capacity. Fortunately, a few pharmaceutical campanies, such as Terumo Co., are going to associate for mass production of HbV. Therefore, preclinical studies, including in middle–or large–sized animals, will be achieved in the near future.

The Society of Blood Substitutes, Japan provided a guidline for clinical trial with new artificial blood six years ago. The project team of Ministry Health & Welfare, Japan, decided to make some protocols for clinical trial of HbV in accordance with accumulation of data from preclinical studies.

DESIGN OF CLINICAL TRIAL

For planning the clinical trial, two important things are that the trial should fully satisfy ethical requirements and also draw definite evidence from its results.

To satisfy the medical ethics, we have to keep in mind three points, as listed in Table 4. First of all, any clinical trial should be done as treatment or therapy for patients. Secondly, the treatment or therapy performed in clinical trials should be equivalent or superior to conventional treatment or therapy. Thirdly, clinical trials must be done holding authorized and proper, informed consent.

Considering medical ethics, two main requirements for selection of subject are considered. One of them is a definite reason that this artificial blood (HbV) is beneficial for treatment or therapy of the subject. In another, certain time will be obtained to receive an informed consent from the subject before action of clinical trial, as shown in Table 5.

For assessment of clinical efficacy of the HbV as a substitute of blood, two crucial items are focused on, namely stabilized circulatory dynamics and sufficient oxygen supply to tissues:

Table 5. Selection of subjects for the clinical trial regarding reception of informed consent

Sufficient Reason for Use of Artificial Blood
(1) without Adverse associated with Ordinary Blood Transfusion
(2) with Oxygen Carrying Property not for Plasma Substitutes
(3) Sufficient Interval between Finding Subject and Operation for Explain & Understanding Protocol and Receiving Consent
Indication for Alternative Transfusion with HbV: Acute Anemia which the HbV could be used for matched to its optimum properties

Table 6. First selection for clinical trial

Replacement for Surgical Blood Loss with 15–20 ml/kg
Hemodilutional Autologous Transfusion
Substitute for Blood Transfusion for Emergent Surgery without Proper Blood prepared
Substitute for Blood Transfusion for Unexpected Bleeding during Operation
Transfusion for Patient with Uncommon Blood Type or with Refusal of Blood Transfusion
Limited for Blood Loss of 15–20 ml/kg during the Clinical Trial but not for Clinical Use in the Future

For achievement of definite and satisfactory results, four items should be considered, as follows.

1. Setting for proper control for procedure (treatment or therapy);
2. Complete achievement of initial determination (control) of physiological parameters;
3. Application of routine laboratory test and simple technique for measurement;
4. Selection of subjects who do not suffer severe illness or are not placed in unstable or complicated pathological conditions.

With those understandings, first clinical trial should be recommended to be done for replacement of surgical blood loss of 16–20 ml/kg, listed in Table 6. Namely, it will be done for hemodilutional autologous blood transfusion, blood transfusion for emergent surgery or unexpected bleeding during surgery in which proper blood for transfusion is not provided, and blood transfusion for patients with an uncommon blood type whose surgery should be done relatively urgently.

Some other applications as shown in Table 7 that might be proposed for the clinical trial. In our opinion, however, it should be better to avoid those, since those pathological conditions or illness and situations per se influence parameters measured. The data obtained from those patients in those situations will mislead and hinder us from making an accurate evaluation on the HbV as artificial blood and the decision to progress further to practical application for the HbV.

The physiological parameters measured in the first clinical trial should be limited in routine laboratory tests if possible and technique should be simple and easy. Those physiological parameters measured are listed in Table 8. Insertion of CVP catheter is possibly acceptable for cases whose surgical bleeding is anticipated to exceed over 12 ml/kg, but Swan Ganz catheter is not always acceptable. If the tip of the CVP catheter is placed in front of the tricuspidal valve, we can judge blood

Table 7. No program projected in clinical trials for new artificial blood

Post-traumatic Hypovolemia
Hemorrhagic Shock
Myocardial & Cerebral Ischemia due to Stenosis
Supplemental Treatment for Idiopathic Anemia
Priming for Extracorporeal Circuit
Superoxygenation for Malignant Neoplasma
Liquid Ventilation
Organ Perfusion & Preservation for Transplation

sampled there as the mixed venous blood. [8]. Then we can determine its oxygen partial pressure, which could estimate mean oxygen partial pressure of whole body tissue. Blood lactate level can be used as an indicator for tissue hypoxia.

Incidentally, an actual protocol for the trial with hemodilutional autologous blood transfusion is illustrated in Figure 1. Namely, after receiving patient's written informed consent, the patient will be anesthetized and an arterial and central venous catheter will be inserted. All physiological parameters listed in Table 8 will measured. Then his autologous blood of 8 ml/kg will be collected and simultaneously the HbV plus colloid solution, such as hydroxyethyl strach solution, or colloid solution alone, will be infused. Selection of the HbV or the colloid solution alone should have been done randomly before the anesthesia. Second physiological measurements as same as the first are performed immediately after the replacement. Second collection of autologous blood and infusion are carried out in the same manner. Again, third physiological measurements will be performed. Then surgery will be commenced and bleeding associated with surgery will be replaced with the autologous blood collected. If total blood loss will not exceed over 20 ml/kg, no additional blood transfusion or any

Table 8. Physiological parameters measured in the clinical trial

Arterial Blood Pressure	Arterial O_2 Partial Pressure
Right Atrial Mean Pressure	Arterial CO_2 Partial Pressure
Pulmonary Artery Blood Pressure*	Arterial pH
Heart Rate	Arterial Lactate Level
Cardiac Output*	Arterial Blood Hb, Hct & HbVcrit
ECG	Mixed Venous O_2 Partial Pressure
Urine Volume	Mixed Venous O_2 Saturation
Routine Urine Analysis	AV O_2 Content Difference (calculation)
Routine Blood Chemical Analysis	O_2 Consumption (calculation)*

*If Swan Ganz Catheter could be allowed to be used.

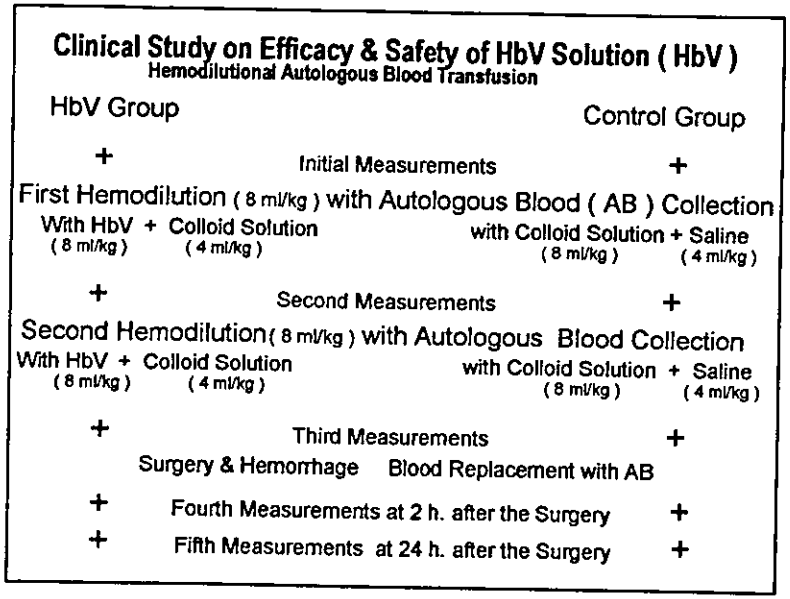

Figure 1

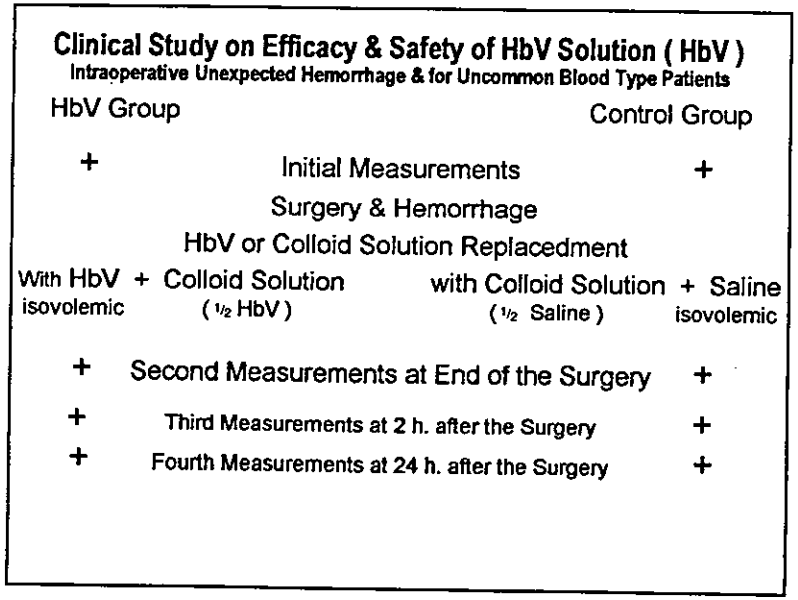

Figure 2

Table 9. Social needs for artificial blood

Disaster Use..... Long-term Shelf Storage
Emergent Use..... Before Arrival of Matched or O(−) Blood
Shortage of Blood for Transfusion
Recycle Use of Hemoglobin from Donated Blood
Application as Universal Blood
Evasion from Transfusion Complications & Human Error
Transfusion for Patients with Uncommon Blood Type or with Refusal of Donated Blood

other treatment may be required. Fourth and fifth measurements will be performed at two hours after the surgery and 24 hours after, respectively. However, if the bleeding will exceed over 20 ml/kg and hemodynamic condition will become unstable, necessitating some drugs or additional homologous blood transfusion, data obtained above will be excluded from the study.

Similary, as shown in Figure 2, study of treatment of unexpected bleeding during surgery, and of transfusion for patients with uncommon blood types, is planned for clinial trial using HbV. First measurements before commencement of surgery will be performed and subsequent blood loss during the surgery will be treated with the HbV plus the colloid solution or the colloid solution alone. Second, third, and fourth measurements will be performed at the end of surgery, two hours and 24 hours after surgery, respectively. In those trials, it can be understood easily that the amount of the HbV used should be equal to that of blood lost. On the other hand, it is hard to decide which amount of colloid solution should be infused, namely to be equal or one-half of volume of blood lost. It will be a concern that excess volume loading will be induced immediately after the infusion when the equal amount will be chosen and that hypovolemia will be caused at 2–3 hours after the infusion, when one-half volume will be used. Nevertheless, Hiippala's report [9] may lead us to use the one-half volume in the first clinical trial.

CONCLUSION

We have to achieve whole preclinical studies associated with mass production of the HbV. And we hope that the clinical trials mentioned in this paper will be carried out as soon as possible. Artificial blood, such as the HbV, is expected to be applied in many clinical practices, as shown in Table 9, in the future. We hope, at least in our country, that the HbV could supplement sufficiently the coming shortage in blood for transfusion.

REFERENCES

1. Takaori, M., Fukui, A. (1996). Treatment of massive hemorrhage with liposome encapsulated human hemoglobin (NRC) and hydroxyethyl starch (HES) in beagles. *Artif. Cell Blood Subst.Immobil. Biotech.* **24** (6): 643–653.
2. Tsutsui, Y., Kimura, T., Ishizuka, T., Oomori, S., Shizawa, T., Goto, H., Ogata, Y., Kaneda, S. (2002). Duration of efficacy of NRC (Neo Red Cell) in arat hemodilution model. *Artificial Blood* **10**: 36–41.
3. Kobayashi, K. (2003). Evaluation of artificial oxygen carrier in vivo—History and perspective of Keio-Waseda Joint Project of oxygen infusions. *Artificial Blood* **11**: 37.
4. Center for Biologic Evaluation and Research. (1994). Points to consider in the safety evaluation of hemoglobin based oxygen carriers. *Transfusion* **31**: 369–371.
5. Center for Bilogic Evaluation and Research. (1994). Points to consider in the safety evaluation of hemoglobin-based and perfluorocarbon-based oxygen carriers. *Transfusion* **34**: 712–713.
6. Przybelski, R.L., Daily, E.K., Stern, K.N., MattiaGoldberg, C.A. (1997.) A grade scale for assessment of safety of blood substitutes. *Transfusion* **37**: 749–751.
7. Fratantoni, J.C. (1998). Red Cell Substitutes: Evolution of Approaches for Demonstrating Efficacy in Blood Substitutes - Present and Future Perspectives. (ed.) Tsuchida, E. Amsterdam, Elsevier Sci., pp. 33–39.
8. Schou, H., de Sa, V.P., Larsson, A. (1998) Central and mixed venous blood oxygen correlate well during acute normovolemic hemodilution in anesthetized pigs. *Acta Anaesthesiol. Scand.* **42**: 172–177.
9. Hiippala, S., Linko, K., Myllylae, G., Lalla, M., Hekali, R., Maekelaeinen, A. (1995). Replacement of major surgical blood loss by hypo-oncotic or conventional plasma substitutes. *Acta Anaesthesiol. Scand.* **39**: 228–235.

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Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model

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Abstract

Phospholipid vesicles encapsulating Hb (Hb-vesicles: HbV) have been developed for use as artificial O_2 carriers (250 nm ϕ). As one of the safety evaluations, we analyzed the influence of HbV on the organ functions by laboratory tests of plasma on a total of 29 analytes. The HbV suspension ([Hb] = 10 g/dl) was intravenously infused into male Wistar rats (20 ml/kg; whole blood = 56 ml/kg). The blood was withdrawn at 8 h, and 1, 2, 3, and 7 days after infusion, and the plasma was ultracentrifuged to remove HbV in order to avoid its interference effect on the analytes. Enzyme concentrations, AST, ALT, ALP, and LAP showed significant, but minor changes, and did not show a sign of a deteriorative damage to the liver that was one of the main organs for the HbV entrapment and the succeeding metabolism. The amylase and lipase activities showed reversible changes, however, there was no morphological changes in pancreas. Plasma bilirubin and iron did not increase in spite of the fact that a large amount of Hb was metabolized in the macrophages. Cholesterols, phospholipids, and β-lipoprotein transiently increased showing the maximum at 1 or 2 days, and returned to the control level at 7 days. They should be derived from the membrane components of HbV that are liberated from macrophages entrapping HbV. Together with the previous report of the prompt metabolism of HbV in the reticuloendothelial system by histopathological examination, it can be concluded that HbV infusion transiently modified the values of the analytes without any irreversible damage to the corresponding organs at the bolus infusion rate of 20 ml/kg.

Keywords: Biomimetic material; Blood; Drug delivery; In vivo test; Liposome; Nanoparticle

1. Introduction

Liposomes or phospholipid vesicles have been extensively studied for the application of drug delivery system, and some are now approved for a clinical use as antifungal or anticancer therapies [1]. Another promising application is to use vesicles for encapsulating a concentrated human Hb. The resulting Hb-vesicle (HbV) can serve as an O_2 carrier with ability comparable to red blood cells (RBC) [2–4]. The advantages of the Hb-based O_2 carriers (HBOCs) are the absence of blood-type antigens and transmission of known and unknown blood-borne disease, the possibility to improve the rheological properties of blood flow according to the needs of patients, and stability for long-term storage. These characteristics will make it possible to use the HBOCs both in elective and emergency situations [5,6]. In this sense, the infusion of HBOCs becomes superior to the conventional blood transfusion that still has the potential of mismatching, infection such as HIV and hepatitis virus, and the problems of only 2–3 week preservation period. The acellular Hb modifications including polymerized Hb and polymer-conjugated Hb are now undergoing the final stages of clinical trials [7,8]. However, the cellular structure of HbV (particle diameter, ca. 250 nm) most closely mimics the characteristics of natural RBC such as the cell membrane function of physically preventing direct contact of Hb

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with the components of blood and vasculature during circulation [9]. In comparison with some acellular Hb modifications, the Hb encapsulation in vesicles suppresses hypertension induced by vasoconstriction, a theory that is suggested to be due to the high affinity of Hb with nitric oxide and carbon monoxide as vasorelaxation factors [10,11]. Moreover, the surface modification of HbV with polyethylene glycol (PEG) chains not only prolongs the circulation half-life [12] but also prevents the intervesicular aggregation and guarantees the homogeneous dispersion in the plasma phase that provides a prompt blood flow in the microcirculation and the resulting sufficient tissue oxygenation [13,14].

According to the clinical conditions HbVs are supposed to be applied for, the organism is faced with the metabolism of a large amount of both Hb and lipids, because the dose rate of HbV is significantly large. The HbV particles, as well as phospholipid vesicles, infused in the blood stream are finally captured by phagocytes in the reticuloendothelial system (RES, or mononuclear phagocytic system, MPS) [4,15]. In a previous report, we clarified by the histopathological studies of rats receiving 20 ml/kg of HbV infusion that the HbV particles were captured and metabolized within 7 days in RES mainly in the spleen and liver [16]. Transmission electron microscopy provided a clear image of the HbV particles in the phagosomes 1 day after infusion, but they disappeared within 7 days. Staining with the anti-human Hb antibody, Berlin blue, and hematoxylin/eosin showed prompt metabolism of Hb molecules with no morphological changes in the liver and spleen. The phagocytic activity decreased and then transiently increased, but tended to return to the original level. From these studies, we did not see any irreversible damage to the organs.

Serum laboratory tests are the most common diagnostic tools to monitor organ functions clinically. However, both the PEG-modified HbV particles and the chemically modified Hb solutions remained in the plasma even after usual centrifugation to remove RBC, showing significant interference effects due to the light absorption by Hb and light scattering by the particles. These interference effects hindered the accurate evaluation of plasma laboratory tests and have been regarded as a serious issue for the development of HBOCs [17,18]. However, quite recently we have clarified by an in vitro experiment that the simple removal of PEG-modified HbV as a precipitate by ultracentrifugation (50,000 *g*, 20 min) or by conventional centrifugation in the presence of a high-molecular-weight dextran diminished most of the interference effects [19]. Using this simple procedure, we aimed to evaluate the safety of HbV by the laboratory tests of plasma after bolus intravenous infusion of HbV at a rate of 20 ml/kg, the same experimental model as in the previous study [16].

2. Materials and methods

2.1. Preparation of PEG-modified HbV

The PEG-modified HbV was prepared in a sterile condition as previously reported in the literature [10, 20–22]. Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and the Society of Red Cross, Japan (Tokyo, Japan). The encapsulated purified Hb (38 g/dl) contained 14.7 mM of pyridoxal 5′-phosphate (PLP, Sigma) as an allosteric effector at a molar ratio of PLP/Hb = 2.5. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate at a molar ratio of 5/5/1 (Nippon Fine Chem. Co., Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) (NOF Co., Tokyo, Japan, 0.3 mol% of the total lipid). The HbCO solution and the lipid powder were mixed and stirred for 12 h at 4°C. The resulting multilamellar vesicles were extruded through membrane filters with a final filter pore size of 0.22 μm. Thus prepared PEG-modified HbV was suspended in saline at the Hb concentration of 10 g/dl, and filtrated (pore size: 0.45 μm). The physicochemical parameters of the HbV are as follows: particle diameter, 251 ± 80 nm; [Hb], 10 g/dl; [metHb], <3%; [HbCO], <2%; phospholipids, 4.0 g/dl; cholesterol, 1.7 g/dl; and oxygen affinity (P_{50}), 30 Torr. The endotoxin content was precisely measured by modified *Limulus* Amebocyte lysate gel-clotting analysis that has been developed by our group recently, and confirmed that the endotoxin content was less than 0.1 endotoxin unit/ml [23].

2.2. HbV infusion and procedure for the plasma laboratory tests

All animal studies were approved by the Animal Subject Committee of Keio University School of Medicine and performed according to NIH guidelines for the care and use of laboratory animals (NIH publication #85-23 Rev. 1985). The experiments were carried out using 40 male Wistar rats (200–210 g, Saitama Experimental Animals, Kawagoe, Japan). They were anesthetized with diethylether inhalation, and the HbV suspension was infused into the tail vein at a dose rate of 20 ml/kg ($n = 5$ for every time point). Ten rats were used to obtain the control values. All the rats were housed in cages and provided with food and water *ad libitum* in a temperature controlled room on a 12 h dark/light cycle.

After 8 h, and 1, 2, 3, and 7 days, the rats were anesthetized with 1.5% sevoflurene inhalation (Maruishi Pharm. Co., Osaka, Japan) using a vaporizer (Model

TK-4 Biomachinery, Kimura Med., Tokyo). Polyethylene tubes (PE-50, Natsume Co., Tokyo) were implanted in the carotid artery for withdrawing blood into heparinized syringes for the Hct, HbV concentration, and plasma laboratory tests. The animals were finally laparotomized and sacrificed with acute bleeding from the abdominal aorta and the liver and spleen were obtained for weight measurements. The control rats received the same procedure for the measurements.

A part of the withdrawn blood (6 ml) was centrifuged to obtain plasma which was turbid and red/brown colored due to the presence of PEG-modified HbV particles especially in the samples taken at 8 h, 1 and 2 days after infusion. The plasma was ultracentrifuged (50,000 g, 20 min) to remove the HbV particles. The obtained transparent plasma specimens were stored at −80°C until the laboratory tests at BML, Inc. (Kawagoe, Japan). The selected analytes were total protein, albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), γ -glutamyltransferase (γ-GTP) alkaline phosphatase (ALP), cholinesterase (ChE), leucin amino peptidase (LAP), creatine phosphokinase (CPK), amylase, lipase, total cholesterol (Total-Chol.), cholesteryl ester (Chol.Ester), free cholesterol (Free-Chol.), HDL-cholesterol (HDL-Chol.), β-lipoprotein, triglyceride (TG), free fatty acid (FFA), phospholipids, total lipids, uric acid (UA), blood urea nitrogen (BUN), creatinine (CRE), K^+, Ca^{2+}, inorganic phosphate (IP), and Fe^{3+}. In our previous study, it was confirmed that the concentrations of the plasma components in terms of the above analytes did not change after the ultracentrifugation at 50,000 g for 20 min [19]. Since rat albumin is slightly insensitive to the bromcresol green method, the values were corrected according to Takano et al. [24].

2.3. Histopathological examination of pancreas

After sacrificing the animals by acute bleeding from the abdominal aorta, the pancreas was resected for a histopathological study. The organs were fixed in a 10% formalin neutral buffer solution (Wako Chem. Co., Tokyo) immediately after the resection, and the paraffin sections were stained with hematoxylin/eosin.

2.4. Data analysis

Differences between the control and a treatment group were analyzed using a one-way ANOVA followed by Fisher's protected least-significant difference (PLSD) test. The changes were considered statistically significant if $p<0.05$.

3. Results

All the rats receiving the bolus infusion of HbV at a dose rate of 20 ml/kg tolerated the infusion and survived until intentional sacrifice. There was no noticeable change in appearance such as piloerection.

3.1. Hct and circulation persistence of HbV

The control Hct was 42±1%, and it decreased slightly to 40±1% at 1 day after HbV infusion. The estimated Hb concentration of HbV in plasma just after infusion was about 6 g/dl, and it gradually decreased to 4.4±0.3 g/dl at 8 h, 1.9±0.2 g/dl at 1 day, 1.3±0.1 g/dl at 2 days, and 0.8±0.01 g/dl at 3 days (Fig. 1). At 7 days, HbV was not detected at all in the plasma phase.

3.2. Spleen and liver weights

The changes in the spleen and liver weights were expressed as percents of the body weight (Fig. 1). The liver weight ratio (control, 4.81±0.17%) showed a significant increase 1 day after the infusion (5.29±0.27%, $p<0.01$), and then it returned to the original level at 2 days. Spleen weight ratio significantly increased from 0.32±0.05% to 0.66±0.06% 3 days after the infusion ($p<0.01$), however, it was reduced to 0.41±0.02% at 7 days.

3.3. Plasma laboratory tests

The plasma fraction after centrifugation of the blood sample for 3 days after the HbV infusion was turbid due to the presence of PEG-modified HbV. However, ultracentrifugation of the plasma produced transparent and light-yellow plasma phase and PEG-modified HbV was precipitated at the bottom in a tube. There was no sign of the presence of Hb in the supernatant, indicating that there was no hemolysis of both RBC and HbV.

As for the analytes that reflect the liver function, the total protein (control, 5.2±0.1 g/dl) and albumin (2.46±0.06 g/dl) slightly decreased to, e.g., 4.9±0.2 and 2.11±0.10 g/dl, respectively, with statistically significant differences ($p<0.01$) for 3 days after the HbV infusion (Fig. 2). They tended to return to its original level at 7 days ($p<0.05$). AST (control, 60±7 U/l) decreased to 46±3 U/l ($p<0.05$) and returned to the original level at 7 days. ALT (control, 32±5 U/l) only slightly increased to 40±8 U/l 1 day after the HbV infusion ($p<0.01$), but it returned to its original level 2 days after the infusion. LDH (control, 150±60 U/l) did not change significantly. ALP (control, 1265±231 U/l) decreased at 2 days (812±149 U/l) and 3 days (872±98 U/l) ($p<0.01$), but it returned to the control level at 7 days. γ-GPT (control, 1.6 U/l) and LAP (31±1 U/l) showed significant but minimal reductions

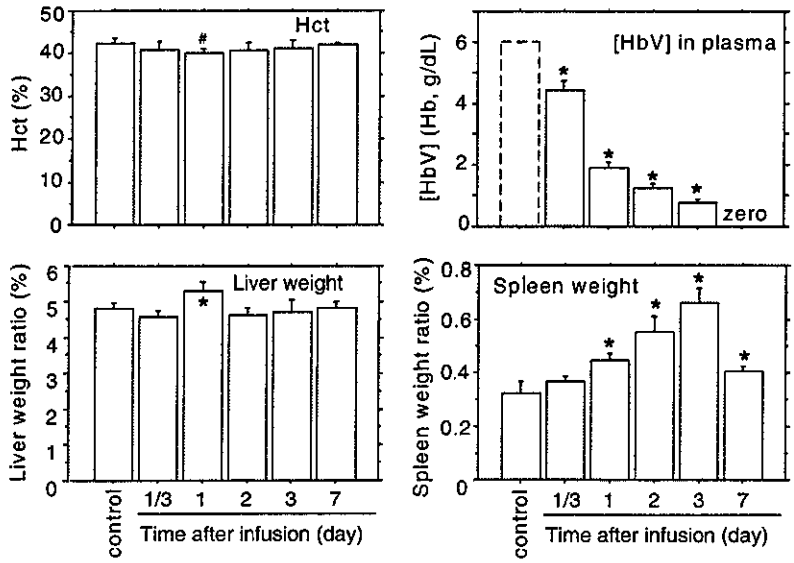

Fig. 1. Changes in hematocrit, concentration of HbV in plasma, and spleen and liver weights after infusion of HbV (20 ml/kg). The values are mean ± SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. The control value of [HbV] is the estimated concentration of HbV immediately after the infusion and expressed as with a dashed line. The spleen and liver weights are expressed as the ratio to the body weight (%).

($p<0.05$). ChE (control, 76 ± 18 U/l) did not show a noticeable change. Plasma total bilirubin (≤0.1 mg/dl) and Fe^{3+} showed some reductions but were maintained at a low level for 7 days in spite of the metabolism of a large amount of Hb.

CRE (control, 0.3 mg/dl) was maintained at a low level for 7 days. BUN (control, 16 ± 3 mg/dl) showed a slight increase at 7 days (21 ± 3 mg/dl) (Fig. 3). UA (control, 0.47 ± 0.19 mg/dl) increased to 0.70 ± 0.16 mg/dl at 3 days, however, it returned to a non significant level at 7 days. Amylase (control, 1613 ± 74 U/l) significantly decreased for 3 days after the infusion ($p<0.01$), but returned to its original level at 7 days. Lipase (control, 9 ± 1 U/l) showed significant increases ($p<0.01$) after the HbV infusion, and it tended to decrease after 3 days, and was reduced to a non-significant level at 7 days. CPK (control, 304 ± 116 U/l) decreased at 7 days ($p<0.05$), but did not show a noticeable increase during the experiment. As for the electrolyte concentrations, K^{+}, Ca^{2+}, and IP did not show any significant changes.

The most consistent changes were seen in the lipid components (Fig. 4). Total-Chol. (control, 73 ± 7 mg/dl), Free-Chol. (18 ± 2 mg/dl), Chol.Ester (59 ± 8 mg/dl), and HDL-Chol. (32 ± 4 mg/dl) showed significant increases and maximum values at 2 days ($p<0.01$). Free-Chol. increased to 39 ± 4 mg/dl, about twice the control value. However, it tended to decrease at 3 days, and returned to its control level at 7 days. β-Lipoprotein (control, 110 ± 42 mg/dl) slightly increased at 1 day (160 ± 33 mg/dl), but returned to its original level at 3 days. TG (control, 64.4 mg/dl) significantly decreased to 12.4 mg/dl at 2 days ($p<0.01$), but tended to increase to its original level at 7 days. Phospholipid (control, 132 ± 8 mg/dl) significantly increased to 150 ± 9 mg/dl at 1 day ($p<0.01$), and then returned to the original level at 3 days.

3.4. Histopathological examination of pancreas

The histology of pancreatic tissue 2 days after the infusion of HbV is shown in Fig. 5. There was no significant morphological change in spite of the increment of the pancreatic lipase activity.

4. Discussion

The clinical indications for the use of the HbV suspension as an artificial O_2 carrying fluid are estimated to be mainly preoperative or perioperative hemodilution, or resuscitation from hemorrhagic shock in emergency situations [25], both of which result in exchanging more than 20% of the original blood with the HbV suspension. Thus, the dose amount is extremely greater than that of stealth liposomes for drug delivery systems. HbV particles in the blood stream are finally captured by RES in the same manner as the conventional phospholipid vesicles [15]. In a previous study, we confirmed by the histopathological examination in a rat model that HbV particles were captured in the phagosomes of liver Kupffer cells and spleen macrophages without tissue damage, and they had completely disappeared within 7 days [16]. The transient splenomegaly and hepatomegaly in Fig. 1 seemed associated with the entrapment of HbV. The total weight change of

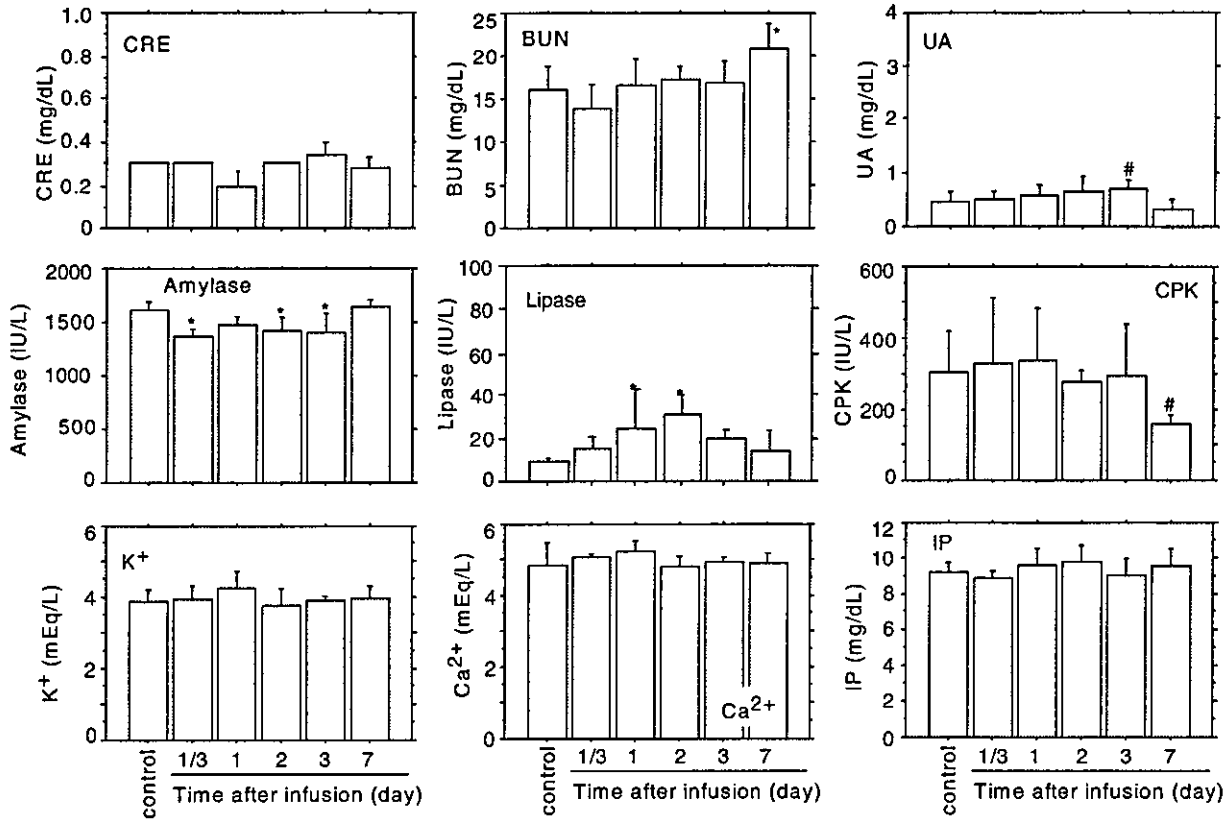

Fig. 3. Plasma laboratory tests representing renal, pancreatic and myocardial function, and electrolytes after infusion of HbV (20 ml/kg). The values are mean ± SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. Abbreviations: creatinine (CRE), blood urea nitrogen (BUN), uric acid (UA), creatine phosphokinase (CPK), inorganic phosphate (IP).

lipoprotein lipase. However, this level of increment was significantly smaller than the reported value for the Wistar rats of pancreatitis. Hofbauer et al. [26] reported that acute necrotising pancreatitis increased lipase activity from 10 to 475–5430 IU/l. It was reported that the injection of liposome amphotericin B raised the serum lipase activity, and one possible reason was speculated to be the enzyme induction in the pancreas by the presence of a large amount of lipids from the liposomes [27], because pancreatic lipase hydrolyze not only TG but also phosphatidylcholine [28]. This speculation was also supported by our results that the profiles of the transient increases in the lipid components coincided with that of lipase, but not with amylase. The cause of this modification is not clear at the present time. Histopathological analysis showed no significant pathological change in the pancreas. However, the pancreatic function should carefully be monitored in the ongoing safety studies.

Significant and consistent increases were seen in the lipid components with maximum at 1 or 2 days. They should be derived from the HbV particles because they contain a large amount of cholesterol (ca. 1200 mg/dl) and DPPC (1840 mg/dl) in the infused suspension ([Hb] = 10 g/dl). The gradual increases in cholesterols by 2 days after infusion and no Hb release from HbV in the plasma indicate that they should be liberated from RES after HbV are captured by RES and destroyed in the phagosomes. This is also supported by the fact that the maximum concentrations were seen at 2 days when the HbV in the plasma had mostly disappeared from the blood. It has been reported that the infused lipid components of the phospholipid vesicles are trapped in the Kupffer cells, and diacylphosphatidylcholine is metabolized and reused as a component of the cell membrane, or excreted in the bile and in the exhaled air [29–31]. Cholesterol is finally catabolized as bile acids in the parenchymal hepatocytes. There should be no direct contact of HbV and the hepatocytes because HbV is so large that it cannot diffuse across the fenestrated endothelium into the space of Disse [11]. Cholesterol from HbV should reappear in the blood mainly as lipoprotein cholesterol after entrapment in the Kupffer cells [32], and then excreted in the bile after entrapment of the corresponding lipoprotein by the hepatocytes [33]. We speculate that the main components of the lipid bilayer membrane of HbV, the phospholipids and cholesterol, would gradually be redistributed

eventually aggregate in mass with an iron content as high as 50%. These are hemosiderins composed of degraded protein and coalesced iron. Both ferritin and hemosiderin release iron molecules, and they are anticipated to induce hydroxyl radical production and succeeding lipid peroxidation [37,38]. However, iron release from hemosiderin is substantially less than that from ferritin, thus iron molecules in hemosiderin are relatively inert [39]. Plasma iron, mostly bound to transferrin, remained constant after HbV infusion. The iron concentration should be coordinately regulated through the "iron regulatory proteins" that sense the levels of iron for hematopoiesis and metabolic needs [40], and the excess amount of iron should be stored in an insoluble and less toxic form as hemosiderin. Together with the time course of the histopathological changes, the results of the plasma laboratory tests indicate that the metabolism of heme and the recycling or excretion of iron molecule is within the physiological capacity and suggested to be on the physiological pathway that has been well characterized for the metabolism of senescent RBC [41].

5. Conclusion

In this study, the plasma laboratory tests after the infusion of HbV (20 ml/kg) did not demonstrate an irreversible sign for a deteriorative damage to the organs. Plasma bilirubin and iron, which were considered to be released during the metabolism of the Hb molecule, did not increase during the observation period. This may be due to the moderate rate of Hb metabolism in RES after the entrapment of HbV with a moderate length of circulation time. The lipid components significantly increased at 2 or 3 days after infusion. These may be derived from the membrane component of HbV entrapped in RES. The complete normalization of the lipid components indicates that they are metabolized in a normal metabolic and/or recycling pathway. The precise biodistribution and fate of the components should be confirmed by a radioisotope technique. Our results have demonstrated the safety of HbV using only healthy rats, while rats in hemorrhagic shock, septic shock, or lipemia have to be tested in the ongoing safety studies. It should also be emphasized that the data cannot be extrapolated to large animals or humans, which may react differently to such a large dose of HbV.

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References

[1] Lian T, Ho RJY. Trends and developments in liposome drug delivery system. J Pharm Sci 1991;90:667–80.
[2] Djordjevich L, Mayoral J, Miller IF, Ivankovich AD. Cardiorespiratory effects of exchanging transfusions with synthetic erythrocytes in rats. Crit Care Med 1987;15:318–23.
[3] Izumi Y, Sakai H, Hamada K, Takeoka S, Yamahata T, Kato R, Nishide H, Tsuchida E, Kobayashi K. Physiologic responses to exchange transfusion with hemoglobin vesicels as an artificial oxygen carrier in anesthetized rats: changes in mean arterial pressure and renal cortical tissue oxygen tension. Crit Care Med 1996;24:1869–73.
[4] Rudolph AS, Cliff RO, Spargo BJ, Spielberg H. Transient changes in the mononuclear phagocyte system following administration of the blood subsitute liposome-encapsulated haemoglobin. Biomaterials 1994;15:796–804.
[5] Tsuchida E. Blood substitutes: present and future perspectives. Amsterdam: Elsevier Science; 1998.
[6] Chang TMS. Blood substitutes: principles, methods, products, and clinical trials. Basel: Karger; 1997.
[7] Levy JH, Goodnough LT, Greilich PE, Parr GV, Stewart RW, Gratz I, Wahr J, Williams J, Comunale ME, Doblar D, Silvay G, Cohen M, Jahr JS, Vlahakes GJ. Polymerized bovine hemoglobin solution as a replacement for allogeneic red blood cell transfusion after cardiac surgery: results of a randomized, double-blind trial. J Thorac Cardiovasc Surg 2002;124:35–42.
[8] Johnson JL, Moore EE, Offner PJ, Partrick DA, Tamura DY, Zallen G, Silliman CC. Resuscitation with a blood substitute abrogates pathologic postinjury neutrophil cytotoxic function. J Trauma 2001;50:449–55.
[9] Takeoka S, Teramura Y, Atoji T, Tsuchida E. Effect of Hb-encapsulation with vesicles on H_2O_2 reaction and lipid peroxidation. Bioconjugate Chem 2002;13:1302–8.
[10] Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension in conscious hamster model. Am J Physiol Heart Circ Physiol 2000;279:908–15.
[11] Goda N, Suzuki K, Naito S, Takeoka S, Tsuchida E, Ishimura Y, Tamatani T, Suematsu M. Distribution of heme oxygenase isoform in rat liver: topographic basis for carbon monoxide-mediated microvascular relaxation. J Clin Invest 1998;101:604–12.
[12] Phillips WT, Klipper RW, Awasthi VD, Rudolph AS, Cliff R, Kwasiborski V, Goins BA. Polyethylene glycol-modified liposome-encapsulated hemoglobin: a long circulating red cell substitute. J Pharmacol Exp Ther 1999;288:665–70.
[13] Sakai H, Takeoka S, Park SI, Kose T, Izumi Y, Yoshizu A, Nishide H, Kobayashi K, Tsuchida E. Surface-modification of hemoglobin vesicles with polyethyleneglycol and effects on aggregation, viscosity, and blood flow during 90%-exchange transfusion in anesthetized rats. Bioconjugate Chem 1997;8:15–22.
[14] Sakai H, Tsai AG, Kerger H, Park SI, Takeoka S, Nishide H, Tsuchida E, Intaglietta M. Subcutaneous microvascular responses to hemodilution with a red cell substitute consisting of

polyethyleneglycol-modified vesicles encapsulating hemoglobin. J Biomed Mater Res 1998;40:66–78.
[15] Rudolph AS, Klipper RW, Goins B, Phillips WT. In vivo biodistribution of a radiolabeled blood substitute: ^{99m}Tc-labeled liposome-encapsulated hemoglobin in an anesthetized rabbit. Proc Natl Acad Sci USA 1991;88:10976–80.
[16] Sakai H, Horinouchi H, Tomiyama K, Iikeda E, Takeoka S, Kobayashi K, Tsuchida E. Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in metabolism. Am J Pathol 2001;159:1079–88.
[17] Glick MR, Ryder KW. Double trouble: hemolysis and stabilized hemoglobins (so you think you're seeing red now?). Clin Chem 2003;39:1761–3.
[18] Kazmierczak SC, Catrou PG, Best AE, Sullivan SW, Briley KP. Multiple regression analysis of interference effects from a hemoglobin-based oxygen carrier solution. Clin Chem Lab Med 1999;37:453–64.
[19] Sakai H, Tomiyama K, Masada Y, Takeoka S, Horinouchi H, Kobayashi K, Tsuchida E. Pretreatment of serum containing Hb-vesicels (oxygen carriers) to avoid interference in clinical laboratory tests. Clin Chem Lab Med 2003;41:222–31.
[20] Takeoka S, Ohgushi T, Terase K, Ohmori T, Tsuchida E. Layer-controlled hemoglobin vesicles by interaction of hemoglobin with a phospholipid assembly. Langmuir 1996;12:1755–9.
[21] Sakai H, Yuasa M, Onuma H, Takeoka S, Tsuchida E. Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. Bioconjugate Chem 2000;11:56–64.
[22] Sou K, Endo T, Naito Y, Takeoka S, Tsuchida E. Effective encapsulation of proteins into size-controlled phospholipid vesicles using freeze-thawing and extrusion. Biotechnol Progr 2003;19:1547–52.
[23] Sakai H, Hisamoto S, Fukutomi I, Sou K, Takeoka S, Tsuchida E. Detection of Lipopolysaccharide in hemoglobin-vesicles by Limulus amebocyte lysate test with kinetic-turbidimetric gell clotting analysis and pretreatment with a surfactant. J Pharm Sci 2004;93:310–21.
[24] Takano S, Komiyama H, Nomura M. A study on the correction of assay variation caused by the reagents in the measurement of rat albumin. Rinshokagaku (Clin Chem) 2001;30:164–6.
[25] Sakai H, Takeoka S, Wettstein R, Tsai AG, Intaglietta M, Tsuchida E. Systemic and microvascular responses to the hemorrhagic shock and resuscitation with Hb-vesicles. Am J Physiol Heart Circ Physiol 2002;283:H1191–9.
[26] Hofbauer B, Friess H, Weber A, Baczako, Kisling P, Schilling M, Uhl W, Dervenis C, Buchler MW. Hyperlipaemia intensifies the course of acute oedematous and acute necrotising pancreatitis in the rat. Gut 1996;38:753–8.
[27] Stuecklin-Utsch A, Hasan C, Bode U, Fleischhack G. Pancreatic toxicity after liposomal amphotericin B. Mycoses 2002;45:170–3.
[28] Rowland RN, Woodley JF. The stability of liposomes in vivo to pH, bile salts and pancreatic lipase. Biochim Biophys Acta 1980;620:400–9.
[29] Verkade HJ, Derksen JT, Gerding A, Scherphof GL, Vonk RJ, Kuipers F. Differential hepatic processing and biliary secretion of head-group and acyl chains of liposomal phosphatidylcholines. Biochem J 1991;275:139–44.
[30] Dijkstra J, van Galen M, Regts D, Scherphof G. Uptake and processing of liposomal phospholipids by Kuppfer cells in vitro. Eur J Biochem 1985;148:391–7.
[31] Matsushita Y, Eshima K, Shindo T, Yamamoto Y, Hasegawa E, Nishide H, Tsuchida E. Clearance of tissue distribution of functionalized polymeric liposomes from the blood stream of rats. Biochim Biophys Acta 1987;901:166–71.
[32] Schwartz K, Lawn RM, Wade DP. ABC1 gene expression and apoA-1-mediated cholesterol efflux are regulated by LXR. Biochem Biophys Res Commun 2000;274:794–802.
[33] Kuipers F, Spanjer HH, Havinga R, Scherphof GL, Vonk RJ. Lipoproteins and liposomes as in vivo cholesterol vehicles in the rat: preferential use of cholesterol carried by small unilamellar liposomes for the formation of muricholic acids. Biochim Biophys Acta 1986;876:559–66.
[34] Braggins PE, Trakshel GM, Kutty RK, Maines MD. Characterization of two heme oxygenase isoforms in rat spleen: comparison with the hematin-induced and constitutive isoforms of the liver. Biochem Biophys Res Commun 1986;141:528–33.
[35] Lenz G, Junger H, Schneider M, Kothe N, Lissner R, Prince AM. Elimination of pyridoxalated polyhemoglobin after partial exchange transfusion in chimpanzees. Biomater Artif Cells Immobilization Biotechnol 1991;19:699–708.
[36] Finch CA, Huebers H. Perspectives in iron metabolism. New Engl J Med 1982;306:1520–8.
[37] O'Connell MJ, Peters TJ. Ferritin haemosiderin in free radical generation, lipid peroxidation and protein damage. Chem Phys Lipids 1987;45:241–9.
[38] Grady JK, Chen Y, Chasteen ND, Harris DC. Hydroxyl radical production during oxidative deposition of iron in ferritin. J Biol Chem 1989;264:20224–9.
[39] O'Connell MJ, Ward RJ, Baum H, Peters TJ. Iron release from haemosiderin and ferritin by therapeutic and physiological chelators. Biochem J 1989;260:903–7.
[40] Ponka P, Beaumont C, Richardson DR. Function and regulation of transferrin and ferritin. Seminar Hematol 1998;35:35–54.
[41] Bennett GD, Kay MM. Homeostatic removal of senescent murine erythrocytes by splenic macrophages. Exp Hematol 1981;9: 297–307.

Reduction of Methemoglobin via Electron Transfer from Photoreduced Flavin: Restoration of O_2-Binding of Concentrated Hemoglobin Solution Coencapsulated in Phospholipid Vesicles

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Ferric methemoglobin is reduced to its ferrous form by photoirradiation either by direct photoexcitation of the heme portion to induce electron transfer from the surrounding media (Sakai at al. (2000) *Biochemistry 39*, 14595−14602) or by an indirect electron transfer from a photochemically reduced electron mediator such as flavin. In this research, we studied the mechanism and optimal condition that facilitates photoreduction of flavin mononucleotide (FMN) to $FMNH_2$ by irradiation of visible light, and the succeeding reduction of concentrated metHb in phospholipid vesicles to restore its O_2 binding ability. Visible light irradiation (435 nm) of a metHb solution containing FMN and an electron donor such as EDTA showed a significantly fast reduction to ferrous Hb with a quantum yield (Φ) of 0.17, that is higher than the method of direct photoexcitation of heme ($\Phi = 0.006$). Electron transfer from a donor molecule to metHb via FMN was completed within 30 ns. Native-PAGE and IEF electrophoresis indicated no chemical modification of the surface of the reduced Hb. Coencapsulation of concentrated Hb solution (35 g/dL) and the FMN/EDTA system in vesicles covered with a phospholipid bilayer membrane (Hb-vesicles, HbV, diameter: 250 nm) facilitated the metHb photoreduction even under aerobic conditions, and the reduced HbV restored the reversible O_2 binding property. A concentrated HbV suspension ([Hb] = 8 g/dL) was sandwiched with two glass plates to form a liquid layer with the thickness of about 10 μm (close to capillary diameter in tissue, 5 μm), and visible light irradiation (221 mW/cm^2) completed 100% metHb photoreduction within 20 s. The photoreduced $FMNH_2$ reacted with O_2 to produce H_2O_2, which was detected by the fluorescence measurement of the reaction of H_2O_2 and *p*-nitrophenylacetic acid. However, the amount of H_2O_2 generated during the photoreduction of HbV was significantly reduced in comparison with the homogeneous Hb solution, indicating that the photoreduced $FMNH_2$ was effectively consumed during the metHb reduction in a highly concentrated condition inside the HbV nanoparticles.

INTRODUCTION

Photoinduced electron transfer is an essential reaction in biological systems especially during photosynthesis by the chlorophyll−protein complex (*1*). Hemoproteins such as hemoglobin (Hb) and myoglobin (Mb) are originally not related to the photoreaction in a biological system; however, photoexcitation and the resulting electron transfer reactions have been extensively studied (*2*−*6*). In our previous report, we clarified the mechanism of reduction of ferric methemoglobin (metHb) to its ferrous form by the direct excitation of the porphyrin N band in the UVA region (*7*). The reduction proceeds by charge transfer from the porphyrin ring to the central ferric iron to form the porphyrin π cation radical and ferrous iron by the N band excitation, and the contribution of the amino acid residues in the globin chain as an electron donor or an electron pathway. Another photochemical process to reduce metHb is an electron transfer from photochemically reduced flavin derivatives. The precise photoreduction mechanism of a flavin derivative to its reduced form has been extensively studied (*8*−*10*), and this has contributed to the understanding of the mechanism of the electron transfer of flavoproteins (*11*−*17*) and their function in the biological systems such as bacterial photosynthesis (*18*) and visual organs (*19*). Irrespective of the flavoproteins, the photoreduction of metMb and metHb by the externally added photoreduced form of the flavin mononucleotide (FMN) was first reported by Brwon and Synder (*20*) and Yubisui et al. (*21*, *22*), respectively.

We have paid special attention to the reduction of hemoproteins that will be applicable to Hb-based artificial red cells (*23*). We focus on the liposome-encapsulated Hb; a concentrated Hb solution is encapsulated in phospholipid vesicles to form Hb-vesicles (HbV)[1] with a particle size of about 250 nm (*24*, *25*). Ferrous state Hb binds oxygen to form HbO_2; however, it gradually converts to ferric metHb and superoxide anion and loses its oxygen binding property both in vivo and in vitro (*26*). A series of thiols was studied as a reductant for metHb,

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[1] Abbreviations: HbV, Hb-vesicles; FMN, flavin mononucleotide; SOD, superoxide dismutase; EDTA, ethylenediamine tetraacetic acid; DTPA, diethylenetriamine pentaacetic acid; IEF, isoelectric focusing; Native-PAGE, Native-polyacrylamide gel electrophoresis; PEG, poly(ethylene glycol); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DHSG, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate; PEG-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000}; HbCO, carbonylhemoglobin; $[FMN]_{in}$, concentration of FMN in HbV; $[EDTA]_{in}$, concentration of EDTA in HbV; $[heme]_{in}$, concentration of heme in HbV; PHA, *p*-hydroxyphenylacetic acid; DBDA, 6,6′-dihydroxy-(1,1′-biphenyl)-3,3′-diacetic acid, FMN*, triplet FMN; $FMNH_2$, reduced form of FMN;

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which is coencapsulated in the vesicles (*27*, *28*). As a result, the functional half-life of the Hb-vesicles is doubled by coencapsulation of the DL-homocysteine and active oxygen scavengers (*27*, *29*). To retard metHb formation, bioconjugation of enzymes such as catalase or superoxide dismutase (SOD) (*30*) and coencapsulation of RBC enzymes including the metHb reductase system, carbonic anhydrase, SOD, or catalase (*29*, *31*) have also been reported.

To restore the O_2 binding property of HbV, we tested utilization of the photoreduction system: the indirect excitation of an externally added electron mediator (*32*), or the direct excitation of metHb absorption in the UV region (*7*). In this study, we have made significant efforts to find out a condition that facilitates metHb reduction by a photoreduced flavin mononucleotide (FMN), because this system was well characterized by Everse (*32*), and the advantages of this system are visible light irradiation and high quantum yield (*9*, *10*). We analyzed the influence of electron donors to FMN, dissolved gases, etc., to find the facilitating condition and elucidate the mechanism for the facilitation of the metHb photoreduction in the HbV nanoparticles, a structure similar to that in red blood cells (RBCs), and this may also help understand the underlying mechanism of the reaction of NADPH-flavin reductase and metHb in RBCs.

EXPERIMENTAL PROCEDURES

Preparation of metHb. Carbonylhemoglobin (HbCO) was purified from outdated donated blood offered by the Hokkaido Red Cross Blood Center as previously reported (*33*, *34*). MetHb was prepared by reacting HbCO with an excess amount of potassium ferricyanide. The unreacted ions and ferrocyanide ions were removed twice by stirring with a mixed bed ion-exchange resin (Bio Rad AG 501-X8), and the solution was then permeated through 0.22 μm-filters (Advantec Co.). The metHb conversion was 99.8% measured by the cyanomethemoglobin method.

Chemicals. Amino acids (Met, Gln, Arg, Glu, Phe, Lys, Tyr, and Trp) were purchased from the Kanto Chem. Co (Tokyo). Peptides (Met-Met and Met-Gly) were from Sigma, and saccharides (mannitol, maltotriose, dextran, glucoseamine, glucron amide), methanol, citric acid sodium salt, ethylenediamine tetraacetic acid (EDTA), and diethylenetriamine pentaacetic acid (DTPA) were from the Kanto Chem. Co. All the chemicals were used without purification.

Photoreduction of FMN in the Presence of an Electron Donor. Three milliliters of phosphate-buffered saline (10 mM PBS, pH 7.4) with an electron donor (e.g., amino acids, peptides, sugars, as listed above, 20 mM) was sealed in a cuvette (2 mm width) with a butyl rubber cap. The solution was bubbled with N_2 for 30 min. A stock solution of FMN prepared in the dark was added at a concentration of 10 μM. The light source was a super high-pressure mercury lamp (USH-250D, 250W, Ushio Co., Tokyo) with a cutoff filter (L-42 and HA-50, Hoya Co., Tokyo) to obtain a single beam with the maximum wavelength of about 435 nm, which is close to λ_{max} of FMN (450 nm). The cuvette was located 2.5 cm away from the light source, and the light intensity was 221 mW/cm^2 that was measured with a power meter (PSV-3102, Gentec Co.). The conversion of the reduction, FMN to $FMNH_2$, was calculated from the reduction of absorbance at 450 nm, measured with an UV/vis spectrophotometer (V-560, Jasco, Tokyo).

Photoreduction of metHb in the Presence of FMN and an Electron Donor. Three milliliters of phosphate-buffered saline (10 mM PBS, pH 7.4) with an electron donor and FMN was sealed in a cuvette (2 mm width) with a butyl rubber cap. The solution was bubbled with N_2 gas for 30 min. A concentrated metHb stock solution deaerated by a gentle N_2 flowing in another bottle (about 3 mM, 10 μL) was injected into the cuvette. This procedure avoided bubbling of a metHb solution that might induce foaming and metHb denaturation. The final concentration of heme was 0.1 mM. The cuvette was exposed to the same visible light (435 nm) as described above. The conversion of the metHb reduction was calculated from the ratio of the Soret band absorption at 405 nm (λ_{max} of metHb) versus 430 nm (deoxyHb) or 415 nm (HbO_2).

A laser flash photolysis system (Tokyo Instr. Co.) was used for the transient spectrum measurement of the reduction of FMN and the succeeding metHb (*7*). The sample solutions were excited at 450 nm with a Pulsed Nd:YAG laser (SL803G-10, Spectron Laser Systems, Ltd.) equipped with an optical parametric oscillator. One irradiation time was 5−8 ns (fwhm) and the interval was 100 ms. A total of 100 accumulations were collected to get an acceptable signal-to-noise ratio. The transient spectra were recorded between 350 and 550 nm using a spectrophotometer (MS257, Oliel Instr. Co.) equipped with an ICCD detector (DH520−18F−WR, ANDOR Technol. Co.). A sample solution was placed in a 10 mm quartz cuvette and purged with N_2. The fastest time point of the measurements was 30 ns. A solution of FMN (100 μM)/Met (20 mM) in a 10 mM phosphate-buffered saline (pH 7.4), and a solution of FMN (5 μM)/Met (20 mM)/metHb ([heme] = 10 μM) in the phosphate-buffered saline were tested.

Quantum Yield Measurement. The ferrioxalate actinometer of $K_3[Fe(C_2O_4)_3]\cdot 3H_2O$ was used to measure the quantum yield (Φ) of metHb photoreduction (*7*, *35*, *36*). In the actinometer, Φ of the photoreduction of Fe^{3+} to Fe^{2+} was assumed to be 1.11 (*35*), and this value was used to calculate the total photons absorbed by the sample solution and Φ of the metHb photoreductions.

Isoelectric Focusing (IEF) and Native Polyacrylaminde Gel Electrophoresis (Native-PAGE). IEF and native-PAGE were performed on PhastGel IEF 3−9 (pH 3−9) and PhastGel Gradient 8−25 (PAGE content, 8−25%) (Amersham Pharmacia Biotech), respectively, with the PhastSystem (Pharmacia). The photoreduced Hbs in N_2 and air in the presence of FMN/EDTA was compared with metHb and the purified HbO_2.

IEF. Forty microliters of sample (1 mg/mL) per one lane was applied on the gel. This was focused and then stained with PhastGel Blue R (Coomassie brilliant blue) in the development unit of the PhastSystem. The marker was the p*I* calibration kit 3−10 (Pharmacia).

Native-PAGE. The samples were applied on the gel and the electrophoresis was automatically performed. The gel was stained with PhastGel Blue R. The marker was HMW Kit E (Parmacia).

Restoration of Oxygen Binding Property. The photoreduced deoxyHb solution ([heme] = 20 mM, [FMN] = 5 μM, [EDTA] = 10 mM) in an Ar atmosphere was bubbled with oxygen, and the UV/vis spectroscopy was measured. The photoreduced Hb solution was permeated through a column of Sephadex G-25 (Pharmacia) to remove FMN, the oxygen equilibrium curve of the obtained Hb solution was obtained at 37 °C with a Hemox Analyzer (TCS Products Inc.), and the oxygen affinity (P_{50}) and Hill number were measured. The Hb samples were diluted in a Hemox phosphate buffer (TCS Products Inc.).

Preparation of Hb-Vesicles Coencapsulating FMN and EDTA, and the Photoreduction of metHb. HbVs were prepared as previously reported (*24*, *34*, *37*). The purified HbO_2 solution (35 g/dL, [heme] = 21.7 mM) contained FMN (5, 10, or 50 mM) and EDTA (10, 20, 30, 50, or 200 mM), this was mixed with the lipid mixtures, and the resulting multilamellar vesicles were extruded through filters to regulate the vesicular size. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (DHSG) at the molar ratio of 5/5/1 (Nippon Fine Chem. Co., Osaka), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (PEG-DSPE, NOF Co., Tokyo) (*38*). Thus the vesicular surface was covered with PEG chains. The molar composition of the DPPC/cholesterol/DHSG/PEG-DSPE was 5/5/1/0.033. HbVs were suspended in a physiologic salt solution at [Hb] = 10 g/dL. The suspension was incubated in the dark at 40 °C for 48 h to facilitate the metHb formation and to prepare metHbV. The concentrations of FMN, EDTA, and heme of Hb in HbV, expressed as $[FMN]_{in}$, $[EDTA]_{in}$, and $[heme]_{in}$, respectively, are assumed to be identical to the fed concentrations for the HbV preparation.

Photoreduction of metHbV was performed in the same manner with a metHb solution in a relatively diluted condition ([heme] = 10 μM) in a 2 mm quartz cuvette. At a higher concentration ([heme] = 5 mM) under aerobic condition, the suspension of metHbV was sandwiched between two glass plates. The optical path length was 10 μm.

Measurement of H_2O_2 in the metHb Photoreduction. The reaction of *p*-hydroxyphenyl acetic acid (PHA) and H_2O_2 to generate a fluorescent dimer, 6,6'-dihydroxy (1,1'-biphenyl)-3,3'-diacetic acid (DBDA), was used to detect H_2O_2 generated during the metHb photoreduction in the metHbV and metHb solutions. During the photoreduction of metHb or metHbV ([heme] = 20 μM in a cuvette) in the presence of FMN (5 μM)/EDTA (50 μM), 1 mL of sample was pipetted out and immediately mixed with horseradish peroxidase (Sigma, 3.7 μM), and PHA (5.8 mM). The mixture was ultracentrifuged in a tube with a filter (Cut off Mw. 30 kDa, Ultrafree, Amicon) at 12 000 rpm for 20 min to remove Hb or HbV and to obtain the filtrate solution. The fluorescence of the filtrate was measured with a fluorometer (JASCO, Ex: 317 nm, Em: 404 nm). The calibration curve was obtained by analysis of a diluted standard H_2O_2 solution (Kanto Chem., Co).

RESULTS

Photoreduction of FMN with an Electron Donor. Figure 1 shows the time course of the conversion of FMN to $FMNH_2$ by irradiation of visible light (435 nm). FMN primarily converts to the photoexcited triplet FMN* and this reacts with two electron donors (D) to generate $FMNH_2$. The reduction can be confirmed by the decrease in the absorption of the characteristics peaks at 370 and 450 nm. Without an addition of an electron donor, photoreduction gradually proceeds (baseline, initial reduction rate = 12 μM/min) (Table 1). A ribityl group in a FMN molecule of itself can be an electron donor. However, further irradiation should induce decomposition that was evident from the phenomena that the spectroscopic curves did not coincide at the isosbestic point. A significantly fast reduction was observed by the addition of EDTA and DTPA that were 88 and 84 times faster than the condition without the addition of an electron donor. Among the amino acids, Met showed the fastest

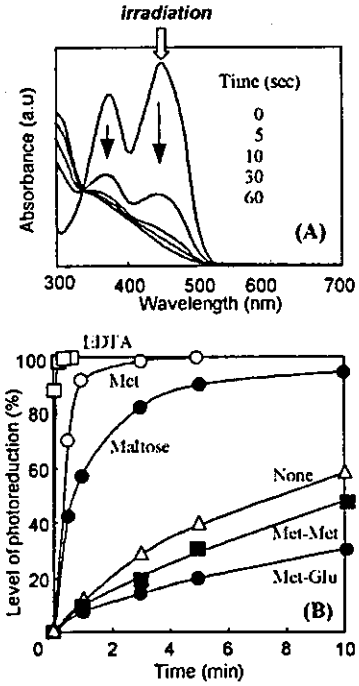

Figure 1. (A) Time course of the spectral changes during the conversion of FMN to $FMNH_2$ in the presence of EDTA (20 mM) by visible light irradiation (435 nm). The characteristic two peaks disappeared with the photoreduction conversion. (B) Time course of the conversion of FMN to $FMNH_2$ with various electron donors. EDTA and Met showed fast photoreduction rate. On the other hand, Met-Met and Met-Glu retarded the reaction. [FMN] = 100 μM, [electron donor] = 20 mM, pH = 7.4, in N_2 atmosphere.

Table 1. Initial Rates of the Photoreduction of FMN to $FMNH_2$ with Various Electron Donors (10 mM)

electron donor	mw	initial reduction rate (μM/min)	comparison with baseline
EDTA	292	1056	88
DTPA	393	1008	84
Met	149	140	11.7
Met-Met (10 mM)	280	5	0.2
Met-Met (20 mM)	280	10	0.8
Met-Glu	278	7	0.6
Arg	174	124	10.3
Phe	165	118	9.8
Lys	146	104	8.7
Gln	146	58	4.8
Glu	147	46	3.8
mannitol	182	45	3.8
maltotriose (10 unit mM)	504	47	3.9
dextran (10 unit mM)	5×10^6	45	3.6
glucoseamine	216	100	8.3
glucron amide	193	72	6.0
methanol	32	42	3.5
citric acid sodium salt	294	40	3.3
hydrogen	2	82	6.8
none (baseline)	---	12	1.0

reduction rate (140 μM/min, 12 times faster than the baseline), while Arg, Phe, Lys, Glu, and Gln showed moderate facilitation. On the other hand, Tyr and Trp showed slower rates of photoreduction. Unexpectedly, Met-Met and Met-Glu lowered the reduction rate. As for the saccharides, mannitol, maltotriose, dextran, glucosamine, and glucron amide showed similar facilitation at the same glucose units. However, they are much slower than Met and EDTA. The presence of H_2 gas

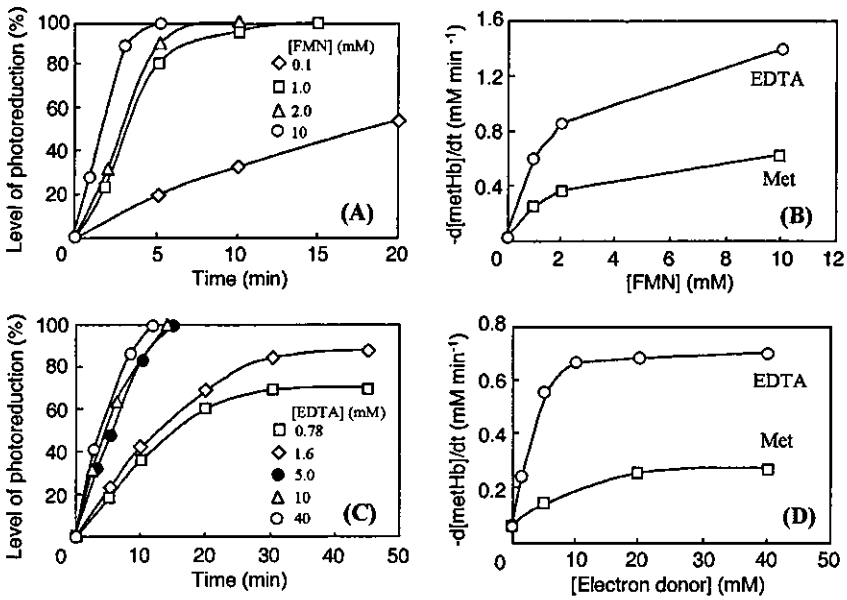

Figure 2. Influence of the concentrations of FMN and EDTA on the rate of metHb reduction. The time course of the level of photoreduction and the initial reaction rates are summarized. (A, B): Influence of the FMN concentration at a constant [EDTA] (20 mM). [FMN] was at 0.1, 1.0, 2.0, or 10 mM. (C, D): Influence of [EDTA] at the constant [FMN] (1.0 mM). [EDTA] was at 0.78, 1.6, 5.0, 10, or 40 mM. The data for the Met addition were inserted as a reference. [heme] = 3.1 mM.

slightly facilitated the reduction. Citric acid and methanol showed a slight facilitation. From these results, EDTA and Met were mainly studied as electron donors.

Reduction of metHb by the Photoreduced FMNH$_2$. The reduction of metHb by the photoreduced $FMNH_2$ was evident from the spectroscopic change of λ_{max} in the Soret and Q-bands. The influence of the concentration of FMN was examined at constant concentrations of metHb (5 g/dL, [heme] = 3.1 mM) and EDTA (20 mM) (Figure 2A). The presence of 100 μM FMN showed 50% reduction of metHb at 20 min; however, 1 mM FMN completed the reduction at 15 min. The influence of the EDTA concentration was examined at constant concentrations of metHb ([heme] = 3.1 mM) and FMN (1.0 mM) (Figure 2B). Without EDTA, the metHb photoreduction proceeded since a ribityl group of FMN and probably globin of Hb can be an electron donor. When the EDTA concentration was less than that of the heme concentration, the reduction rate was very slow, and the reduction could not be completed. However, 5 mM EDTA and higher showed a faster rate and the reduction was completed within 15 min. Similar results were obtained with Met; however, the initial rates were much slower than with EDTA.

The transient spectrum of the photoreduction of FMN in the presence of Met after laser flash irradiation showed the reduction of the absorbances at 445 and 373 nm at 30 ns, and the spectral profile was the same at 5 ms (data not shown here). Therefore, the photoreduction of FMN to $FMNH_2$ was completed within 30 ns. In the presence of metHb, a total of 30 ns was enough to observe the reduced deoxyHb (λ_{max} = 430 nm) and the spectrum was the same for 5 ms.

The influence of the presence of O_2 was examined (Figure 3). The metHb photoreduction in the presence of EDTA and FMN in the N_2 atmosphere completed the reduction within 15 min. The metHb photoreduction under the aerobic conditions became slightly slower, and the level of reduction reached 95% and then showed a plateau. In the case of the addition of Met, the reduction was completed within 40 min in the N_2 atmosphere that

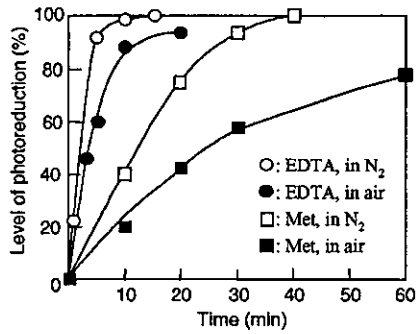

Figure 3. The influence of the presence of O_2 on the rate of photoreduction of metHb ([heme] = 3.1 mM) with FMN (1.0 mM) and an electron donor (20 mM) at pH 7.4. The data for Met addition (1.0 mM) were also inserted as a reference. The presence of O_2 retarded the metHb photoreduction.

was much slower in comparison with the EDTA addition. Under the aerobic condition, the reduction in the presence of Met was significantly slow and did not reach 80% at 60 min.

Native-PAGE of the photoreduced Hb both in the N_2 and aerobic atmospheres showed identical bands with the normal oxyHb and meHb (Figure 4A). Even though the Mw of Hb is 64.5 kDa, it showed a higher relative Mw than albumin (67 kDa) as one of the markers in the Native-PAGE in the absence of sodium dodecyl sulfate, SDS, because the surface charge of the protein directly affect on the traveling distance during the electrophoresis. IEF of the photoreduced Hbs showed the presence of HbO_2 at pI = 7.0 as a dense band and a weak band at pI = 7.2 of a partially reduced Hb (Figure 4B). There was no band at 7.4 that corresponds to metHb.

The oxygen dissociation curve of the photoreduced Hb was identical with that of the normal HbO_2 (Data not shown here). The P_{50} and Hill number of the photoreduced Hb were 10.5 Torr and 1.8, respectively, and they were almost identical with the normal HbO_2 (11 Torr and 1.7, respectively).

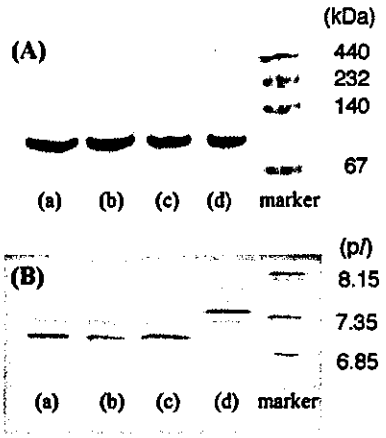

Figure 4. Native-PAGE (A) and IEF (B) of the photoreduced Hb in the presence of EDTA and FMN both in N_2 and aerobic atmospheres: (a) photoreduced Hb in N_2, (b) photoreduced Hb in air, (c) oxyHb, (d) metHb. In A, there was no change in the molecular weight of the Hb subunits. Since Native-PAGE does not include sodium dodecyl sulfate, the surface property of the protein directly affect on the traveling distance during electrophoresis. Therefore, Mw of Hb (Mw = 64.5 kDa) seemed much larger than albumin marker (Mw = 67 kDa). In B, the band at 7.4, which corresponded to metHb, almost disappeared in lanes a and b. No other bands were observed.

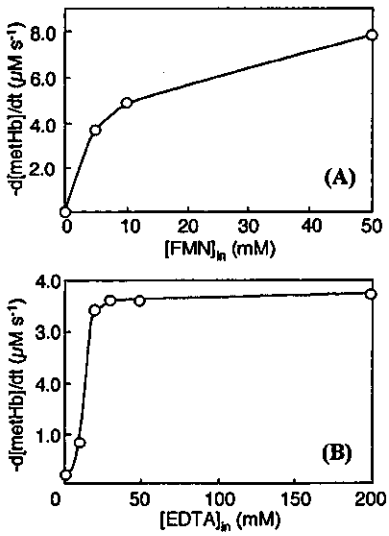

Figure 5. Influence of the concentrations of FMN and EDTA inside HbV on the initial rate of metHb reduction. (A) Influence of $[FMN]_{in}$ at the constant $[EDTA]_{in}$ = 20 mM. (B) Influence of $[EDTA]_{in}$ at the constant $[FMN]_{in}$ = 5 mM. [heme] = 10 μM in the cuvette, $[heme]_{in}$ = 21.7 mM. When $[EDTA]_{in}$ was higher than $[heme]_{in}$, the initial rate of metHb reduction was plateau.

Reduction of metHb in Hb-Vesicles. At first a diluted metHbV suspension ([heme] = 10 μM in a cuvette; $[heme]_{in}$ = 21.7 mM) was tested for photoreduction to analyze the kinetics. The initial rate of metHb reduction increased with increasing $[FMN]_{in}$ at a constant $[EDTA]_{in}$ (20 mM); however, the initial rate at $[FMN]_{in}$ = 10 mM was lower than twice that at $[FMN]_{in}$ = 5 mM (Figure 5A). At a constant $[FMN]_{in}$ (5 mM), increasing the $[EDTA]_{in}$ significantly facilitated the metHb photoreduction, however, the photoreduction rate did not increase above 20 mM (Figure 5B). This critical concentration is almost identical to $[heme]_{in}$ (21.7 mM). From these results, the rate-determining step of this system should be the electron transfer from an electron donor to the photoexcited FMN.

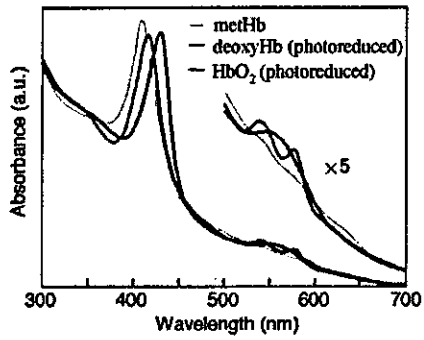

Figure 6. UV−visible spectra of HbV before irradiation (metHb), after photoreduction (deoxyHb), and its oxygenated form (HbO_2). $[EDTA]_{in}$ = 50 mM, $[FMN]_{in}$ = 5 mM, $[heme]_{in}$ = 21.7 mM. These spectra indicate the successful restoration of O_2-binding property of HbV.

The absorption spectra of the metHbV and the photoreduced HbV (λ_{max} = 430 nm) are shown in Figure 6. Due to the light scattering effect of the HbV particles, the turbidity was higher at a lower wavelength (*39*). Bubbling with an O_2 gas in a photoreduced HbV solution reversibly converted deoxyHb to HbO_2 with a characteristic shift of λ_{max} from 430 to 415 nm, indicating that the oxygen binding ability was successfully restored.

The concentration of [heme] in an HbV suspension for the intravenous infusion should be estimated to about 3−6 mM, which is significantly higher in comparison with 10 μM in a cuvette for the absorption spectral analysis. To test the photoreduction at a practical Hb concentration, a metHbV suspension ([heme] = 5.0 mM) was sandwiched between two glass plates and irradiated with visible light. The photoreduction proceeded quite promptly (Figure 7). Due to the thin liquid layer (ca. 10 μm in thickness), the effect of light scattering seen in Figure 6 is minimized. At the constant $[FMN]_{in}$ (5 mM) condition, the $[EDTA]_{in}$ of 10 and 20 mM were not enough to complete the reduction. At $[EDTA]_{in}$ = 50 mM, the photoreduction was significantly fast and the reaction was completed within 20 s with the characteristic λ_{max} of deoxyHb (430 nm). At $[FMN]_{in}$ = 100 mM and $[EDTA]_{in}$ = 20 mM, the initial reduction rate was the fastest; however, the reduction was not completed which was evident from the fact that the absorption at 430 nm in the Soret band was not high enough. The value of $[EDTA]_{in}$ should at least be higher than $[heme]_{in}$ (21.7 mM).

Quantum Yield of the Photoreduction Reactions. Table 2 summarizes the quantum yield, Φ, of various photoreduction conditions. The combination of metHb/FMN/EDTA showed the highest value (0.17) in an Ar atmosphere at [heme] = 0.1 mM. This was about 28 times higher than that for the photoreduction by the direct excitation of the N-band irradiating by near UV light (365 nm, Φ = 0.003−0.006) (*7*), and 4 times higher than the condition without an electron donor (0.04). In the case of HbV that coencapsulates FMN and EDTA, the concentrations of the components in the cuvette were much smaller, however, the concentrations in the nanoparticles (HbV) are much higher and the Φ for HbV was also very high (0.09−0.11). Probably due to the light scattering effect of HbV, Φ for HbV is slightly lower than that for the homogeneous Hb solution, but significantly higher than that for the N-band excitation (0.003−0.006).

Measurement of H_2O_2 in the metHb Photoreduction. Visible light irradiation to metHb([heme] = 20 μM)/FMN(5 μM)/EDTA(50 μM) under aerobic conditions

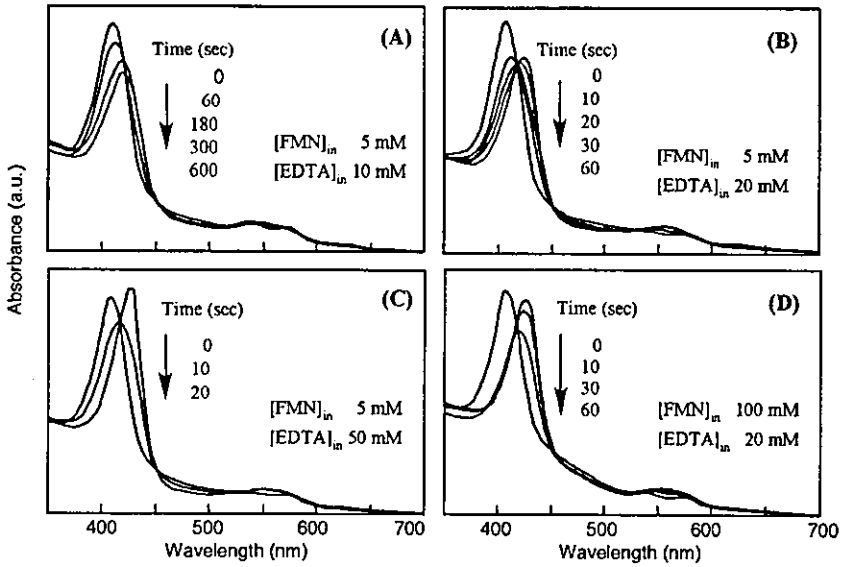

Figure 7. UV–visible spectral changes of HbV in a liquid layer sandwiched between two glass plates during photoreduction under aerobic conditions. The thickness of the layer was approximately 10 μm. Condition (C) ($[FMN]_{in}$ = 5 mM, $[EDTA]_{in}$ = 50 mM) showed the fastest rate of photoreduction, and the reaction was completed within 20 s. The arrows indicate the decrease in absorbance at 405 nm of MetHb with irradiation time.

Table 2. Quantum Yield (Φ) of Photoreduction of metHb and metHbV

	heme (mM)	FMN (mM)	electron donor (mM)	condition	λ_{ex} (nm)	Φ
metHb	0.1	0.01	EDTA (20)	in Ar	435	0.17
	0.1	0.01	Met (20)	in Ar	435	0.11
	0.1	0.01	no addition	in Ar	435	0.04
metHbV	0.01 (21.7)[a]	2.3×10^{-3} (5)[a]	EDTA (9.2×10^{-3}) (20)[a]	in Ar	435	0.09
	0.01 (21.7)[a]	46×10^{-3} (100)[a]	EDTA (9.2×10^{-3}) (20)[a]	in Ar	435	0.11
metHb	0.01	–	Trp (1.0)	in Ar	365	0.006[b]
	0.01	–	mannitol (100)	in CO	365	0.006[b]
	0.01	–	no addition	in CO	365	0.003[b]

[a] Concentrations of the components inside HbV; $[heme]_{in}$, $[FMN]_{in}$, $[EDTA]_{in}$. [b] Data from ref 7.

produced H_2O_2, and the fluorescent intensity of DBDA (λ_{em} = 404 nm) significantly increased (Figure 8a). The amount of H_2O_2 reached 40 μM at 120 s (Figure 8b). Irradiation to FMN alone produced 100 μM H_2O_2 for 120 s without any formation of $FMNH_2$. We confirmed that the irradiation to metHb alone did not produce H_2O_2 (data not shown here). The level of metHb photoreduction was less than 20% at 120 s (Figure 8c). A significant suppression of H_2O_2 generation was confirmed for the irradiation to metHbV and the H_2O_2 generation decreased to less than 20 μM, and the level of metHb photoreduction reached 50% at 120 s. A further increase in the level of photoreduction to 80% was confirmed when the partial oxygen pressure in the cuvette was regulated to 40 Torr; however, the amount of H_2O_2 could not be significantly reduced.

DISCUSSION

We found for the first time that the coencapsulation of concentrated Hb solution and the FMN/EDTA system in phospholipid vesicles (HbV) significantly facilitated the reduction of metHb by visible light irradiation (435 nm). This was evident from the Φ of the reaction, i.e., 0.17 for the Hb solution and 0.10 for the HbV suspension. The lowered Φ for HbV in comparison with that for a Hb solution is probably due to the light scattering of the illuminated visible light due to the particle of HbV (diameter, 250 nm) (*39*). However, they are much higher than that for the metHb photoreduction via direct photoexcitation of the N-band of the porphyrin ring in the UVA region (Φ = 0.006) (*7*). Even though the concentrations of the components in the cuvette were much lower for HbV than for the homogeneous Hb solution as shown in Table 2, the concentrations inside HbV were significantly higher and this condition facilitated the desired reactions (photoreduction of FMN and metHb) and suppressed the unwanted side reactions (generation of active oxygen species).

The reaction mechanism is that the photoexcited triplet FMN* rapidly receives an electron from the donor molecule, EDTA, to transform to the semiquinone followed by disproportionation to the two electron reduced form, $FMNH_2$. They are effective reducing agent to offer an electron to metHb. According to Yubisui et al., $FMNH_2$ reduces metHb with the rate constant of 5.5×10^6 M^{-1} s^{-1} (*22*), which is significantly faster than do glutathione (rate constant = 2.5×10^{-3} M^{-1} s^{-1}) (*27*) and ascorbic acid (3.0×10^{-3} M^{-1} s^{-1}) (*22*). The transient spectrum of the reduction of metHb by the photoreduced form of FMN demonstrated the completion of the reaction at 30 ns. Our result may be plausible because it is reported that a flavocytochrome showed complete photoreduction within 100 ns (*14*), measured by a laser flash-induced transient absorption difference spectra. The externally added FMN should more freely access to the protoporphyrin IX (heme) in the Hb molecule and would show a faster electron transfer. It is reported that the direct chemical conjugation of flavin to the propionic acid residue of heme significantly facilitates the electron transfer from flavin to heme in a reconstituted myoglobin (*40*, *41*). Therefore,

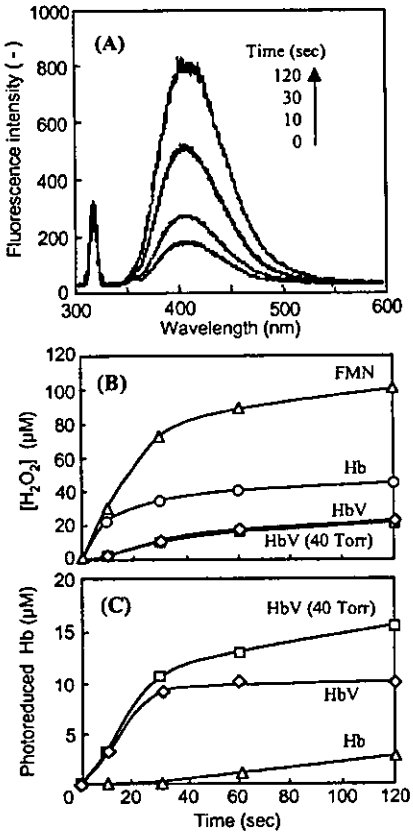

Figure 8. Detection of H_2O_2 using the fluorescence of DBDA during the photoreduction of Hb and HbV in the presence of FMN and EDTA. (A) An example of the fluorescence spectroscopy of the DBDA. The fluorescence intensity (λ_{em} = 404 nm) increased with time during the photoreduction of metHb solution under aerobic conditions (pO_2= 150 Torr). (B) Time course of the generation of H_2O_2 during the photoreduction of Hb and HbV under aerobic conditions (pO_2= 150 Torr), and HbV at pO_2 = 40 Torr. Irradiation to FMN alone was also tested as a reference (top curve) that produced 100 μM H_2O_2 for 120 s. Liberation of H_2O_2 from HbV was significantly suppressed in comparison with Hb solution. (C) The levels of metHb photoreduction during the measurement of H_2O_2 generation. The concentrations of heme (20 μM), FMN (5 μM), and EDTA (50 μM) in the cuvette were identical between the metHb solution and HbV suspension. For HbV, $[heme]_{in}$ = 21.6 mM, [FMN]in = 5 mM, and $[EDTA]_{in}$ = 50 mM.

two propionic acid groups of a heme that directly face the outer aqueous phase of an Hb molecule should contribute to the electron transfer from the externally added FMN to the heme.

The side reaction of $FMNH_2$ is the reaction with O_2 to generate singlet O_2 ($^1O_2$) or H_2O_2 (*11*, *42*), due to the low redox potential of reduced flavin (E_m = −209 mV). However, according to the quantitative measurement of H_2O_2, photoreduction of metHbV significantly reduced the side reaction in comparison with the metHb solution. This effect is due to the highly concentrated condition inside metHbV: the photoexcited FMN* readily reacts with EDTA to generate $FMNH_2$, and it also readily reacts with concentrated metHb inside the HbV nanoparticle. However, for the complete removal of H_2O_2, further coencapsulation of catalase would be effective (*29*) in the presence of O_2. Of course, in the absence of O_2, only the metHb reduction proceeds.

We tried to find other optimal electron donors instead of EDTA, because it has been reported that the oxidized and decomposed EDTA elements contain acetaldehyde that might react with the lysine residues on a protein molecule (*10*), and EDTA is a strong chelator of Ca^{2+} as an anticoagulant and may require caution when using a large dosage. We confirmed that Met was effective secondary to EDTA, as reported by other researchers (*9*, *32*). Arg was also effective, but it was not stable against oxidation during incubation under aerobic conditions at 37 °C for 3 days. Met was stable against oxidation. However, the small amino acid, Met (Mw = 149), gradually leaks out from the HbV across the phospholipid bilayer membrane (data not shown). To minimize the leakage of an electron donor, larger molecules, Met-Met and Met-Glu, were tested. Unexpectedly, they did not show any contribution as an electron donor and retarded the reduction of FMN. The ribityl phosphate group in the FMN molecule can be an electron donor, because the photoreduction of FMN proceeds without the addition of an electron donor. The retardation by the peptides should be probably due to some interaction of these peptides with the ribityl phosphate group that may hinder the electron transfer to the isoalloxazine ring. Other amino acids such as Phe and Lys, and saccharides such as mannitol or maltotoriose, are effective as an electron donor; however, their reduction rates of FMN were much lower in comparison with EDTA. Interestingly, methanol and gaseous H_2 also showed facilitation. DTPA, a structure similar to EDTA, showed an effectiveness comparable with EDTA. EDTA is a well-known electron donor, and its larger size (Mw = 292) and four negative charges prevent leakage from the vesicles. We could not find a more effective electron donor in our study, but confirmed that IEF and native-PAGE did not demonstrate any change in the chemical modification of the photoreduced Hb in the presence of EDTA/FMN, and the O_2 binding property was successfully restored. Therefore, we tested coencapsulation of FMN/EDTA in HbV for the other studies.

When HbV is intravenously infused for the substitution of blood, the concentrations of Hb and the heme of HbV in plasma should reach 5 g/dL and 3.1 mM, respectively, or higher (*43*). These are much higher than the experimental conditions in Figures 1−4, and it is impossible to test such a highly concentrated solution in a cuvette because of the strong light scattering by the particles and absorption by Hbs. We thus tested sandwiching the solution with two glass plates, thus making a thin liquid layer between the glass plates. The thickness of the liquid membrane is approximately 10 μm, about twice the capillary diameter in in vivo peripheral tissues. Irradiation of visible light onto the liquid membrane of HbV coencapsulating FMN and EDTA showed significantly fast rates for the metHb photoreduction. Especially, the coencapsulation of FMN (5 mM) and EDTA (50 mM) completed the metHb photoreduction within only 20 s. This significantly fast photoreduction system would be applicable to the transcutaneous irradiation of visible light to the body for the rejuvenation of HbV when the metHb content increased after the infusion of HbV.

In our study we established an efficient photoreduction system in a nanoparticle as shown in Figure 9. The illuminated visible light excites FMN to convert it to FMN*, and this reacts with an electron donor and transforms to $FMNH_2$, that subsequently reduces ferric metHb to its ferrous form. The reduced Hb can then reversibly bind O_2. Irrespective of the blood substitutes, one advantage of coencapsulation in a nanoparticle is that the concentrations of the components in the vesicles (nanoenvironment) are very high. Accordingly, the desired reactions are significantly accelerated and the

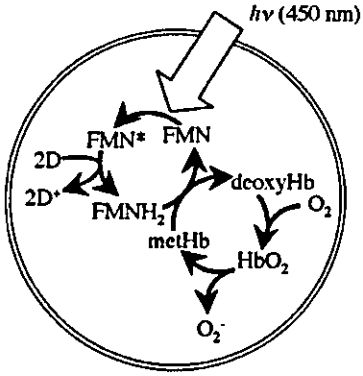

Figure 9. MetHb photoreduction system in a nanoparticle (HbV) using FMN and an electron donor (D), and recovery of the O_2-binding property.

unwanted side reaction is minimized in comparison with the homogeneous solution. To completely eliminate the side reaction of $FMNH_2$ and O_2, photoreduction under anaerobic conditions or coencapsulation of a radical scavenger, such as catalase, would be effective (*29, 30*). RBC contains NADPH-flavin reductase to reduce metHb (*21*), and the reduced form of flavin is susceptible to react with O_2 as a side reaction. However, our results imply that the highly concentrated condition in RBCs and well-organized radical scavenging system should contribute to the effective metHb reduction in RBCs.

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LITERATURE CITED

(1) Tracewell, C. A., Cua, A., Stewart, D. H., Bocian, D. F., and Brudvig, G. W. (2001) Characterization of carotenoid and chlorophyll photooxidation in photosystem II. *Biochemistry 40*, 193−203.

(2) Vorkink, W. P., and Cusanovivh, M. A. (1974) Photoreduction of horse heart cytochrome *c*. *Photochem. Photobiol. 19*, 205−215.

(3) Adar, F., and Yonetani, T. (1978) Resonance Raman spectra of cytochrome oxidase. Evidence for photoreduction by laser photons in resonance with the Soret band. *Biochim. Biophys. Acta 502*, 80−86.

(4) Kitagawa, T., and Nagai, K. (1979) Quaternary structure-induced photoreduction of haem of haemoglobin. *Nature 281*, 503−504.

(5) Kitagawa, T., Chihara, S., Fushitani, K., and Morimoto, H. (1984) Resonance Raman study of subunit assembly dependent photoreduction of heme of extracellular giant hemoglobin. *J. Am. Chem. Soc. 106*, 1860−1862.

(6) Pierre, J., Bazin, M., Debey, P., and Santus, P. (1982) One-electron photoreduction of bacterial cytochrome P-450 by ultraviolet light. *Eur. J. Biochem. 124*, 533−537.

(7) Sakai, H., Onuma, H., Umeyama, M., Takeoka, S., and Tsuchida E. (2000) Photoreduction of methemoglobin by irradiation in the near-ultraviolet region. *Biochemistry 39*, 14595−14602.

(8) McCormick, D. B., Koster, J. F., and Veeger, C. (1967) On the mechanism of photochemical reductions of FAD and FAD-dependent flavoproteins. *Eur. J. Biochem. 2*, 387−391.

(9) Heelis, P. F., Parsons, B. J., Phillips, G. O., and McKellar, J. F. (1979) The photoreduction of flavins by amino acids and EDTA. A continuous and flash photolysis study. *Photochem. Photobiol. 30*, 343−347.

(10) Traber, R., Kramer, H. E. A., and Hemmerich, P. (1982) Mechanism of light-induced reduction of biological redox centers by amino acids. A flash photolysis study of flavin photoreduction by ethylenediaminetetraacetate and nitrilotriacetate. *Biochemistry 21*, 1687−1693.

(11) Massey, V., Strickland, S., Mayhew, S. G., Howell, L. G., Engel, P. C., Matthews, R. G., Schuman, M., and Sullivan, P. A. (1969) The production of superoxide anion radicals in the reaction of reduced flavins and flavoproteins with molecular oxygen. *Biochem. Biophys. Res. Commun.* 36, 891−897.

(12) Massey, V., Stankovich, M., and Hemmerich, P. (1978) Light-mediaated reduction of flavoproteins with flavins as catalyst. *Biochemistry 17*, 1−8.

(13) Cusanovich, M. A., Meyer, T. E., and Tollin, G. (1985) Flavocytochrome *c*. Transient kinetics of photoreduction by flavin analogues. *Biochemistry 24*, 1281−1287.

(14) Sharp, R. E., Moser, C. C., Rabanal, F., and Dutton, P. L. (1998) Design, synthesis, and characterization of a photoactivatable flavocytochrome molecular maquette. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10465−10470.

(15) Hazzard, J. T., Govindaraj, S., Poulos, T. L., and Tollin, G. (1997) Electron transfer between the FMN and heme domains of cytochrome P450 μ_B-3. *J. Biol. Chem. 272*, 7922−7926.

(16) Shumyantseva, V. V., Bulko, T. V., Schmid, R. D., and Archakov, A. I. (2002) Photochemical properties of a riboflavins/cytochrome P450 2B4 comples. *Biosensors Bioelecronics 17*, 233−238.

(17) Shumyantseva, V. V., Bulko, T. V., Schmid, R. D., and Archakov, A. I. (2000) Flavocytochrome P450 2B4 photoreduction. *Biophysics 45*, 982−987 (Translated from *Biofizika*).

(18) Masuda, S., and Bauer, C. E. (2002) AppA is a blue light photoreceptor that antirepresses photosynthesis gene expression in Rhodobacter sphaeroides. *Cell 110*, 613−623.

(19) Tsubota, K., Laing, R. A., and Kenyon, K. R. (1987) Noninvasive measurements of pyridine nucleotide and flavoprotein in the lens. *Invest. Ophthalmol. Vis. Sci. 28*, 785−789.

(20) Brown, W. D., and Synder, H. E. (1969) Nonenzymatic reduction and oxidation of myoglobin and hemoglobin by nicotinamide adenine dinucleotides and flavins. *J. Biol. Chem. 244*, 6702−6706.

(21) Yubisui, T., Takeshita, M., and Yoneyama, Y. (1980a) Reduction of methemoglobin through flavin at the physiological concetration by NADPH-flavin reductase of human erythrocytes. *J. Biochem. 87*, 1715−1720.

(22) Yubisui, T., Matsukawa, S., and Yoneyama, Y. (1980b) Stopped flow studies on the nonenzymatic reduction of methemoglobin by reduced flavin mononucleotide. *J. Biol. Chem. 255*, 11694−11697.

(23) Tsuchida, E. (1998) *Blood Substitutes: present and future perspectives* (Tsuchida, E., Ed.) Elsevier Science, New York.

(24) Takeoka, S., Ohgushi, T., Ohmori, T., Terase, T., and Tsuchida, E. (1996) Layer controlled hemoglobin vesicles by interaction of hemoglobin with phospholipid assembly. *Langmuir 12*, 1775−1779.

(25) Sakai, H., Takeoka, S., Park, S. I., Kose, T., Nishide, H., Izumi, Y., Yoshizu, A., Kobayashi, K., and Tsuchida, E. (1997) Surface modification of hemoglobin vesicles with poly(ethyleneglycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. *Bioconjugate Chem. 8*, 23−30.

(26) Szebeni, J., Hauser, H., Eskelson, C. D., Watson, R. R., and Winterhalter, K. H. (1988) Interaction of hemoglobin derivatives with liposomes. Membrane cholesterol protects against the changes of hemoglobin. *Biochemistry 27*, 6425−6434.

(27) Takeoka, S., Sakai, H., Kose, T., Mano, Y., Seino, Y., Nishide, H., and Tsuchida, E. (1997) Methemoglobin formation in hemoglobin vesicles and reduction by encapsulated thiols. *Bioconjugate Chem. 8*, 539−544.

(28) Sakai, H., Tomiyama, K., Sou, K., Takeoka, S., and Tsuchida, E. (2000) Poly(ethyleneglycol)-conjugation and

deoxygenation enable long-term preservation of hemoglobin vesicles as oxygen carriers. *Bioconjugate Chem. 11*, 425−432

(29) Teramura, Y., Kanazawa, H., Sakai, H., Takeoka, S., and Tsuchida, E. (2003) The prolonged oxygen -carrying ability of Hb vesicles by coencapsulation of catalase in vivo. *Bioconjugate Chem. 14*, 1171−1176.

(30) D'Agnillo, F., and Chang, T. M. (1998) Polyhemoglobin-superoxide dismutase-catalase as a blood substitute with antioxidant properties. *Nat. Biotechnol. 16*, 667−71.

(31) Chang, T. M., Powanda, D., and Yu WP. (2003) Analysis of polyethylene-glycol-polylactide nano-dimension artificial red blood cells in maintaining systemic hemoglobin levels and prevention of methemoglobin formation. *Artif. Cells Blood Substitutes Immobilization Biotechnol. 31*, 231−47.

(32) Everse, J. (1994) Photochemical reduction of methemoglobin and methemoglobin derivatives. *Methods Enzymol. 231*, 524−536.

(33) Sakai, H., Takeoka, S., Yokohama, H., Seino, Y., Nishide, H., and Tsuchida, E. (1993) Purufication of concentrated Hb using organic solvent and heat treatment. *Protein Expression Purif. 4*, 563−569.

(34) Sakai, H., Yuasa, M., Onuma, H., Takeoka, S., and Tsuchida, E. (2000) Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. *Bioconjugate Chem. 11*, 56−64.

(35) Hatchard, C. G., and Parker, C. A. (1956) A new sensitive chemical actinometer II. Potasium ferrioxalate as a standard chemical actinometer. *Proc. R. Soc. London 235*, 518−536.

(36) Wegner, E. E., and Adamson, A. W. (1966) Photochemistry of complex ions. III. Absolute quantum yields for the photolysis of some aqueous chromium (III) complexes. Chemical actinometry in the long wavelength visible region. *J. Am. Chem. Soc. 88*, 394−404.

(37) Sou, K., Naito, Y., Endo, T., Takeoka, S., and Tsuchida, E. (2003) Effective encapsulation of proteins into size-controlled phospholipid vesicles using freeze−thawing and extrusion. *Biotechnol. Prog. 19*, 1547−1552.

(38) Sou, K., Endo, T., Takeoka, S., and Tsuchida, E. (2000) Poly(ethylene glycol)-modification of the phospholipid vesicles by using the spontaneous incorporation of poly(ethylene glycol)-lipid into the vesicles. *Bioconjugage Chem. 11*, 372−379.

(39) Sakai, H., Tomiyama, K., Masada, Y., Takeoka, S., Horinouchi, H., Kobayashi, K., and Tsuchida, E. (2003) Pretreatment of serum containing Hb-vesicels (oxygen carriers) to avoid interference in clinical laboratory tests. *Clin. Chem. Lab. Med. 41*, 222−231.

(40) Hamachi, I., Nomoto, K., Tanaka, S., Tajiri, Y., and Shinkai, S. (1994) Self-sufficient electron injection from NADH to the active center of flavin-pendant myoglobin. *Chem. Lett. 1994*, 1139−1142.

(41) Matsuo, T., Hayashi, T., and Hisaeda, Y. (2002) Reductive activation of dioxygen by a myoglobin reconstituted with a flavohemin.*J. Am. Chem. Soc. 124*, 11234−11235.

(42) Frati, E., Khatib, A., Front, P., Panasyuk, A., Aprile, F., and Mitrovic, D. R. (1997) Degradation of hyaluronic acid by photosensitized riboflavin in vitro. Modulation of the effect by transition metals, radical quenchers, and metal chelators. *Free Rad. Biol. Med. 22*, 1139−1144.

(43) Sakai, H., Horinouchi, H., Masada, Y., Yamamoto, M., Takeoka, S., Kobayashi, K., Tsuchida, E. (2004) Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit. Care Med. 32*, 539−545.

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Physiological Capacity of the Reticuloendothelial System for the Degradation of Hemoglobin Vesicles (Artificial Oxygen Carriers) after Massive Intravenous Doses by Daily Repeated Infusions for 14 Days

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ABSTRACT

A hemoglobin vesicle (HbV; diameter 252 ± 53 nm) or liposome-encapsulated Hb is an artificial oxygen carrier developed for use as a transfusion alternative, and its oxygen-transporting capacity has been well characterized, although critical physiological compartments for the Hb degradation after a massive infusion of HbV and the safety outcome remain unknown. In this study, we aimed to examine the compartments for its degradation by daily repeated infusions (DRI) of HbV, focusing on its influence on the reticuloendothelial system (RES). Male Wistar rats intravenously received the HbV suspension at 10 ml/kg/day for 14 consecutive days. The cumulative infusion volume (140 ml/kg) was equal to 2.5 times the whole blood volume (56 ml/kg). The animals tolerated the DRI well and survived, and body weights continuously increased. One day after DRI, hepatosplenomegaly occurred significantly through the accumulation of large amounts of HbV. Plasma clinical chemistry was overall normal, except for a transient elevation of lipid components derived from HbV. These symptoms subsided 14 days after DRI. Hemosiderin deposition and up-regulation of heme oxygenase-1 coincided in the liver and spleen but were not evident in the parenchyma of these organs. Furthermore, the plasma iron and bilirubin levels remained unchanged, suggesting that the heme-degrading capacity of the RES did not surpass the ability to eliminate bilirubin. In conclusion, phospholipid vesicles for the encapsulation of Hb would be beneficial for heme detoxification through their preferential delivery to the RES, a physiological compartment for degradation of senescent RBCs, even at doses greater than putative clinical doses.

Phospholipid vesicles or liposomes have been extensively studied as a carrier of functional (macro)molecules for a drug delivery system, and some are now approved for clinical use as antifungal or anticancer therapies (Lian and Ho, 2001). Vesicles encapsulating concentrated hemoglobin (Hb), so-called Hb vesicles (HbV) or liposome-encapsulated Hb, have been developed as artificial oxygen carriers, and their sufficient ability to transport oxygen comparable with blood has been well clarified (Djordjevich et al., 1987; Chang et al., 1992; Izumi et al., 1997; Phillips et al., 1999; Sakai et al., 2004c). The advantages of an artificial oxygen carrier are the absence of blood-type antigens and transfusion-related transmission of infections, and stability during long-term storage. In this sense, the infusion of oxygen carriers becomes superior to the conventional blood transfusion that still has the potential of mismatching, the risk of infections secondary to the infusion of contaminated blood, and the problem of only a few weeks' storage life.

In a series of safety studies of HbV, it has been clarified that the cellular structure and the size of the HbV are advantageous for maintaining a steady blood circulation with-

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ABBREVIATIONS: HbV, Hb vesicle(s); Hct, hematocrit; RES, reticuloendothelial system; PEG, poly(ethylene glycol); RBC, red blood cell; DRI, daily repeated infusion(s); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DHSG, 1,5-*O*-dihexadexyl-*N*-syccynyl-L-glutamate; MAP, mean arterial pressure; HR, heart rate; UA, uric acid; BUN, urea nitrogen; CRE, creatinine; PT, prothrombin time; APTT, activated partial thromboplastin time; HO-1, heme oxygenase-1.