weight for rat (Frank, 1976; Petty, 1982) and 12 times the femur weight for rabbit (Dietz, 1944).

Estimation of the Biodistribution in Humans. The total Hb or lipids per organ (Ws) was calculated from the %ID and ID of Hb or lipids in terms of weight.

$$Ws(\text{mg}) = \frac{\%\text{ID} \times \text{ID}}{100} \quad (2)$$

The organ weight (Wo) of experimental animals was measured by an electronic balance and the Hb per organ weight (R) was calculated.

$$R(\text{mg/g}) = \frac{Ws}{Wo} \quad (3)$$

Ws was calculated from eq. 3 for humans, where the weights of liver, spleen, and bone (Wo) were estimated as 1.8, 0.18, and 5.0 kg, respectively, for average humans (70 kg) (International Commission on Radiological Protection, 1984), and the R value was applied as an average value between rats and rabbits shown in Table 4 for each organ. The ID of HbV ([Hb] = 9.5 g/dl, [lipids] = 4.75 g/dl) was calculated to be 25% of the blood volume (4.9 liters, 70 ml/kg b.wt.), and the %ID was calculated from eq. 2. The half-life times ($t_{1/2\beta}$) were estimated from eq. 4, where, constant value (C) was determined as a slope of the fitting line in this study and $\%\text{ID}_{\text{total}}$ was sum values of %ID for liver, spleen, and bone.

$$t_{1/2\beta} = \frac{C}{\%\text{ID}_{\text{total}}} \quad (4)$$

Statistical Methods. Values are reported as mean ± S.E.M. Statistical analysis was performed using Microsoft Excel for Windows. The image analysis and biodistribution data were compared using the Student's unpaired t test. A P value <0.01 or 0.05 was considered statistically significant.

Results

Labeling Efficiencies. The labeling efficiencies of ^{99m}Tc-HbV and ^{99m}Tc-EV were 69.1 ± 2.0% (n = 2) and 75.6 ± 5.1% (n = 3) for the rat studies, and 62.0 ± 4.8% (n = 5) and 70.9 ± 2.1% (n = 2) for the rabbit studies. Labeling efficiencies were similar for both ^{99m}Tc-HbV and ^{99m}Tc-EV, even though ^{99m}Tc-HbV used homocysteine and ^{99m}Tc-EV used glutathione. The ^{99m}Tc would be located in the inner aqueous phase of vesicles, and both homocysteine and Hb of HbV, and glutathione of EV would possibly bind the ^{99m}Tc (Rudolph et al., 1991; Phillips et al., 1992). The incubation of labeled HbV and EV in serum for 48 h revealed that 5 and 4% of the ^{99m}Tc dissociated from HbV and EV, indicating that the labeling was very stable and the contents were stably encapsulated inside the vesicles.

Circulation Kinetics. To determine the circulation kinetics as shown in Fig. 1, a and b, the radioactive counts of blood samples were plotted as a percentage of the counts for blood sample collected immediately at the end of the infusion with

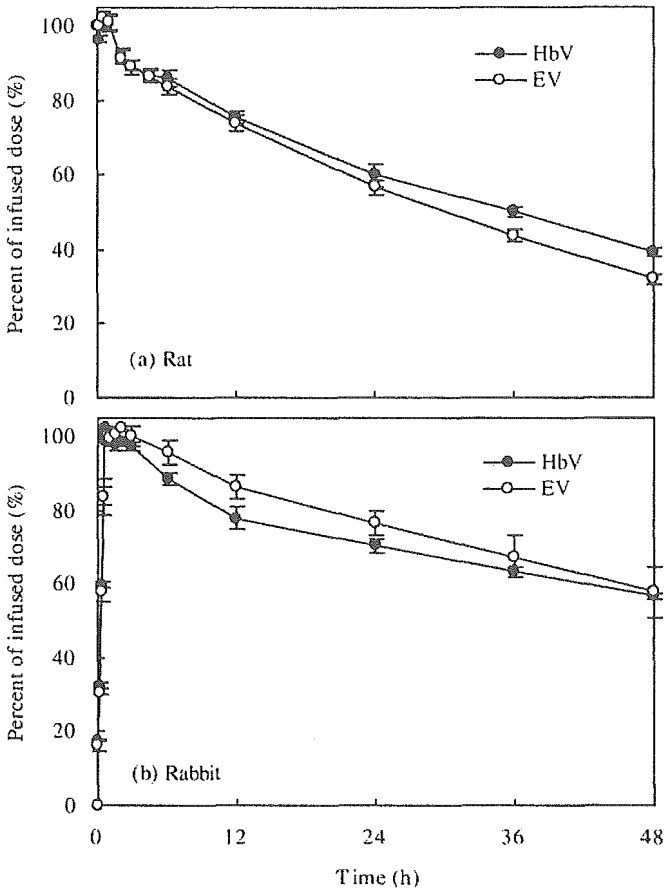

Fig. 1. Circulation kinetics of HbV and EV after top-loading intravenous infusion (14 ml/kg) in rats and rabbits. The radioactivity was determined by scintillation counting of blood samples with time. The percentage of radioactivity is calculated as a percentage of baseline radioactivity in a blood sample withdrawn just after HbV or EV infusion.

time. The elimination profiles of infused HbV showed two components with an initial fast clearance followed by a slower clearance phase, which is regarded as a distribution (α) phase in the mononuclear phagocyte system (MPS) and an elimination (β) phase, respectively. The clearance rate constant in the distribution phase of HbV was equal to that of EV, and k_β was 1.3 times smaller than that of EV in rats as shown in Table 2. The circulation half-life times ($t_{1/2}$ values) associated with both the distribution and elimination phases of HbV and EV in rats were 34.8 and 29.3 h, respectively. The clearance rates of HbV and EV were slower in rabbits compared with those in rats, especially for the distribution phase. The k_α of HbV was 0.0226 h^{-1} in rabbit, which was one-quarter of that in rats and 1.4 times larger than that of EV in rabbit. k_β for HbV was 1.3 times smaller than that of EV. The $t_{1/2}$ values of HbV and EV were 62.6 and 57.3 h in rabbits, respectively.

Imaging Study. The gamma camera images of rats or rabbits receiving HbV were acquired at various times to determine the organ distribution profiles with time. As shown in Figs. 2 and 3, radioactivity was observed over the whole body of animals and in the heart, demonstrating that HbV were circulating. Immediately after infusion, the heart, liver, and spleen were identified because these organs had a large blood pool volume, and the relative intensities of the liver and spleen increased in comparison with the heart with time. The %ID in liver and spleen calculated from gamma camera images with decay correction and correction for blood pool contribution are shown in Fig. 4. The %ID in liver was increased during the infusion and decreased after the infusion ended, especially in HbV as shown in Fig. 4, a and c. This initial decrease was most likely due to the adjustment of blood volume after top-loading. The values of %ID in liver and spleen were quickly increased during the first 6 to 12 h after infusion and reached a plateau at 48 h. At 48 h, the liver had 10.9 ± 0.8 and 7.6 ± 1.0% of HbV in rats and rabbits, respectively, whereas the spleen had 6.6 ± 0.3 and 0.98 ± 0.14% of HbV in rats and rabbits, respectively.

Biodistribution. The detailed biodistribution data of HbV at 48 h are shown in Table 3. HbV could be precipitated easily by ultracentrifugation of blood sample, and no Hb was detected in the supernatant serum in the blood sample for 48 h. In addition, no Hb was detected in urine for 48 h supporting that the Hb was not eluted from vesicles during circulation. HbV and EV were mainly distributed in liver, bone marrow, and spleen, and the %ID values for HbV were smaller than those of EV in these organs. Relatively high values for the bowel, feces, and urine were likely due to metabolism during excretion of HbV. The sum values of %ID for liver, spleen, and bone ($\%ID_{total}$), which are the main organs for MPS uptake, were 26.60 and 13.64% for HbV and 36.36 and 17.84% for EV in rats and rabbit, respectively. The corresponding $t_{1/2\beta}$ values given in Table 2 were 39.1, 79.2, 30.1, and 60.2 h, respectively. These $t_{1/2\beta}$ values are in proportion to the reciprocal of $\%ID_{total}$ as shown in Fig. 5, and the constant value (C) in eq. 4 was determined to be 1074.1 as a slope of the fitting line.

The calculated total lipids and Hb doses (in milligrams) delivered to the liver, bone, and spleen are summarized in Table 4. These values are independent of the species dependence of relative weight balances of organs in whole body and represent the amount of uptake of the HbV in a gram of each organ. The spleen had 14.43 ± 0.54 and 14.92 ± 1.25 mg of Hb per gram in rat and rabbit, and the liver and bone also had similar values in rat and rabbit.

Discussion

The improvement in oxygen-carrying capacity of HbV as a RBC substitute requires longer circulation and a higher en-

TABLE 2
Kinetic parameters of HbV and EV clearance from blood in rats and rabbits (25% top-loading)

Animal	Sample	Distribution (α) Phase		Elimination (β) Phase		$t_{1/2}$
		k_α	$t_{1/2\alpha}$	k_β	$t_{1/2\beta}$	
		h^{-1}	h	h^{-1}		h
Rat	HbV	0.0894	7.8	0.0177	39.1	34.8
	EV	0.1004	6.9	0.0230	30.1	29.3
Rabbit	HbV	0.0226	30.7	0.0088	79.2	62.6
	EV	0.0159	43.6	0.0115	60.2	57.3

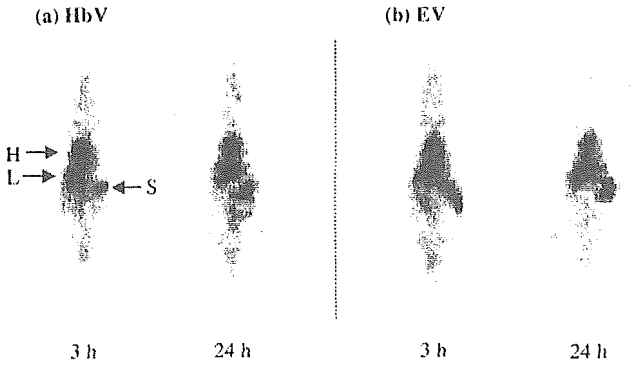

Fig. 2. Static gamma camera images of whole body of rats infused with HbV or EV acquired at 3 and 24 h after infusion. The images were acquired for 1 min at 3 h and 2 min at 24 h. The arrows show heart (H), liver (L), and spleen (S).

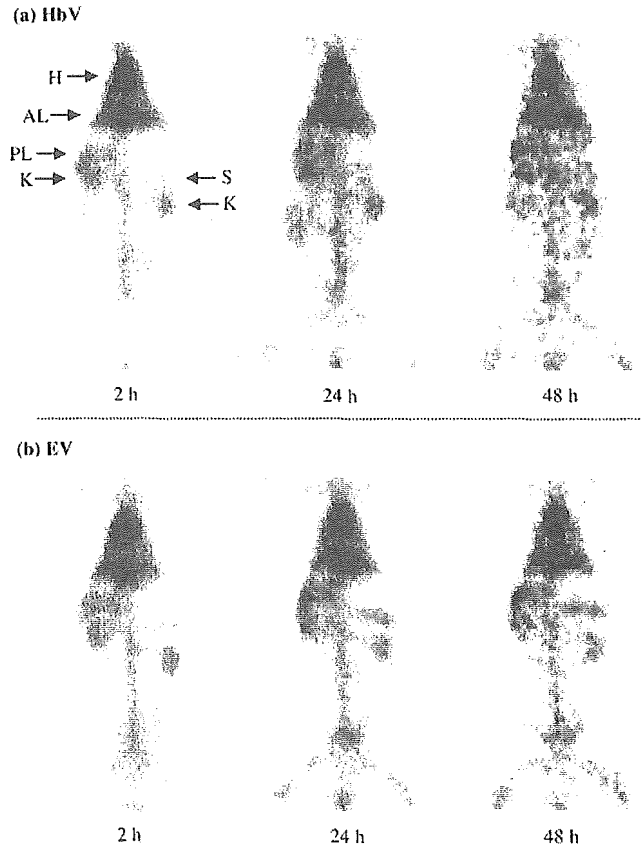

Fig. 3. Static gamma camera images of rabbits acquired at 2, 24, and 48 h after HbV or EV infusion. The images were acquired for 1 min at 2 h, 2 min at 24 h, and 5 min at 48 h. The arrows indicate heart (H), anterior liver (AL), posterior liver (PL), spleen (S), and kidney (K).

capsulation efficiency of Hb. The HbV formulation described in this study has high encapsulation efficiency ([Hb]/[lipid] = 2.0), and a circulatory half-life time of 34.8 and 62.6 h in rats and rabbits, respectively. This value is equal to the 65-h circulation half-life time for a PEG-liposome-encapsulated Hb formulation with a long circulation time in rabbits (Phillips et al., 1999). Other long-circulation vesicle formulations are successful for therapeutic uses such as cancer therapy or antibacterial treatment (Papahadjopoulos et al., 1991; Gabizon et al., 2003). However, the characteristics of small size (below 200 nm), neutral surface, and incorporation of significant amounts of PEG-lipid (5–10 mol%) of these formulations are ineffective in encapsulating Hb into vesicles (Perkins et al., 1993; Nicholas et al., 2000). The HbV formulation described in the present study is mainly composed of DPPC and cholesterol, only 0.3 mol% of PEG-lipid to prevent aggregation of the vesicles (Sakai et al., 2000a; Sou et al., 2000), and 9 mol% of anionic DPEA to reduce the lamellarity of the bilayer membrane (Sou et al., 2003). In general, anionic phospholipids such as phosphatidylglycerol or phosphatidylserine are used for the preparation of anionic vesicles; however, some side effects such as complement and platelet activations have been reported (Reinish et al., 1988). These immunological responses accelerate plasma protein adsorption on the surface of vesicles (opsonins) and then those vesicles are rapidly trapped into MPS. Our DPEA has a carboxylic group to negatively charge the surface of vesicles instead of a phosphate group of anionic phospholipids, and it does not have side effects like those reported for phosphatidylglycerol-containing vesicles (Wakamoto et al., 2001). The safety studies of HbV are underway, and the initial results in rats suggest that the DPEA vesicles have fewer side effects on immunological responses such as complement activation and thrombocytopenia compared with vesicles containing other anionic phospholipids. This bioinactive surface imparted by DPEA contributes to the stable circulation of HbV.

The diameter of vesicles is also an important factor for circulation kinetics and encapsulation efficiency. Recently, Awasthi et al. (2003) reported that the maximum size to show long circulation characteristics of PEG vesicle was around 240 nm in rabbits. The larger size of HbV is advantageous for the encapsulation efficiency of Hb; however, 250-nm HbV is of maximum and reasonable size to satisfy both long circulation and high Hb content requirements. We satisfied both long-circulation and high encapsulation efficiency of Hb by developing the lipid formulation and strictly regulating the diameter by the extrusion method. The clear effect of encapsulated Hb on the circulation time of vesicles was prolongation of the β phase for both animals. This is most likely due to greater saturation of the MPS by the encapsulated Hb.

Biodistribution data showed that HbV and EV were mainly distributed into liver, spleen, and bone. We have already clarified that Hb and phospholipid from HbV readily disappeared from the Kupffer cells in liver and macrophages in spleen in rats within a week after administration (Sakai et al., 2001). The trapping of HbV in MPS is regarded as a normal physiological pathway for removal of aged RBC; therefore, this should be a reasonable pathway for the elimination and metabolism of Hb-based RBC substitutes. The importance of the biodistribution of Hb-based RBC substitutes has been discussed and a vasoconstrictive effect of modified Hbs has been indicated (Sakai et al., 2000b). These side effects are triggered by the unusual biodistribution of small-sized modified Hb (<100 nm) to smooth muscle across the endothelium or the space of Disse in fenestrated endothelium of hepatic sinusoids, where the vasorelaxation factors nitric oxide and carbon monoxide are bound to Hb (Goda et al., 1998). The smaller vesicles might be effective for longer circulation of encapsulated Hb, but this would have the risk of causing similar or unusual side effects as those observed for modified Hb.

As summarized in Table 3, the %ID of HbV and EV in biodistribution data at 48 h is significantly different between

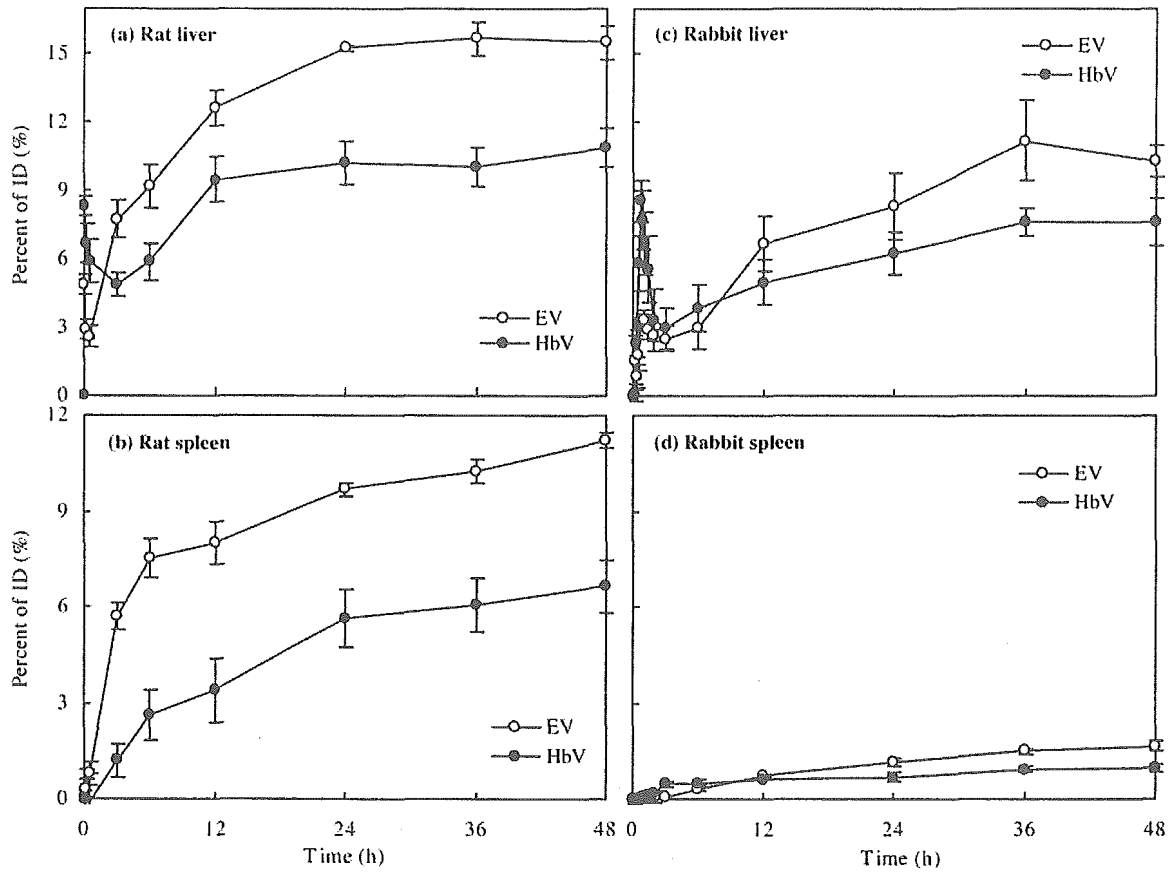

Fig. 4. %ID for liver and spleen calculated from the gamma camera image acquired at particular times and after decay correction. The blood pool contribution was corrected using values of 17 and 6% of the total blood volume for liver and spleen in rats, respectively. For rabbit, the liver was corrected by 25.4% of the total blood volume, and the spleen was individually corrected by 1.047 ± 0.076% for HbV and 1.592 ± 0.049% of the total blood volume for EV as %ID of just after infusion, respectively.

rats and rabbits ($P < 0.05$ for many organs). The rat had more HbV and EV in liver, bone, and especially spleen and less HbV and EV in blood. These data suggest that the biodistribution pattern of vesicles was not specifically changed by the encapsulation of Hb or the animal species tested; however, the quantitative values of %ID were significantly affected by these factors. Image analysis showed that the %ID required for saturating the liver and spleen with time was as shown in Fig. 4. The former liposome encapsulated Hb, which had non-PEG, showed significantly greater %ID (liver, 15.4 ± 2.1% ID; spleen, 18.1 ± 3.3% ID) in rabbit (Rudolph et al., 1991). The "saturated" level observed at those infusion doses would be determined by the balance between rate of uptake from the circulation, which was strongly affected by the HbV formulation and the rate of metabolic processing. The full saturation of MPS by the increased infusion dose of HbV might diminish the difference of pharmacokinetics between HbV formulations because the metabolic processing should become dominant factor. At 48 h, the blood clearance was in the slower β phase (Fig. 1) so that the inverse proportion between %ID and $t_{1/2\beta}$ is reasonable, and the determined constant C is available to estimate the $t_{1/2\beta}$ from the %ID. In addition, we have discovered that the most important factor for explaining the difference of %ID accumulating in the organs of the MPS between species is due to the different ratio of organ weight to body weight between species. For example, the average spleen weights of the experimental animals for HbV were 0.65 ± 0.07g in rats (216 ± 20 g b.wt., $n = 5$) and 0.87 ± 0.21 g in rabbits (2670 ± 97 g b.wt., $n = 5$). Therefore, the ratio of organ weight to body weight of rats is 9 times larger than that of rabbits, which means that rats have a 9 times larger mass capacity in spleen at the same infusion dose based on body weight. When the uptake of HbV is calculated in terms of mg of lipid and Hb per gram of MPS organ, the values in rats and rabbits are very close to each other as summarized in Table 4, indicating that the concentration of HbV in these organs was species-independent in this case. These values can be used to quantitatively estimate biodistribution of HbV based on organ weight. By using these two factors of C values and milligrams of lipids per organ weight, we were able to roughly estimate the biodistribution and circulation time of HbV in humans (see *Materials and Methods*). Laverman et al. (2000) reported that the distribution pattern of PEG-liposomes in humans was similar to that of rats and rabbits, with high uptake in liver, spleen, and bone marrow. Other biodistribution studies of vesicles also suggested a high uptake in liver, spleen, and bone marrow in humans (Dams et al., 2000; Gabizon et al., 2003), and these reports support our estimation. Based on the MPS organ weights of average humans and the milligrams of uptake of lipid and hemoglobin per gram MPS organs at 48 h (human liver weight, 1.8 kg; human spleen, 0.18 kg; and human bone, 5.0 kg) (International Commission on Radiological Protection, 1984), we estimated that %IDs of HbV are 5.4% (liver), 4.5% (spleen), and 6.4% (bone), and a $t_{1/2\beta}$ of approximately 66 h in humans after a 25% top-loading

TABLE 3
Biodistribution of HbV and EV as a percentage of the infused dose per organ (%ID/organ) and percentage of the infused dose per gram of organ (%ID/g organ) at 48 h after 25% top-loading in rats or rabbits

Organ	Rat		Rabbit	
	HbV	EV	HbV	EV
%ID/organ ± S.E.M.				
Blood	33.27 ± 1.11*	24.13 ± 0.65	50.95 ± 2.02†	52.76 ± 4.80‡
Liver	10.04 ± 0.86*	14.13 ± 0.40	7.55 ± 0.46†	8.64 ± 0.34‡
Bone	10.06 ± 0.21*	13.05 ± 0.38	5.37 ± 0.33*†	7.36 ± 0.28‡
Spleen	6.50 ± 0.30*	9.18 ± 0.37	0.72 ± 0.10*†	1.84 ± 0.28‡
Bowels	7.30 ± 1.59	4.16 ± 0.35	9.61 ± 2.31	8.62 ± 4.42
Skin	2.37 ± 0.33	2.29 ± 0.12	0.88 ± 0.05†	1.09 ± 0.21‡
Kidney	2.40 ± 0.10*	3.35 ± 0.08	1.47 ± 0.13†	1.69 ± 0.21‡
Muscle	1.94 ± 0.28	1.98 ± 0.27	2.51 ± 0.31	2.62 ± 0.76
Lung	0.62 ± 0.03	0.54 ± 0.03	0.55 ± 0.02	0.43 ± 0.06
Heart	0.17 ± 0.01	0.16 ± 0.01	0.12 ± 0.01†	0.13 ± 0.02
Brain	0.16 ± 0.01*	0.09 ± 0.01	0.08 ± 0.01*†	0.05 ± 0.00‡
Testis	0.12 ± 0.01*	0.09 ± 0.01	0.06 ± 0.02†	0.07 ± 0.01
Feces	9.50 ± 1.17	6.95 ± 0.29	5.06 ± 2.56	2.02 ± 0.55‡
Urine	13.61 ± 0.31	12.87 ± 0.41	11.30 ± 1.22	7.81 ± 1.44‡
%ID/g organ ± S.E.M.				
Blood	2.919 ± 0.032	1.706 ± 0.044	0.356 ± 0.017	0.354 ± 0.030
Liver	1.244 ± 0.096	1.378 ± 0.045	0.093 ± 0.004	0.131 ± 0.019
Bone	0.497 ± 0.021	0.518 ± 0.020	0.043 ± 0.003	0.062 ± 0.002
Spleen	10.059 ± 0.072	10.790 ± 0.402	0.823 ± 0.072	1.483 ± 0.072
Bowels	0.390 ± 0.073	0.202 ± 0.008	0.031 ± 0.006	0.029 ± 0.014
Skin	0.091 ± 0.014	0.070 ± 0.004	0.004 ± 0.000	0.004 ± 0.001
Kidney	1.604 ± 0.057	1.839 ± 0.055	0.089 ± 0.005	0.110 ± 0.017
Muscle	0.024 ± 0.003	0.020 ± 0.003	0.002 ± 0.000	0.002 ± 0.001
Lung	0.619 ± 0.022	0.458 ± 0.014	0.068 ± 0.004	0.057 ± 0.012
Heart	0.264 ± 0.009	0.187 ± 0.012	0.026 ± 0.002	0.027 ± 0.006
Brain	0.111 ± 0.010	0.062 ± 0.003	0.011 ± 0.001	0.006 ± 0.001
Testis	0.042 ± 0.002	0.027 ± 0.001	0.013 ± 0.002	0.016 ± 0.003

* Difference is statistically significant from EV in same species at $P < 0.01$.
† Difference is statistically significant from HbV in rat at $P < 0.05$.
‡ Difference is statistically significant from EV in rat at $P < 0.05$.

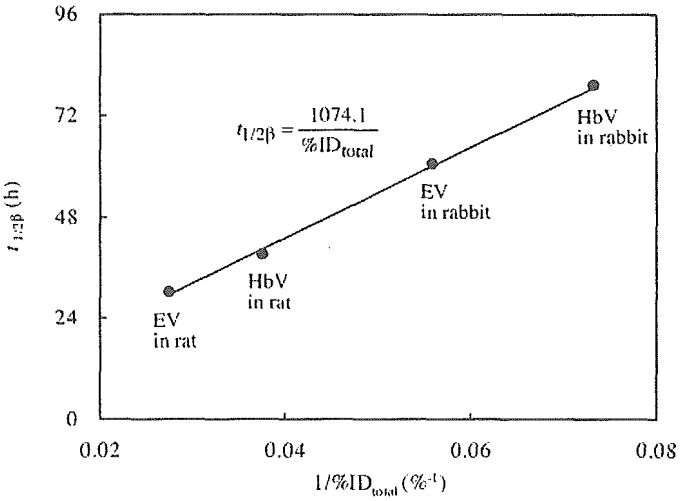

Fig. 5. Proportional relationship between the circulation half-life time ($t_{1/2\beta}$) and the reciprocal of $\%ID_{total}$ in the elimination phase. The $\%ID_{total}$ was calculated as a sum value of %ID in liver, bone, and spleen at 48 h. The fitting line was determined by the regression analysis (coefficient of determination; $R^2 = 0.9985$).

([Hb], 9.5 g/dl; [lipid], 4.75 g/dl). The normal range of human organ weight is relatively wide such as 1.4 to 1.8 kg (liver) and 0.08 to 0.3 kg (spleen), so the $t_{1/2\beta}$ would be varied around 3 days. This $t_{1/2\beta}$ is approximately two times larger than that of rat, and this ratio almost follows that derived from empirical speculation (Gabizon et al., 2003). This method of estimating vesicle circulation kinetics and organ uptake in different animal species may be useful for all types of vesicle (liposome) formulations that are currently under development as drug delivery vehicles. More studies will be required to further validate this method of estimating circulation kinetics and organ uptake in different animal species.

The development of RBC substitutes is progressing, and some modified Hbs have been studied in clinical trials. The reported $t_{1/2}$ value was 23 h for polymerized bovine Hb (Hughes et al., 1995), 16 to 20 h for *o*-raffinose-cross-linked and polymerized human Hb (Carmichael et al., 2000), and 24 h for glutaraldehyde-cross-linked and polymerized human Hb (Gould et al., 1998). Even though HbV have not yet been tested clinically, we have demonstrated in the present report that HbV have significantly improved properties, based on their circulation kinetics and biodistribution, suggesting their improved safety and efficacy as a RBC substitute. In addition, the successful application of vesicles as RBC substitutes at this large infusion dose suggests a promising future for vesicles (liposomes), and the present formulation would potentially be available not only as a RBC substitute but also for various applications such as drug delivery systems.

Acknowledgments

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TABLE 4
Comparison of HbV and EV as milligrams of lipids per gram of organ and milligrams of Hb per gram of organ at 48 h after 25% top-loading in rats or rabbits

Organ	HbV in Rat		HbV in Rabbit		EV in Rat	EV in Rabbit
	mg lipids/g organ[a]	*mg Hb/g organ*[b]	*mg lipids/g organ*[a]	*mg Hb/g organ*[b]	*mg lipids/g organ*[a]	
Blood	4.23 ± 0.20	8.40 ± 0.40	6.47 ± 0.24[†]	12.93 ± 0.48[†]	2.94 ± 0.06	6.55 ± 0.64[†]
Liver	1.79 ± 0.12	3.56 ± 0.23	1.68 ± 0.06	3.36 ± 0.12	2.38 ± 0.06	2.24 ± 0.18
Bone	0.72 ± 0.01	1.42 ± 0.02	0.78 ± 0.05	1.57 ± 0.09	0.89 ± 0.04	1.09 ± 0.08
Spleen	14.43 ± 0.54	28.63 ± 1.06	14.92 ± 1.25	29.85 ± 2.50	18.58 ± 0.51	25.83 ± 1.43[†]

[†] Difference is statistically significant from HbV in rat at $P < 0.05$.
[a] Calculated values from ID of lipids and %ID/g organ.
[b] Calculated values from ID of Hb and %ID/g organ.

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PAT

Oxygen infusions (hemoglobin-vesicles and albumin-hemes) based on nano-molecular sciences†

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Since the discovery of a red-colored saline solution of a heme derivative that reversibly binds and releases oxygen (1983), significant efforts have been made to realize an oxygen infusion as a red cell substitute based on the sciences of both molecular assembling phenomena and macromolecular metal complexes. The authors have specified that hemoglobin (Hb)-vesicles (HbV) and recombinant human serum albumin-hemes (rHSA-heme) would be the best systems that meet the clinical requirements. (A) Hb is rigorously purified from outdated, donated red cells via pasteurization and ultrafiltration, to completely remove blood type antigen and pathogen. The HbV encapsulates thus purified concentrated Hb solution with a phospholipid bimolecular membrane (diameter, 250 nmø), and its solution properties can be adjusted comparable with blood. Surface modification of HbV with a water-soluble polymer ensures stable dispersion state and storage over a year at 20°C. *In vivo* tests have clarified the efficacy for extreme hemodilution and resuscitation from hemorrhagic shock, and safety in terms of biodistribution, metabolism in reticuloendothelial system (RES), clinical chemistry, blood coagulation, etc. The HbV does not induce vasoconstriction thus maintains blood flow and tissue oxygenation. (B) rHSA is now manufactured in Japan as a plasma-expander. The rHSA can incorporate eight heme derivatives (axial base substituted hemes) as oxygen binding sites, and the resulting rHSA-heme is a totally synthetic O_2-carrier. Hb binds endothelium-derived relaxation factor, NO, and induces vasoconstriction. The rHSA-heme binds NO as Hb does, however, it does not induce vasoconstriction due to its low pI (4.8) and the resulting low permeability across the vascular wall (1/100 of Hb). A 5%-albumin solution possesses a physiologic oncotic pressure. Therefore, to increase the O_2-transporting capacity, albumin dimer is effective. Albumin dimer can incorporate totally 16 hemes with a regulated oncotic pressure. The rHSA-heme is effective not only as a red cell substitute but also for oxygen therapeutics (e.g. oxygenation for tumor). Significant efforts have been made to produce HbV and rHSA-heme with a facility of Good Manufacturing Practice (GMP) standard, and to start preclinical and finally clinical trials.

KEYWORDS: oxygen infusion; blood substitutes; surface modification; water-soluble polymers; biomaterials

INTRODUCTION

For human beings to survive, it is necessary to continuously deliver O_2 that is needed for the respiration of all tissue cells. Blood, a so-called moving internal-organ, reversibly binds and releases O_2 under physiological conditions. From this point of view, realization of red blood cell (RBC) substitutes, or O_2-infusions, would contribute significantly to human health and welfare. In this research field, the basic sciences for macromolecular complexes, molecular assemblies, and nano-molecular sciences play fundamental roles. The authors have systematically studied the metal complexes (synthetic heme derivatives) embedded into a hydrophobic cluster in aqueous medium, and clarified that the electronic processes of the active sites are controlled by the surrounding molecular environment. As a result, the reaction activity is observed as cooperative phenomena with the properties of the molecular atmosphere. In other words, the development of our O_2-infusion has been based on "the regulation of the electronic process on macromolecular metal complexes".[1,2]

To reproduce the O_2-binding ability of RBCs, that is, the development of a synthetic O_2-carrier that does not need hemoglobin (Hb), was the starting point of the idea for this study. In general, central ferric iron of a heme is immediately oxidized by O_2 in water, preventing the O_2 coordination process from being observed. Therefore, the electron transfer

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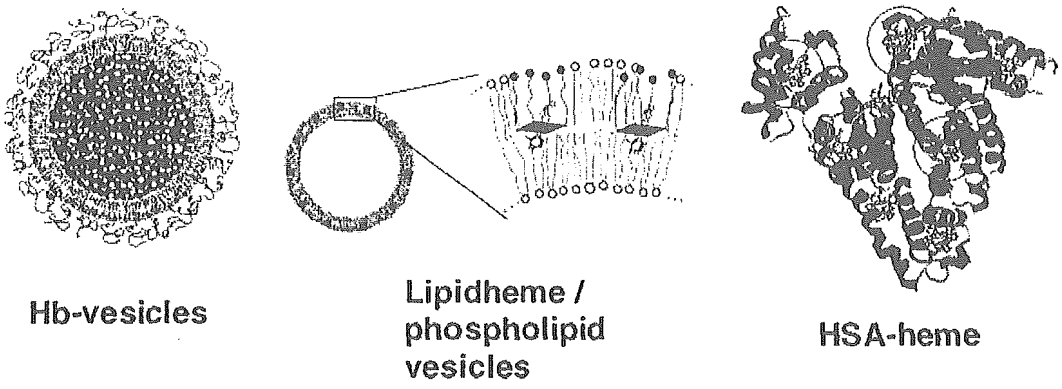

Figure 1. Schematic representation of lipidheme-vesicle, hemoglobin-vesicle, and albumin-heme.

must be prevented. Fortunately, the formation of the O_2-adduct complex could be detected but for only several nano-seconds by utilizing the molecular atmosphere and controlling the electron density in the iron center. Based on this finding, the authors succeeded in reversible and stable O_2-coordination in 1983 and preparing phospholipid vesicles embedded amphiphilic-heme, known as lipidheme/phospholipids vesicles (Fig. 1).[3–5] This was the first example of reversible O_2-binding taking place under physiological conditions. For example, human blood can dissolve about 27 ml of O_2 per dl, however a 10 mM lipidheme-phospholipid vesicle solution can dissolve 29 ml of O_2 per dl. This material is suitable for "O_2-infusion". Thus over hundred types of heme derivatives have been synthesized, and recently new lipidheme bearing phospholipid groups have been synthesized, which completes self-organization in water to form stable vesicles.[6]

In 1985, Dr Sekiguchi at Hokkaido Red Cross Blood Center proposed Waseda group to consider the utilization of Hb in outdated RBCs. Thus the research of Hb-vesicles (HbV) based on molecular assembly technologies was started. In the latter 1990s, a mass-production system for recombinant human serum albumin (rHSA) was established and then albumin-heme hybrids (rHSA-heme) using its non-specific binding ability was prepared, which is now considered to be a promising synthetic material. Based on the effective integration of nano-molecular science and technologies for functional materials developed by Waseda University, and the outstanding evaluation system of safety and efficacy developed by Keio University using animal experiments, strong progress on the research of the O_2-infusion project has been made. In the near future, mass production and clinical tests of O_2-infusion will be started by the pharmaceutical industry.

DEVELOPMENT OF Hb-BASED O_2-CARRIERS AND THE CHARACTERISTICS OF HbV

Historically, the first attempt of Hb-based O_2-carrier in this area was to simply use stroma-free Hb (Fig. 2). However, several problems became apparent, including dissociation into

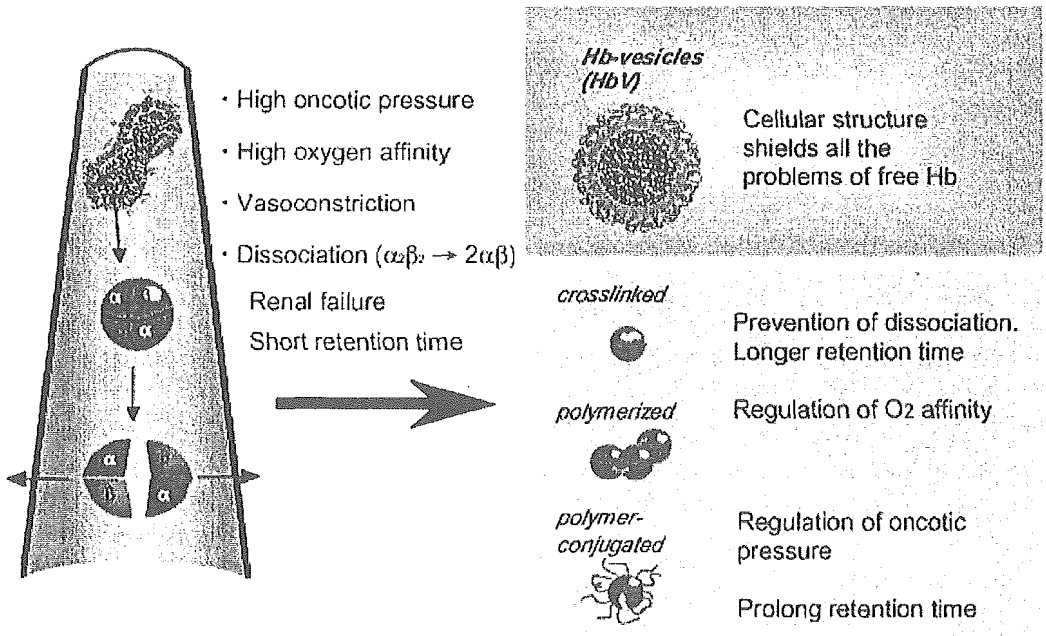

Figure 2. Approaches to solve the problems of utilization of Hb as an O_2-carrier, chemical modification or encapsulation of Hb.

dimers that have a short circulation time, renal toxicity, high oncotic pressure and high O_2-affinity. Since the 1970s, various approaches were developed to overcome these problems.[7,8] This includes intra-molecular crosslinking, polymerization and polymer-conjugation. However, in some cases the significantly different structure in comparison with RBCs resulted in side effects such as vasoconstriction.[9]

Another idea is to encapsulate Hb with a lipid bilayer membrane to solve all the problems of molecular Hb.[10] RBCs have a biconcave structure with a diameter of about 8000 nm. RBCs can deform to a parachute-like configuration to pass through narrow capillaries. The possibility of infection and blood-type mismatching, and short shelf life are the main problems. The idea of Hb encapsulation with a polymer membrane mimicking the structure of RBC is originated from Dr Chang at McGill University.[7] After that, the encapsulation of Hb within a phospholipid vesicle was studied by Dr Djordjevich at the University of Illinois in the 1970s.[11] However, it was not so easy to make HbV with a regulated diameter and adequate O_2-transport capacity, the authors made a breakthrough in routinely producing HbV by using fundamental knowledge of macromolecular and supramolecular sciences.[12–19] Several liters of HbV are routinely prepared in a completely sterile condition. Hb is purified from outdated RBCs, and concentrated to 40 g/dl. Virus removal is performed using a combination of pasteurization at 60° and filtration with a virus removal filter. The Hb encapsulation with phospholipids bilayer membrane and size regulation was performed with an extrusion method. The vesicular surface is modified with polyethylene glycol (PEG) chains. The suspension of Hb-vesicles is deoxygenated at the final stage.

The particle diameter of HbV is regulated to about 250 nm, therefore, the bottle of HbV is turbid. One vesicle contains about 30,000 Hb molecules so that it does not show oncotic pressure. There is no chemical modification of Hb. O_2-affinity is controllable with an appropriate amount of allosteric effectors, pyridoxal 5-phosphate. Hb concentration is regulated to 10 g/dl, and the weight ratio of Hb to total lipid approaches 2.0 by using an ultra pure and concentrated Hb solution of 40 g/dl, which is covered with a thin lipid bilayer membrane. The surface is modified with 0.3 mol% of PEG-lipid. Viscosity, osmolarity, and oncotic pressure are regulated according to the physiological conditions.

HbV can be stored for over 2 years in a liquid state at room temperature.[17] There is little change in turbidity, diameter, and P_{50}. Methemoglobin (MetHb) content decreases due to the presence of reductant inside the HbV, which reduces the trace amount of metHb during storage. This excellent stability is obtained by deoxygenation and PEG-modification. Deoxygenation prevents metHb formation. The surface modification of HbV, with PEG chains prevents vesicular aggregation and leakage of Hb and other reagents inside the vesicles. Liquid state storage is convenient for emergency infusion compared to freeze-dried powder or the frozen state.

IN VIVO EFFICACY OF HbV

The efficacy of HbV has been confirmed mainly with isovolemic hemodilution and resuscitation from hemorrhagic shock.[20–28] In this review two important cases are described. One is isovolemic hemodilution with 90% blood exchange in a rat model. The other is resuscitation from hemorrhagic shock in a hamster model.

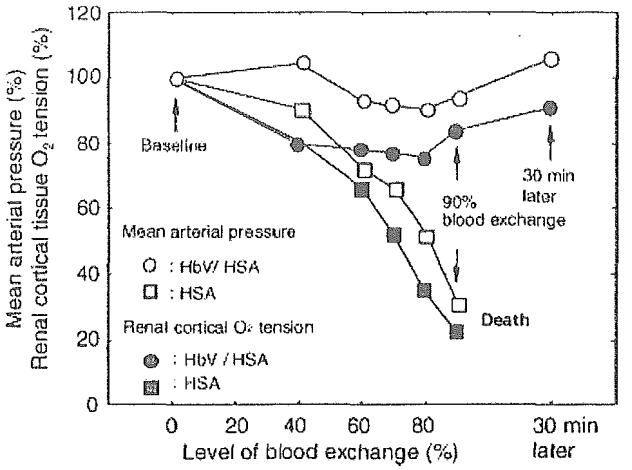

Figure 3. Ninety per cent exchange-transfusion with HbV suspended in HSA (HbV/HSA), or HSA alone. Mean arterial pressure and renal cortical oxygen tension were monitored.

To confirm the O_2-transporting ability of HbV, extreme hemodilution was performed with HbV suspended in human serum albumin (HSA)[21,23] (Fig. 3). The final level of blood exchange reached 90%. Needle-type O_2 electrodes were inserted into the renal cortex, and the blood flow rate in the abdominal aorta was measured with the pulsed Doppler method. Hemodilution with albumin alone resulted in significant reductions in mean arterial pressure and renal cortical O_2 tension, and finally all the rats died of anemia. However, hemodilution with HbV, suspended in HSA sustained both blood pressure and renal cortical O_2 tension, and all the rats survived. These results clearly demonstrate that HbV has sufficient O_2 transporting capability.

To observe the microcirculatory response to the infusion of Hb products, intravital microscopy was used equipped with all the units to measure blood flow rates, vascular diameter, O_2 tension, and so on, in collaboration with Dr Intaglietta at the University of California, San Diego. The hamster dorsal-skin fold preparation allows observation of blood vessels from small arteries down to capillaries. The HbV suspension, as a resuscitative fluid for hemorrhagic-shocked hamsters was evaluated.[26] About 50% of the blood was withdrawn, and the blood pressure was maintained at around 40 mmHg for 1 hr, and the hamsters either received HbV suspended in HSA (HbV/HSA), HSA alone, or shed blood (Fig. 4). Immediately after infusion, all the groups showed increases in mean arterial pressure. However, only the albumin infusion resulted in incomplete recovery. However, the HbV/HSA group showed the same recovery with the shed autologous blood infusion. During the shock period, all the groups showed significant hyperventilation that was evident from the significant increase in arterial O_2 tension. Simultaneously, base excess and pH decreased significantly. Immediately after resuscitation, all the groups tended to recover. However, only the HSA group showed sustained hyperventilation. Base excess for the HSA group remained at a

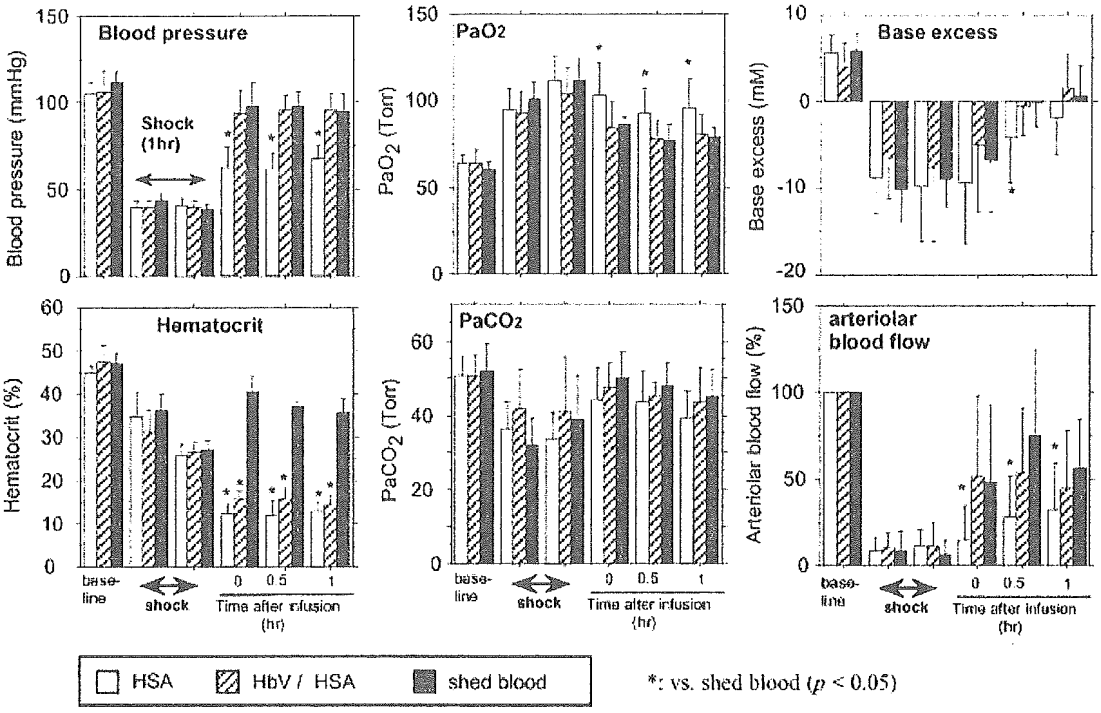

Figure 4. Resuscitation from hemorrhagic shock with HbV suspended in HSA (HbV/HSA) in hamster dorsal skinfold model. Mean ± SD.

significantly lower value 1 hr after resuscitation. Blood flow decreased significantly in arterioles to 11% of basal value during shock. The HbV/HSA and shed autologous blood groups immediately showed significant increases in blood flow rate after resuscitation, while the albumin group showed the lowest recovery.

SAFETY EVALUATION OF HbV

The safety profile of HbV such as cardiovascular responses, pharmacokinetics, influence on RES, influence on clinical measurements and daily repeated infusions were further examined.[29–37]

The microvascular responses to the infusion of intramolecularly crosslinked Hb (XLHb) and HbV were studied using conscious hamsters. XLHb (7 nm in diameter) showed a significant increase in hypertension equal to 35 mmHg, and simultaneous vasoconstriction of the resistance artery equal to 75% of the baseline levels[30] (Fig. 5). However, HbV with diameter of 250 nm showed minimal changes. The small acellular XLHb is homogeneously dispersed in the plasma, and it diffuses through the endothelium layer of the vascular wall and reaches the smooth muscle. XLHb traps nitric oxide (NO) as an endothelium-derived relaxation factor, and induces vasoconstriction, and hypertension. However, the large HbV stay in the lumen and does not induce vasoconstriction. Several mechanisms are proposed for Hb-induced vasoconstriction. These include NO-binding, excess O_2 supply, reduced shear stress, or the presence of Hb recognition site on the endothelium. But it is clear that Hb-encapsulation shields against the side effects of acellular Hbs.

Professor Suematsu at Keio University has revealed the effects of Hb-based O_2 carriers in hepatic microcirculation[29,32] (Fig. 6). On the vascular wall of the sinusoid in hepatic microcirculation, there are many pores, called fenestration, with a diameter of about 100 nm. The small Hb

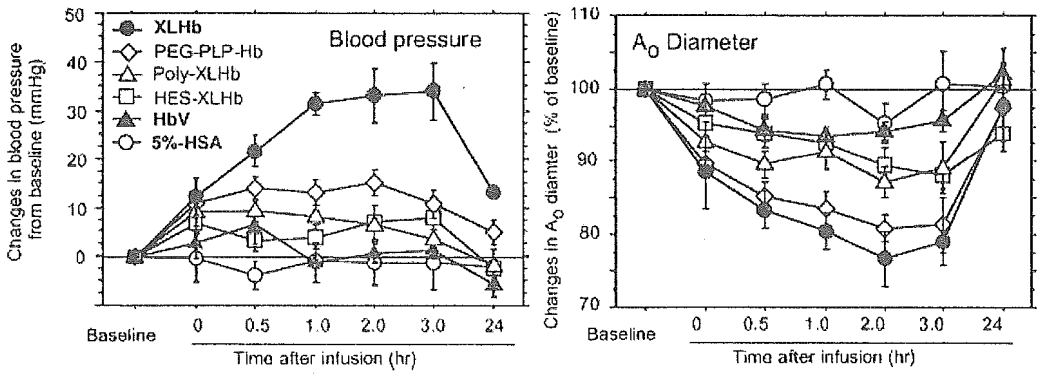

Figure 5. Changes in mean arterial pressure and the diameters of the resistance artery in hamster dorsal skin microcirculation after the bolus infusion of Hb-based O_2-carriers. Mean ± SD.

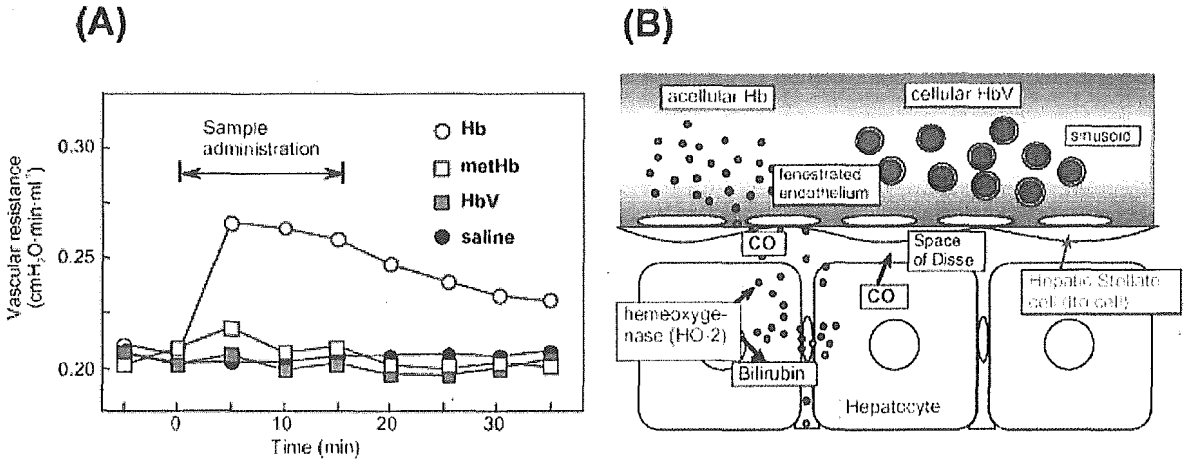

Figure 6. (A) Changes in vascular resistance during perfusion of exteriorized rat liver with HbV, Hb, metHb, or saline. (B) Schematic representation of hepatic microcirculation: the small Hb molecule extravasate across the fenestrated endothelium to reach to the space of Disse, where heme of Hb is catabolized by hemeoxygenase-2 (HO-2) and CO is released as a vasorelaxation factor. However, the excess amount of the extravasated Hb traps CO and induces vasoconstriction and the resulting higher vascular resistance. However, the larger HbV retains in the sinusoid and there is no extravasation and vasoconstriction.

molecules with a diameter of only 7 nm extravasate through the fenestrated endothelium and reach the space of Disse. However, HbV particles, which are larger than the pores, do not extravasate. Heme of extravasated Hb is excessively metabolized by hemeoxygenase-2 in hepatocyte to produce CO and bilirubin. Even though CO acts as a vasorelaxation factor in the liver, the excess amount of Hb rapidly binds CO, resulting in the vasoconstriction and an increase in vascular resistance. Furthermore, HbV (250 nm in diameter) is large enough to remain in the sinusoid, and the vascular resistance is maintained.

From these results, the optimal molecular dimension of Hb-based O_2 carriers can be proposed. The upper limitation is below the capillary diameter to prevent capillary plugging, and for sterilization by membrane filters (Fig. 7). However, smaller sizes exhibit a higher rate of vascular wall permeability with side effects such as hypertension and neurological disturbances. HbV exhibits a very low level of vascular wall permeability. Therefore, the HbV appears to be appropriate from the viewpoint of hemodynamics. However, the influence of HbV on the RES has to be clarified, because the fate of HbV is RES trapping.

Circulation persistence was measured by monitoring the concentration of radioisotope-labeled HbV in collaboration with Dr Phillips at the University of Texas at San Antonio. The circulation half-life is dose dependent, and when the dose rate was 14 ml/kg, the circulation half-life was 35 hr in rats. The circulation time in the case of the human body can be estimated to be twice as long; or about 3 days at the same dose rate. Gamma camera images of radioisotope-labeled HbV

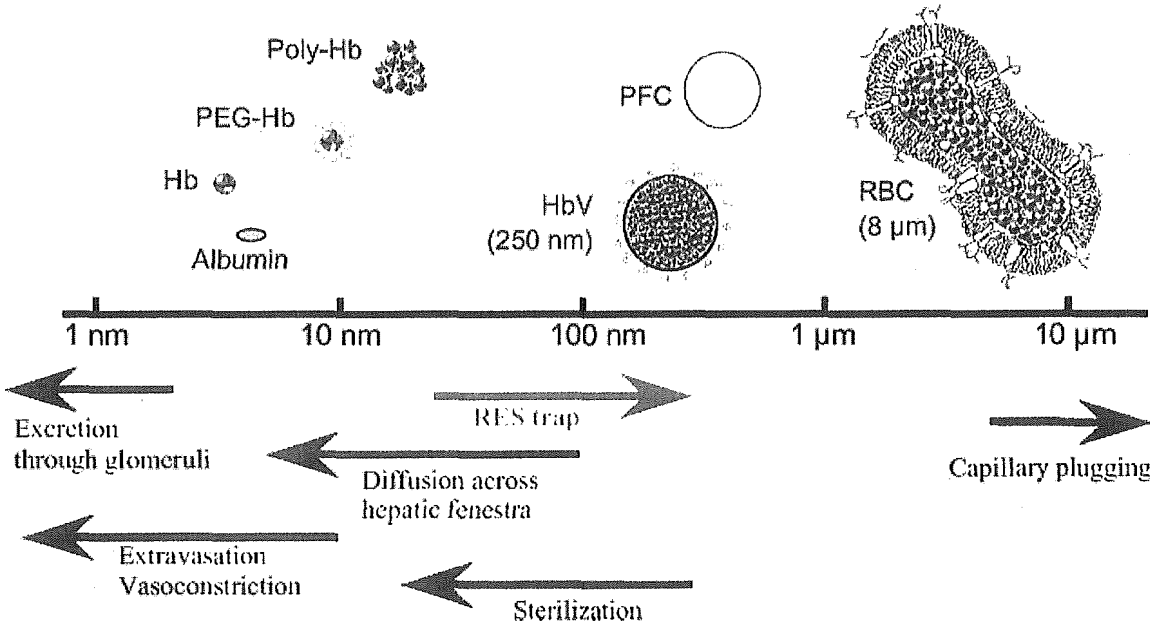

Figure 7. Optimal diameter of Hb-based oxygen carriers from the view point of physiological response and production process.

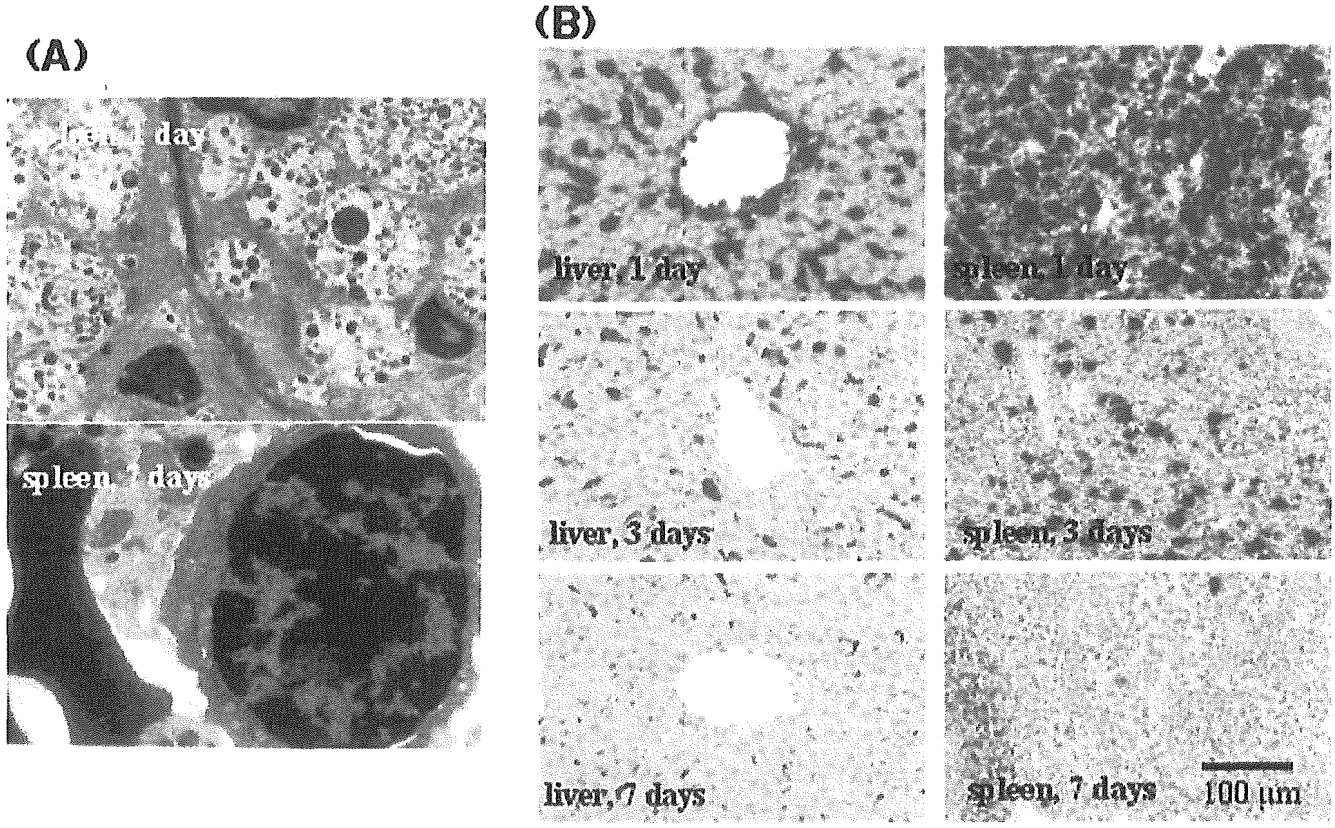

Figure 8. (A) TEM of rat spleen 1 day after the infusion of HbV (20 ml/kg) and after 7 days. Black dots are HbV particles captured in phagosomes in the spleen macrophages, and they disappeared at 7 days. (B) Staining with anti-human Hb antibody showed the presence of HbV in spleen and liver. HbV particles disappeared within 7 days.

showed the time course of biodistribution. After HbV finished playing its role in O_2-transport, a total of 35% of HbV are finally distributed mainly in the liver, spleen and bone marrow. The transmission electron microscopy (TEM) of the spleen 1 day after infusion of HbV clearly demonstrated the presence of HbV particles in macrophages, where HbV particles that appear as black dots are captured by the phagosomes[34] (Fig. 8). RBCs and HbV contain a lot of ferric ion with a high electron density, so that they show strong contrast in TEM. However, after 7 days, the HbV structure cannot be observed. There were no abnormalities in the tissues and no irreversible damages to the organs. A polyclonal anti-human Hb antibody was used as the marker of Hb in the HbV. This antibody does not recognize rat Hb. The red colored parts indicate the presence of Hb in HbV, and they have almost disappeared after 7 days in both the spleen and liver. Therefore, this shows that HbV can be metabolized quite promptly.

One issue of the Hb-based O_2-carriers is that they have a significant influence on clinical laboratory tests. They remain in the plasma phase in hematocrit capillaries after centrifugation of blood samples, and interfere with the colorimetric and turbidimetric measurements. However, HbV can be simply removed from blood plasma either by ultracentrifugation or centrifugation in the presence of a high-molecular-weight dextran to enhance precipitation. A very clear supernatant for accurate analyses can be obtained.[35] This is one advantage of HbV in comparison with acellular Hb solutions. Accordingly, the influence on organ functions by serum clinical laboratory tests after the bolus infusion of HbV at a dose rate of 20 ml/kg was examined. Albumin, alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase, which reflect the liver function, moves their values within normal range.[36] Concentrations of bilirubin and ferric ion are maintained at a low level. The concentration of lipids transiently changed. In particular, the cholesterols increased significantly. And phospholipids slightly increased, however, they returned to the original level after 7 days. These results indicate that the membrane components of HbV, once they reappear from RES, are metabolized on the physiological pathway.

A test of daily repeated infusion is required to evaluate the safety of a new drug. The daily repeated infusion of HbV in Wistar rats at a dose rate of 10 ml/kg/day for 14 days, everyday was tested.[37] The total infusion volume (140 ml/kg) was 2.5 times as much as the volume of the whole blood (56 ml/kg), however, all rats tolerated it well and survived. The body weight showed a monotonous but slightly depressed increase in comparison with the saline. However, after 2 weeks there was no significant difference with the saline control group. All the rats seemed very healthy and active. Histopathological examination 1 day after the final infusion of HbV showed significant accumulation of HbV in spleen macrophages, and liver Kupffer cells, and they mostly disappeared after 14 days. There were no irreversible other morphological abnormalities, and the serum clinical chemistry indicated transient but reversible increases in lipid components. AST and ALT were within the normal range. From these results the authors are confident with the safety of HbV.

DESIGN AND PHYSICOCHEMICAL PROPERTIES OF rHSA-HEME

In this study research on totally synthetic O_2-carriers, or so-called albumin-heme that does not require Hb has been conducted. HSA is the most abundant plasma protein in our blood stream, but its crystal structure has not been elucidated for a long time. In 1998, Dr Stephen Curry of the Imperial College London first elucidated the crystal structure of the HSA complexed with seven molecules of myristic acids.[38] He found that the dynamic conformational changes of albumin take place by the binding of fatty acid. However, in Japan, rHSA is now manufactured on a large scale by expression in the yeast *Pichia pastoris*, and it will appear on the market soon.[39] A large-scale plant, which can produce one million vials per year, has been already established. From the viewpoint of clinical application, O_2-carrying albumin is quite exciting and may be of extreme medical importance. With this background, it has been found that synthetic heme derivative is efficiently incorporated into rHSA, creating a red-colored rHSA-heme hybrid. This rHSA-heme can reversibly bind and release O_2 molecules under physiological conditions in the same manner as Hb. In other words, the rHSA-heme hybrid is a synthetic O_2-carrying hemoprotein, and it is believed that its saline solution will become a new class of RBC substitute.[40–51]

Figure 9 summarizes the structure of the rHSA-heme molecule. The maximal binding numbers of heme to one albumin are eight, and the magnitude of the binding constants ranged from 10^6 to 10^4 (M^{-1}). The isoelectric point of rHSA-heme was found to be 4.8, independent of the binding numbers of heme. This value is exactly the same as that of albumin itself. Furthermore, the viscosity and density did not change after the incorporation of heme molecules, and the obtained solution showed a long shelf life of almost 2 years at room temperature. Since the O_2-binding sites of rHSA-heme are iron-porphyrin, the color of the solution changed in a similar way to Hb. Upon addition of O_2 gas through this solution, the visible absorption pattern immediately changed to that of the O_2-adduct complex. Moreover, after bubbling carbon monoxide gas, rHSA-heme formed a very stable carbonyl complex.

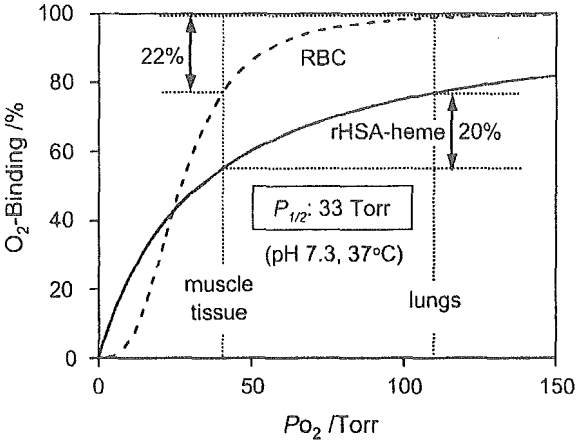

Figure 10. O_2-binding equilibrium curve of albumin-heme.

Figure 10 shows the O_2-binding equilibrium curve of rHSA-heme. The O_2-binding affinity of rHSA-heme is always constant independent of the number of heme, and the O_2-binding profile does not show cooperativity. However, the O_2-transporting efficiency of rHSA-heme between the lungs measuring 110 Torr and muscle tissue measuring 40 Torr increases to 22%, which is identical to the 22% efficiency for RBCs. The O_2-binding property of rHSA-heme can be controlled by changing the chemical structure of heme derivatives incorporated. More recently, it has been found that a protoheme derivative is also incorporated into albumin and can bind and release O_2 as well.[52]

IN VIVO SAFETY AND EFFICACY OF rHSA-HEME

Based on these findings, it can be said that rHSA-heme can become an entirely synthetic O_2-carrier, and satisfy the initial clinical requirements for a RBC substitute. However, there is another problem to solve before this material can be used as an O_2-carrier in the circulatory system. This problem is NO scavenging. Of course, rHSA-heme can bind NO, and it may be anticipated that the injection of rHSA-heme also induce hypertensive action. The authors have evaluated the

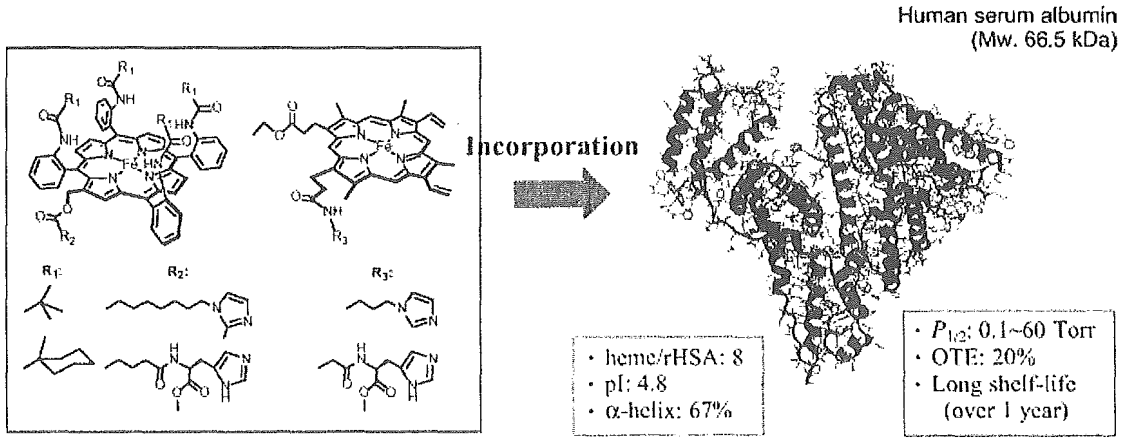

Figure 9. Structure of the albumin-heme molecule.

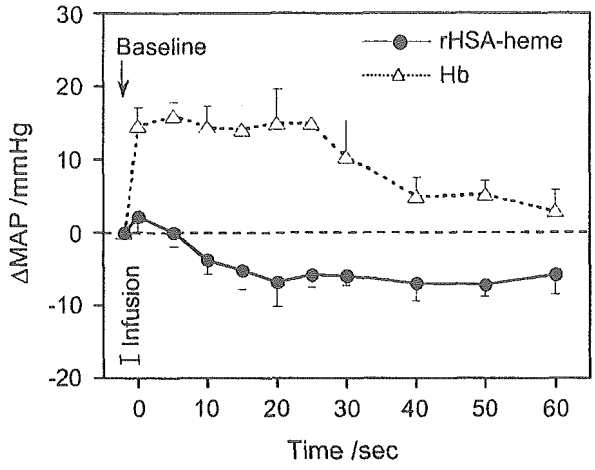

Figure 11. Change of MAP after the administration of rHSA-heme solution in the anesthetized rats ($n=5$). All data are shown as changes from the basal values (ΔMAP) just before the infusion and expressed as mean ± SE. Basal value is 90.1 ± 3.0 mmHg.

efficacy and safety of this rHSA-heme solution with animal experiments.

As described earlier, small Hb molecules extravasate through the vascular endothelium and react with NO, thus inducing vasoconstriction and acute increases in systemic blood pressure. Contrary to the expectations, the observation of the intestinal microcirculation after the infusion of rHSA-heme into an anesthetized rat revealed that the diameters of the venules and arterioles were not deformed at all.[53] Indeed, only a small change in the mean arterial pressure was observed after the administration of the rHSA-heme solution (Fig. 11). In contrast, the infusion of Hb elicited an acute increase in blood pressure. Why does rHSA-heme not induce vasoconstriction or hypertension? The answer probably lies in the negatively charged molecular surface of albumin. One of the unique characteristics of serum albumin is its low permeability through the 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.

Thus the authors are now evaluating the O_2-transporting ability of this rHSA-heme molecule in the circulatory system with further animal experiments.[54] First, the physiological responses to exchange transfusion with rHSA-heme solution into rats after 70% hemodilution and 40% hemorrhage was determined (Fig. 12). The declined mean arterial pressure and blood flow after a 70% exchange with albumin and further 40% bleeding of blood showed a significant recovery of up to 90% of the baseline values by the infusion of the rHSA-heme solution. However, all rats in the control group only injected with albumin died within 30 min. Furthermore, muscle tissue O_2-tension significantly increased. These responses indicate the *in vivo* O_2-delivery of the rHSA-heme solution.

More recently, HSA dimer, which can incorporate 16 hemes in its hydrophobic domain has been synthesized.[55] The human serum rHSA-heme dimer solution dissolves 1.3-times more O_2 compared to that of RBC and keeps its colloid osmotic pressure at the same level as the physiological value.

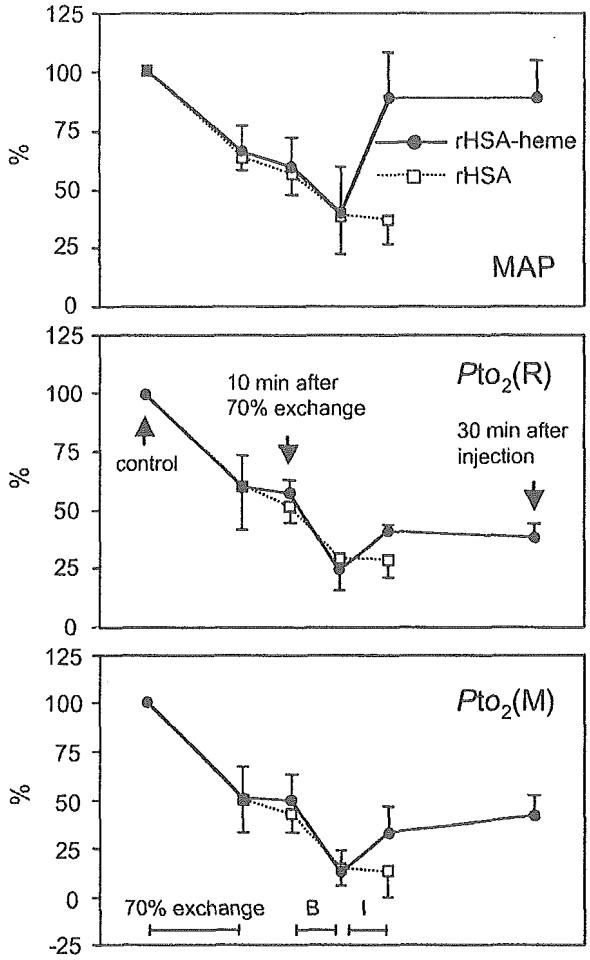

Figure 12. Change of (a) MAP and (b) O_2-tension in renal cortex during the 70% hemodilution with 5 wt% rHSA and further 40% exchange transfusion with rHSA-heme in anesthetized rats ($n=5$). All data are shown as changes from the basal values and expressed as mean ± SE.

POTENTIAL APPLICATIONS OF ARTIFICIAL O_2 CARRIERS

As described earlier the primary application of artificial O_2-carriers would be the resuscitative fluid for hemorrhage. Since some of the characteristics of artificial O_2-carriers overwhelm those of donated blood, there are many potential applications other than blood substitutes.

Tumor oxygenation

Unlike vessels in normal tissues, the development of a vasculature in a tumor lacks regulation and is hence, highly heterogeneous. Consequently, areas of hypoxia are quite common in tumors. In these hypoxic regions, it can be added that tumor cells acquire resistance to treatments such as chemotherapy and radiation. The rHSA-heme was injected into the responsible artery that supplies circulation to an implanted tumor (Fig. 13).[56] O_2-tension of the tumor rises immediately after intra-arterial infusion of albumin heme up to 2.4 times that of the baseline value. The findings in animals indicate that tumor tissue O_2-levels can be elevated by the administration of artificial O_2-carriers due to the

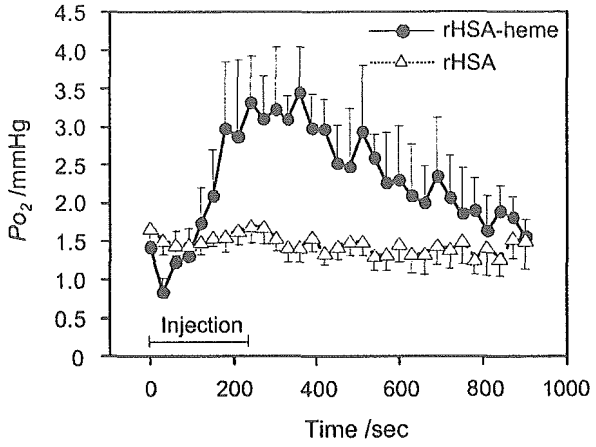

Figure 13. Changes in the O_2 tension of the hypoxic region of the ascites hepatoma LY80 solid tumor after the administration of the O_2 saturated rHSA-heme or rHSA solutions in the anesthetized rats ($n=4$ each). All data are shown as changes from the basal values (P_{O_2}) just before the infusion and expressed as mean ± SE.

difference in O_2-transporting properties from RBCs. Whether this increase in tissue O_2 can potentiate cancer treatment is currently under investigation.

Oxygenation of ischemic tissue

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

Organ preservation

One of the most important agenda in transplantation medicine is long-term organ preservation and circumvention of ischemia reperfusion injuries. It is believed that artificial O_2-carriers can be applied as a perfusate for donor tissue in order to overcome these problems. In particular, its O_2 carrying capacity has the potential to significantly extend the preservation period. This will make it easier to transport organs. Also, utilizing the extra time, it may be possible in the future to perform additional organ tests for better compatibility, or even perform genetic modifications during this period. It is believed that through these applications, the concept of organ preservation can be expanded to culture organs, and furthermore to include the preservation of cells derived from donor tissues.

Extracorporeal circulation

Extracorporeal circulation is quite common in cardiac surgery. Improvements are being made in the priming solutions but red cells are often still required to fill the device circuit, particularly in compromised cases and in children.[57] It is believed that the use of artificial O_2-carriers in the priming solution can decrease or completely eliminate the need for a transfusion in such cases, and hence reduce the incidence of infection or graft-versus-host disease (GVHD).

Liquid ventilation for acute lung injury

For patients who present acute lung injury or acute respiratory distress syndrome (ARDS), gas exchange in the lung exhibits severe deterioration and sometimes even the newest mechanical ventilation method fails to establish adequate oxygenation of the blood. In this type of critical case, liquid ventilation using an artificial O_2-carrier can establish optimal oxygenation of the blood and may reproduce the integrity of lung parenchyma.[58] Briefly explained, oxygenated liquid ventilation fluid is administered into the lung through trachea and O_2 molecules are transferred through diseased alveolus by diffusion and oxygenate the blood. Currently, this method is thought to be effective for patients with congenital diaphragmatic herniation. Efficacy for adult acute lung injuries is now under investigation. Perfluorochemicals are the main fluid used for clinical use, however, aqueous artificial O_2-carriers may have the potential to be used for liquid ventilation.

FUTURE SCOPE

The research field of the red cell substitutes is moving forward very rapidly, and the paradigm in this field is expanding from red cell substitutes to "O_2 therapeutics". Significant efforts have been made to produce HbV and albumin-heme with a facility of GMP standard, and to start preclinical and finally clinical trials. We look forward to the day that our research will play an effective role in treating patients.

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