Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin-vesicles as an artificial oxygen carrier

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Running Title: Pharmacokinetics of hemoglobin and lipid component in HbV

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Abbreviations: Hb, hemoglobin; HbV, hemoglobin vesicle; $^{125}$I-HbV, $^{125}$I-labeled hemoglobin vesicle; $^3$H-HbV, $^3$H-labeled hemoglobin vesicle; RBC, red blood cell; rHSA, recombinant human serum albumin; MPS, mononuclear phagocyte system; AUC, area under the concentration-time curve; CL, clearance; CL$_{uptake}$, uptake clearance; Kp, the tissue-to-plasma partition coefficient; Hp, haptoglobin
Abstract

Hemoglobin-vesicle (HbV) is an artificial oxygen carrier that encapsulates a concentrated Hb solution in lipid vesicles (liposomes). The pharmacokinetic properties of HbV were investigated in mice and rats. Using HbV in which the internal Hb was labeled with iodine-125 (\(^{125}\text{I-HbV}\)) and cell-free \(^{125}\text{I-Hb}\), it was found that encapsulation of Hb increased the half-life by 30 times, accompanied by a decreased distribution in both the liver and kidney. The half-life of HbV was increased, and the uptake clearance for the liver and spleen were decreased with increasing dose of HbV. In an \textit{in vitro} study, the specific uptake and degradation of HbV in RAW 264.7 cells was found, but this was not the case for parenchymal and endothelial cells. The pharmacokinetics of HbV components (internal Hb and liposomal lipid) were also investigated using \(^{125}\text{I-HbV}\) and \(^{3}\text{H-HbV}\) (liposomal cholesterol was radiolabeled with tritium-3). The time courses for the plasma concentration curves of \(^{125}\text{I-HbV}\), \(^{3}\text{H-HbV}\) and iron derived from HbV suggest that HbV maintains an intact structure in the blood circulation up to 24 hr after injection. \(^{125}\text{I-HbV}\) and \(^{3}\text{H-HbV}\) were mainly distributed to the liver and spleen. Internal Hb disappeared from both the liver and spleen 5 days after injection, and the liposomal cholesterol disappeared at about 14 days. Internal Hb was excreted into the urine and cholesterol into feces \textit{via} biliary excretion. These results suggest that HbV has a reasonable blood retention, metabolic and excretion performance and could be used as an oxygen carrier.
Introduction

Blood transfusions are absolutely essential for resuscitation from massive bleeding after a surgical procedure. However, it has the potential of mismatching and can introduce certain infectious diseases such as hepatitis, HIV or West Nile virus etc., which are threats, in spite of the development of the nucleic acid amplification test. In addition, donated red blood cells (RBCs) for blood transfusions can only be stored for a period of 3 weeks in Japan. To overcome these problems, blood substitutes would be highly desirable and have been under development worldwide (Keipert, 1995; Winslow, 2005).

The hemoglobin vesicle (HbV) is an artificial cellular hemoglobin-based oxygen carrier with polyethylene glycol (PEG), in which phospholipid vesicles encapsulating highly concentrated human hemoglobin (Hb) serve as an oxygen carrier with oxygen transport characteristics that are comparable to RBCs. In fact, the pharmacological effects of HbV have been reported to be equivalent to that of RBCs, injected into rats with hemorrhagic shock (Sakai et al., 2004b; Sakai et al., 2009). In addition, HbV has been shown to possess a number of positive characteristics as follows, the absence of viral contamination (Sakai et al., 1993; Abe et al., 2006), a long-term storage period of over 2 year at room temperature (Goda et al., 1998; Sakai et al., 2000b; Abe et al., 2007) and a low toxicity (blood compatability; suppressed nephrotoxicity induced by the dimeric form of Hb and hypertension induced by the direct interaction of Hb with NO and CO) (Sakai et al., 2000a). Moreover, HbV suspended in a solution of human serum albumin (HSA) can be used to regulate rheological properties (e.g. viscosity and colloid osmotic pressure) (Sakai et al., 2000c; Sakai et al., 2004b). Based on these facts, the use of HbV would be predicted to be superior to that of a conventional blood transfusion.

The preclinical pharmacokinetic studies of HbV are essential to evaluate the safety
and efficacy of HbV. To sustain the pharmacological effect of HbV, prolonged oxygen delivery is a required property for an artificial oxygen carrier. In fact, the plasma retention time of free Hb, when isolated from RBCs, is surprisingly short (half-life of ~0.5-1.5 hr). In clinical situations, the total infused dose of RBCs given to patients can be considerable (e.g., for hemorrhagic shock or transfusion during an operation). Because the lipid content of HbV is more than a hundred times higher than that of other liposomal preparations such as AmBisome® or doxil®, massive amounts of Hb and lipid components can be infused when HbV is substituted for RBCs.

Free Hb molecules can trigger numerous side effects, such as renal toxicity, hypertension as well as tissue damage induced by the Fenton reaction, which is mediated by heme (iron) (Balla et al., 2005). On the other hand, high levels of lipid components in the bloodstream, especially cholesterol, are risk factors for kidney disease, arterial sclerosis and hyperlipidemia (Grone and Grone, 2008). Despite the large body of pharmacological evidence for HbV as an artificial oxygen carrier, little is known concerning its pharmacokinetic properties, especially the fate of each component after injection. As of this writing, metabolism studies of HbV components have only been done by histopathological examination and blood serum biochemistry (Sakai et al., 2001; Sakai et al., 2009). However, it is difficult to distinguish between exogenous lipid components, derived from HbV, from endogenous substances. Biodistribution of HbV has been examined by 99mTc-labelled HbV (Sou et al., 2005), however, no pharmacokinetic studies of Hb and lipids, from administration to excretion, were performed.

In the present study, we report on an evaluation of the pharmacokinetic properties of HbV and its components, from the standpoint of its stability in the blood circulation, the metabolism and excretion of each HbV component, in support of its use as an oxygen carrier.
For this purpose, we used two different radiolabeled HbVs, $^{125}$I-HbV in which the enclosed Hb was radiolabeled and $^3$H-HbV in which the lipid component (cholesterol) was radiolabeled.
Materials and Methods

Materials.

An Hb solution was purified from outdated donated blood that was provided by the Japanese Red Cross Society (Tokyo, Japan). Pyridoxal 5'-phosphate (PLP) was purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,5-bis-O-hexadecyl-N-succinyl-L-glutamate (DHSG) were purchased from Nippon Fine Chemical Co. Ltd. (Osaka, Japan). 1,2-distearyl-sn-glycero-3-phosphatidyl-ethanolamine-N-PEG (PEG-DSPE) was purchased from NOF Corp. (Tokyo, Japan). Recombinant human serum albumin (rHSA) was given by Nipro Corp. (Osaka, Japan). Williams’ Medium E and RPMI 1640 medium were purchased from Sigma Chemical Corp. (St. Louis, MO). Dulbecco’s Modified Eagle’s Medium and Penicillin-Streptomycin were purchased from GIBCO (Gaithersburg, MD).

Preparation of HbV.

HbVs were prepared under sterile conditions as previously reported (Sakai et al., 1997). The resulting encapsulated Hb (38 g/dl) contained 14.7 mM of PLP as an allosteric effector to regulate P_{50} to 25–28 torr. The lipid bilayer was comprised of a mixture of DPPC, cholesterol, and DHSG at a molar ratio of 5/5/1, and PEG-DSPE (0.3 mol%). The averaged particle diameter was 250-280 nm. The HbVs were suspended in a physiological salt solution at [Hb] 10 g/dL and [lipids] 6-7 g/dL, filter-sterilized (Dismic, Toyo-Roshi, Tokyo, Japan; pore size, 450 nm), and purged with N2 prior to storage.

In vivo experiment.

Animals
All animal experiments were undertaken in accordance with the guideline principle and procedure of Kumamoto University for the care and use of laboratory animals. Experiments were carried out with male ddY mice (28-30 g body weight; Japan SLC, Inc. Shizuoka Japan) and male Sprague-Dawley (SD) rats (180-210 g body weight; Kyudou Co. Kumamoto, Japan). All animals were maintained under conventional housing conditions, with food and water ad libitum in a temperature-controlled room with a 12-hr dark/light cycle. Before the pharmacokinetic studies using $^{125}$I-Hb or $^{125}$I-HbV, all of the animals were given water containing 5 mM sodium iodide (NaI) to avoid specific accumulation of the isotope in the glandula thyreoidea until the end of the experiment.

**Preparation of radiolabeled Hb and HbV**

$^{125}$I-Hb or $^{125}$I-HbV were prepared by incubating 200 μL of Hb or HbV (100 mM Hb) with iodine-125 as Na$^{125}$I (10 μL) (Piscataway, NJ, USA) in an Iodo-Gen (1, 3, 4, 6-tetrachoro-3α, 6 α- diphenylglycoluril) tube for 30 min at room temperature. $^{125}$I-Hb and $^{125}$I-HbV were then isolated from free $^{125}$I by passage through a PD-10 column (Pharmacia Biotech., Uppsala, Sweden). Over 97% of the total iodine was bound to internal Hb in HbV. $^{3}$H-HbV were prepared to mix HbV (1 mL) with $[1,2$-$^{3}$H(N)]-cholesterol solution (40 μL), (PerkinElmer, Yokohama, Japan) and incubated for 12 hr at room temperature. $^{3}$H-HbV were filtered through a sterilized filter to remove aggregates (pore size, 450 nm). The incubation of $^{3}$H-HbV in serum (24 hr, 37°C) revealed that $^{3}$H failed to completely dissociate from the HbV. Before use in pharmacokinetic experiments, all of the samples were mixed with unlabeled protein (Hb or HbV) to adjust the target Hb concentration. In addition, rHSA was added to correspond to the colloid osmotic pressure (Sakai et al., 1997).
Administration and collecting blood and organs in mice

DdY mice received a single injection of $^{125}$I-Hb (1 mg/kg), $^{125}$I-HbV (1, 10, 200 and 1400 mg Hb/kg) or $^3$H-HbV (1400 mg Hb/kg) containing 5% rHSA in the tail vain under ether anesthesia. At each time after the injection of labeled protein, blood was collected from the inferior vena cava, and plasma was obtained by centrifugation (3000 g, 5 min). After collecting blood, the animal was sacrificed for excision of organs. Urine and feces were collected at fixed intervals in a metabolic cage.

Administration and collecting blood and organs in rats

All of the SD rats were anesthetized with pentobarbital and received a single injection of $^{125}$I-HbV (10, 200 and 1400 mg/kg) or $^3$H-HbV (1400 mg/kg) containing 5% rHSA. The blood was collected from the tail vein, and plasma was obtained by centrifugation (3000 g, 5 min). At each time after an injection of $^{125}$I-HbV or $^3$H-HbV, the three animals were sacrificed for collecting organs, which were rinsed with saline. Urine and feces were collected at fixed intervals in a metabolic cage.

Measurement of $^{125}$I radioactivity

1% bovine serum albumin (BSA) and 40% trichloroacetic acid (TCA) were added to the plasma to remove degraded protein and free $^{125}$I, and pellets were obtained by centrifugation (1000 g, 10 min). The organs, urine and feces were weighed on an electronic balance. $^{125}$I radioactivity was counted using a liquid scintillation counter (ARC-5000, Aloka, Tokyo, Japan).

Measurement of $^3$H radioactivity
The plasma samples were ultracentrifuged to collect intact HbV (50000 g, 30 min) (Sakai et al., 2001). The collected HbV was solubilized in a mixture of Soluene-350 (Perkin Elmer, Yokohama, Japan) and isopropyl alcohol (at a ratio of 1/1) for 24 hr at 50°C, and decolorized by treatment with H2O2. The organ samples were rinsed with saline, minced, and solubilized in Soluene-350 for 24 hr at 50°C. Urine and feces were also weighed and solubilized in Soluene-350. Radioactivity was determined by liquid scintillation counter (LSC-5121, Aloka, Tokyo, Japan) with Hionic Flour (Perkin Elmer, Yokohama, Japan).

**Measurement of iron concentration**

The iron concentration was calculated from atomic emission spectrometric analysis (Ubest-35, Jasco, Tokyo, Japan) using an absorbance of 415 nm. The specific iron concentration derived from HbV was calculated by subtraction of the plasma iron concentration without HbV injection from that with HbV injection. At the same time, we confirmed that the plasma iron level hardly changed without HbV in mouse during the experimental periods.

**In vitro experiment.**

**Separation of hepatic cell**

Separation of hepatic cells from ddY mice was performed using a method similar to that described in a previous report (Nakajou et al., 2005). The portal vein was cannulated with a polyethylene catheter, and the liver was perfused with Gey’s balanced salt solution (GBSS) buffer, pH 7.5, without Ca2+ for 10 min at flow rate of 5 ml/min, and then with GBSS buffer, pH 7.5, containing 125 units/mL collagenase and Ca2+ for 3 min at 37°C. The liver was suspended in ice-cold 1% BSA. The cell suspension was centrifuged at 20 g for 2 min, and
the precipitate and supernatant were collected. Parenchymal and endothelial cells were isolated from the precipitate and supernatant as follows.

**Isolation and culture of hepatic parenchymal cell**

The precipitation was resuspended in 1% BSA, and again centrifuged at 20 g for 2 min. The cells in the pellet were resuspended in 10 mL of phosphate-buffer saline (PBS) and layered on top of a two step Percoll gradient. The gradient consisted of 25% (v/v) Percoll (top) and 50% (v/v) Percoll (bottom). The gradient was centrifuged 3000 g for 30 min and the intermediate zone collected. The enriched hepatic parenchymal cells were suspended in Williams’ Medium E (WE medium, FCS+) supplemented with 0.1 mg/ml streptomycin and 100 i.u./mL penicillin. Aliquots of the cell suspension were seeded into each well (5 × 10^5 per well) of collagenase-coated 24-multiwell plates. The multiple plates were incubated for 60 min at 37°C in a CO2 incubator. Each well was washed three times with 1 mL of PBS to remove non-adherent cells, then further incubated for 12 hr with 1 ml of WE medium (FCS+) and replaced by 1 ml of fresh WE medium (FCS-) followed by a 4 hr incubation before the experiment.

**Isolation and culture of hepatic endothelial cell**

The supernatant was suspended in 10 mL of RPMI 1640 medium (FCS-) and cultured on a plastic plate for 20 min (37°C) to remove Kupffer cells. The supernatant in the plastic plate was then seeded into each well (2 × 10^6 per well) of fibronectin-coated 24-multiwell plates. The multiple plates were incubated for 60 min at 37°C in a CO2 incubator. Each well was washed twice with 1 mL of PBS to remove non-adherent cells and parenchymal cells, then further incubated for 2 hr with 1 ml of RPMI 1640 medium (FCS+).
and replaced by 1ml of fresh RPMI 1640 medium (FCS-) followed by a 4 hr incubation before the experiment.

Culture of RAW 264.7 Cells

RAW 264.7 cells suspended in Dulbecco’s Modified Eagle’s Medium (DMEM medium, FCS+) were seeded into each well (5 × 10⁵ per well) of 24-multiwell plates. The multiple plates were incubated for 24 hr at 37°C in a CO₂ incubator. Each well was washed twice with 1 mL of PBS, and replaced by 1 ml of fresh DMEM medium (FCS-) followed by a 4 hr incubation before the experiment.

Cell assays

The parenchymal, endothelial and RAW 264.7 cells in each well were incubated with various concentrations of ¹²⁵I-HbV for examination, with or without an excess of corresponding unlabelled HbV. After 6 hr incubation, 0.575 ml of culture medium was removed from each well, and mixed with 0.15 ml of 40 % TCA and 0.15 ml of 0.7 M AgNO₃ in a vortex mixer, followed by centrifugation (1000 g, 10 min). The resulting supernatant (0.25 mL) was used to determine TCA-soluble radioactivity, which was taken as an index of the extent of cellular degradation. The remaining cells in each well were washed five times with 1 ml of PBS. The cells were lysed at 37°C for 30 min with 1 ml of 0.1 M NaOH. One portion was used to determine the radioactivity as the cell-associated ligand; the other was used to determine the cellular protein content.

Data Analysis

Pharmacokinetic analyses after HbV or Hb administration proceeded based on a
two-compartment model. Pharmacokinetic parameters were calculated by fitting using MULTI, a normal least-squares program (Yamaoka et al., 1981). The uptake clearance (CL\textsubscript{uptake}) was calculated, as described in a previous report using integration plot analysis at designated times (from 1 min to 30 min) during which the efflux and/or elimination of radioactivity from tissues were negligible (Murata et al., 1998). Data are shown as means ± SD for the indicated number of animals. The overall differences between groups were determined by one-way of analysis of variance (ANOVA). A probability value of p<0.05 was considered to indicate statistical significance.
Results

Comparison of Pharmacokinetic Properties between Hb and HbV.

The fates of the $^{125}$I-Hb and $^{125}$I-HbV administered to mice were evaluated as residual TCA-precipitable radioactivity in the plasma. Figure 1A shows the time courses for the plasma concentration of $^{125}$I-Hb and $^{125}$I-HbV in mice at a dose of 1 mg/kg. The blood circulation of HbV was notably increased. According to the pharmacokinetic parameters based on a two-compartment model analysis, the clearance (CL) of the $^{125}$I-HbV was significantly decreased ($3.4\pm0.1$ mL/hr) compared with that of $^{125}$I-Hb ($12.7\pm2.1$ mL/hr, $p<0.001$). The half-life ($t_{1/2}$) and the area under the concentration-time curve (AUC) of $^{125}$I-HbV were increased ($t_{1/2}$: $3.1\pm1.0$, $0.1\pm0.1$ hr, $p<0.01$: AUC: $29.4\pm9.2$, $7.9\pm3.9$ hr*% of dose/mL, $p<0.001$, for $^{125}$I-HbV and $^{125}$I-Hb, respectively) with decreasing CL, whereas the distribution volume ($V_1$) remained unchanged ($2.3\pm0.1$, $2.6\pm0.3$ mL for $^{125}$I-HbV and $^{125}$I-Hb, respectively). Fig. 1B shows the tissue distribution in each organ at 3 min after an injection of $^{125}$I-Hb or $^{125}$I-HbV. As expected $^{125}$I-Hb was mainly distributed in the kidney and liver. In contrast, the distribution of $^{125}$I-HbV was significantly decreased, while its distribution in the spleen and lung were increased significantly.

Dose-dependency of HbV Pharmacokinetics.

Table 1 shows the pharmacokinetic parameters for $^{125}$I-HbV administered to mice at doses of 1, 10, 200 and 1400 mg/kg. As the dose was increased, $t_{1/2\beta}$ was increased and CL was decreased. The AUC was increased in proportion to $t_{1/2\beta}$. Table 2 shows the dose dependent uptake clearance (CL$_{uptake}$) in each organ. CL$_{uptake}$ in liver and spleen were much higher than that in the other organs. As the dose was increased, CL$_{uptake}$ in liver and spleen was decreased. These results suggest that the distribution of HbV to the liver and spleen,
which contained the majority of HbV, were saturated at higher doses.

**Cell Assay**

It is well-known that liposomes are scavenged and degraded by the mononuclear phagocyte system (MPS) such as Kupffer cells or macrophages (Kiwada et al., 1998). Since HbV was mainly distributed in the liver and spleen, where MPS is mainly localized, we examined the issue of whether HbV is scavenged and degraded by MPS using RAW 264.7 cells, which has been used as an alternative to Kupffer cells. In these experiments, primary parenchymal and endothelial cells from mouse livers were used as a control. As shown in Fig. 2, the specific uptake and degradation of $^{125}$I-HbV was observed only in the RAW 264.7 cells and not in primary parenchymal and endothelial cells. This *in vitro* study supports that HbV is captured by Kupffer cells in the liver and macrophages in the red pulp zone of the spleen, as previously reported in an *in vivo* study (Sakai et al., 2001).

**Pharmacokinetics of HbV Component in mice**

In order to investigate the pharmacokinetics of each HbV component, Hb, enclosed in HbV was radiolabeled with $^{125}$I ($^{125}$I-HbV) or cholesterol, in the lipid component vesicles of HbV was radiolabeled with $^3$H ($^3$H-HbV). As shown in Fig. 3 and Table 3, similar plasma concentration curves and pharmacokinetic parameters of $^{125}$I-HbV were observed for $^3$H-HbV. Furthermore, after administering non-labeled HbV to mice, time course for the plasma iron concentration curve derived from HbV, was consistent with the plasma concentration curves for both labeled-HbV (Fig. 3). These data indicate that HbV is likely to maintain an intact structure in the blood circulation for periods of up to 24 hr after injection. We also examined the issue of whether the injected HbV had any influence on the production of reactive
oxidative stress (ROS) because, if free iron were released from vesicles, it would enhance the production of ROS by the Fenton reaction, as has been previously demonstrated (Anraku et al., 2004; Anraku et al., 2008). To quantitatively evaluate the extent of oxidative stress in the blood circulation, we monitored the ratio of the mercapt-form (non-oxidised form) to the nonmercapt-form (oxidized form) of human serum albumin, which serves as a marker of oxidative stress in the circulation system (Kadowaki et al., 2007; Shimoishi et al., 2007). No significant differences in these ratios were found between HbV and the saline administration groups for periods of up to 7 days after administration (data not shown).

Moreover, the tissue distribution of $^{125}$I-HbV was evaluated using the tissue-to-plasma partition coefficient (Kp). Figure 4 shows the Kp values in organs (kidney, liver, spleen, lung and heart) 8 hr after the administration of $^{125}$I-HbV or $^3$H-HbV to mice. Among these organs, the Kp values for both the liver and spleen reached >1 for both radiolabeled HbVs. Therefore, we examined the time course for the tissue distribution in the liver (Fig. 5A) and spleen (Fig. 5B) after the administration of both labeled-HbVs. At an early time period (Fig. 5, small window), the time course distributions for $^{125}$I-HbV and $^3$H-HbV were consistent with each other, and the CL$_{uptake}$ in liver and spleen were also similar between the two (Liver: 256±37, 301±41; Spleen; 51±6, 43±12 μL/hr, for $^{125}$I-HbV and $^3$H-HbV, respectively). However, the radioactive $^{125}$I was more rapidly eliminated from each organ, and the activity essentially disappeared within 7 days. On the other hand, the elimination of radioactive $^3$H was delayed compared to that of $^{125}$I, and nearly disappeared after 14 days in both the liver and spleen. These data indicate that HbV was mainly distributed to liver and spleen in the form of intact HbV, and that it was degraded by MPS, followed by different routes of excretion for the internal Hb and the lipid component.

In order to identify the excretion pathway of HbV, the levels of radioactivity $^{125}$I and
$^3$H in urine and feces were measured (Fig. 6). The radioactive $^{125}$I was excreted mainly in the urine (84.2±4.1 % of ID at 7 day after injection), but was low in feces (5.1±2.3 % of ID at 7 day after injection). In addition, neither Hb nor protein urea or hemoglobinuria were detected in the urine (data not shown). On the other hand, the majority of the radioactive $^3$H was excreted in the feces (71.1±3.6% % of ID at 7 day after injection), and a small portion was excreted into the urine (19.8±3.4 % of ID at 7 day after injection). At 7 days after injection, a high level of radioactivity from $^3$H was detected in plasma lipoprotein and bile, but $^{125}$I was not (data not shown).

**Pharmacokinetics of the HbV Component in rats**

Since altered pharmacokinetics of liposome have been reported for different animal species, we carried out a pharmacokinetic analysis in rats as well. The rats showed a similar dose-dependency for HbV pharmacokinetics as was observed for mice, except the half-life of HbV was doubled ($t_{1/2}$: 8.8±0.7, 11.5±0.3 and 30.6±4.0 hr at doses of 10, 200 and 1400 mg/kg, respectively).

Table 3 shows data for the pharmacokinetic analysis of $^{125}$I- and $^3$H-HbV in rats. Although the pharmacokinetic behaviors of both radiolabeled HbVs were similar to those for mice, the maximum hepatic distributions of labeled-HbVs in rats were decreased by nearly half of those in mice (13.5±0.5, 17.7±3.0 % of injected dose (ID), for $^{125}$I- and $^3$H-HbV, respectively). Moreover, the radioactive $^{125}$I was excreted mainly into the urine (77.9±6.1, 4.8±1.6 % of ID at 7 day after injection, for urine and feces, respectively). On the other hand, the majority of the radioactivity of $^3$H was excreted into feces (10.9±4.4, 66.3±14.8 % of ID at 7 day after injection, for urine and feces, respectively).
Discussion

HbV has been developed as an artificial oxygen carrier, and has considerable promise for use in clinical settings because of its superb functionality, such as its ability to regulate rheological properties, cardiocirculatory dynamics and its oxygen carrier ability (Izumi et al., 1996; Sakai et al., 2008). Previously, free Hb and perfluorocarbon, which had also been developed for use as artificial oxygen carriers, were excluded from possible candidates for artificial oxygen carriers, because their systemic half-lives were too short (~0.5-1.5hr) or long (1 year or longer) for them to effectively function as an optimal oxygen carrier, respectively (Savitsky et al., 1978; Nose, 2004). Therefore, it is necessary to characterize the pharmacokinetic properties of HbV and its components to demonstrate the efficacy and safety of this preparation because the short half-life of HbV leads to a diminished pharmacological effect, while a long half-life increases its bioaccumulative potential.

In this study, the half-life of HbV was found to be 30 times higher than that of stroma-free Hb at a dose rate of 1 mg Hb/kg due to a decreased distribution to the liver and kidney (Fig. 1). This could reflect physicochemical differences, such as diameter, the absence or presence of a membrane structure and PEG-modification between HbV and Hb. In physiological conditions, Hb that is released from ruptured RBC becomes rapidly bound to haptoglobin (Hp), which promotes CD163 recognition in the liver (Kristiansen et al., 2001). When the Hb concentration exceeds the Hp binding capacity, unbound Hb is filtered through the kidney (Savitsky et al., 1978). Thus, the reduction in HbV distribution in the liver and kidney could be due to the encapsulation of Hb by the liposome because it might not only suppress the binding of internal Hb to Hp but also inhibit renal glomerular filtration.

In the case of HbV, approximately 10 % of the dose was distributed to the liver. Since HbV possesses a liposome structure, it would be expected to be captured by MPS in the
liver and spleen (Kiwada et al., 1998). In fact, as the dose is increased, $CL_{uptake}$ in the liver and spleen were decreased (Table 2) and the half-lives were increased (Table 1). In addition, specific uptake and degradation were observed only in macrophage cells but not in parenchymal and endothelial cells (Fig. 2). These results strongly suggest that HbV is scavenged by MPS such as Kupffer cells or the red pulp zone, and that this ability became saturated at high doses of HbV. These results are in reasonably good agreement with previous in vivo findings that HbV is taken up by Kupffer cells, the red pulp zone and mesangial cells (Sakai et al., 2001; Sakai et al., 2004a). However, because of the increased distributions of HbV into spleen and lung as compared to free Hb, further study will be needed to demonstrate the effect of HbV administration on immune and respiratory system.

In clinical situations, a massive dose of HbV would be administered to patients such as for hemorrhagic shock or in the case of an intensive surgical procedure. Because of this, it is important to confirm the safety of HbV components from the view point of pharmacokinetics, because Hb molecules can trigger a number of side effects such as renal toxicity and hypertension derived by scavenging endothelium-derived NO (synthesized by NOS3) (Yu et al., 2008) and tissue damage induced by the Fenton reaction mediated by heme (iron) (Balla et al., 2005). In addition, the long-term circulation of high amounts of lipids, especially cholesterol, contribute to cardiovascular and kidney disease (Grone and Grone, 2008). In this study, we were able to confirm the safety of HbV components, including Hb, iron, lipids, based on pharmacokinetic analyses, as evidenced by the following procedures.

First, the findings herein clearly showed that up to 24 hr after injection, the plasma concentration curves of $^{125}$I-HbV, $^3$H-HbV and iron derived from HbV exhibited similar behaviors (Fig. 3). In addition, the $CL_{uptake}$ in liver and spleen were also similar between $^{125}$I-HbV and $^3$H-HbV (see result section). These results indicate that the HbV circulates in the
bloodstream as stable, intact vesicles until degraded by MPS.

Secondly, nearly 80% of the radioactive $^{125}\text{I}$ is excreted into the urine by 7 days after its injection. This radioactivity is likely due to the degradation of Hb enclosed in liposomes, because $^{125}\text{I}$ binds covalently to tyrosine residues of protein (Burger et al., 1983). In fact, neither Hb nor protein urea and hemoglobinuria were detected in the urine. Moreover, a previous study showed that multiple or a single high dose treatment of HbV failed to induce any detectable pathological injury in the kidney or any change in arteriolar or venular diameters, which are induced by the dimeric form of Hb or any direct interaction of Hb with NO (Sakai et al., 2001; Sakai et al., 2004a; Cabrales et al., 2005). These results indicate that $^{125}\text{I}$-HbV is degraded by MPS, and that the $^{125}\text{I}$ is excreted in the urine in the form of a degradation product of $^{125}\text{I}$-Hb derived from HbV.

Third, an excess of heme (iron) could cause hemosiderosis and oxidative stress via the Fenton reaction (Buehler and Alayash, 2004). Because of this, the disposition of heme (iron) derived from HbV needs to be clarified. Our study showed that the plasma concentration curve for heme (iron) derived from HbV was similar to that for $^{125}\text{I}$-HbV and $^{3}\text{H}$-HbV (Fig. 3). In addition, after an HbV injection, systematic oxidative stress, as estimated by the oxidized albumin ratio, was not increased (data not shown). These results suggest that excess amount of free heme (iron) derived from HbV was not released in the plasma. In fact, a previous study reported that a daily repeated infusion of HbV (1000 mg Hb /kg/day) for 14 days had no effect on plasma iron and bilirubin levels. Therefore, phospholipid vesicles for the encapsulation of Hb would be beneficial for heme detoxification through their preferential delivery to the MPS as described in a previous report (Sakai et al., 2004a). Furthermore, the deposition of hemosiderin, which is siderosis as heme detoxification, was detected in the liver and spleen after the administration of 2000 mg Hb /kg of HbV, but, this deposition completely
disappeared after 14 days (Sakai et al., 2001). In the future, it will be necessary to study ferrokinetics to confirm this issue by using Hb with radiolabeled iron (e.g. $^{55}$Fe and $^{59}$Fe). It is also interesting to know whether the deposited iron is effectively utilized for hematopoiesis after a massive blood loss and HbV administration.

Finally, the findings herein show that $^{3}$H-cholesterol in HbV was mainly distributed to the liver and spleen, and subsequently excreted into feces around 14 days after $^{3}$H-HbV injection (Fig. 4 and Fig. 5). These results are in good agreement with histopathological examinations, using oil red O staining on liver tissue after an injection of 2000 mg Hb/kg of HbV, which revealed that slight stains were confirmed 3 days after injection, and this staining disappeared within 7 days (Sakai et al., 2001). In addition, Sakai et al. found that daily repeated infusion of HbV for 14 days temporally elevated the plasma lipid components which are one of the risk factor of atherosclerosis (Sakai et al., 2004a). However, cholesterol of the vesicles should reappear in the blood mainly as lipoprotein cholesterol after entrapment in the Kupffer cells and should then be excreted in bile after entrapment of the lipoprotein cholesterol by the hepatocytes (Kuipers et al., 1986). In fact, the high level of radioactivity of $^{3}$H in plasma lipoproteins and bile, which are related to carrying and excretion of endogenous cholesterol by physiological pathways were observed (data not shown). Therefore, it would be desirable to know that cholesterol in the lipid components in HbV behaved the same as endogeneous cholesterol after the metabolization of HbV in MPS.

On the other hand, we did not directly examine the disposition of phospholipid, DPPC, in HbV. Previous reports have shown that phospholipids in the liposome are metabolized in MPS and reused as cell membranes or are excreted into the bile (Dijkstra et al., 1985; Verkade et al., 1991). Therefore, it is also possible that phospholipids in HbV are also metabolized and excreted in the same manner as mentioned above. However, further study...
will be needed to demonstrate this fact.

Base on the present findings, we propose that the disposition of HbV and its components, after circulating in the form of stable HbV, are distributed to the liver and spleen, where they are degraded by MPS. Finally, the enclosed Hb and outer lipid components are mainly eliminated to the urine and feces, respectively, in the same manner as endogeneous substances. In addition, pharmacokinetic study using different animal species enables to predict the pharmacokinetic in human. In fact, we previously reported the half-life of HbV in human was estimated to be approximately 3-4 day by using allometric equation (Taguchi et al., 2009). The above findings provide further support for the effectiveness and safety of HbV for use as an oxygen carrier.
References.


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system for the degradation of hemoglobin vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J Pharmacol Exp Ther* **311**:874-884.


objective comparison between cellular and acellular types. *Bioconjug Chem* 11:56-64.


Footnotes.

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K.T. and Y.U. contributed equally to this work.
Figure legends.

Fig. 1. A, Time course for the plasma level of $^{125}$I-Hb (open symbols) and $^{125}$I-HbV (filled symbols) after administration to mice. DdY mice received a single injection of $^{125}$I-Hb or $^{125}$I-HbV from the tail vein at a dose of 1 mg/kg. Blood was collected from the inferior vena cava under ether anesthesia, and a plasma sample was obtained. Each point represents the mean ± SD (n=3-6). B, Tissue distributions of $^{125}$I-Hb (open symbol) and $^{125}$I-HbV (filled symbol) at 3 min after administration to mice. DdY mice received a single injection of $^{125}$I-Hb or $^{125}$I-HbV from the tail vein at a dose of 1 mg/kg. At 3 min after injection, each organ was collected. Each bar represents the mean ± SD (n=3-6). *p< 0.05, **p<0.01 and ***p<0.001 vs $^{125}$I-Hb.

Fig. 2. A, Dose-dependent endocytic (a) uptake and (b) degradation of $^{125}$I-HbV by RAW 264.7 cells. B and C, endocytic uptake of $^{125}$I-HbV by parenchymal cells and endothelial cells, respectively. RAW 264.7 cells, primary parenchymal and endothelial cells were incubated at 37 °C for 6 hr with the indicated concentration of $^{125}$I-HbV in the presence (filled squares) or absence (filled circles) of 50-fold unlabeled HbV. Specific uptake or degradation (open circle) were calculated by subtracting the non-specific values from the total values. Results are the mean ± SD of three separate experiments.

Fig. 3. Time course for the plasma level of $^{125}$I-HbV (open circles), $^{3}$H-HbV (filled circles) and iron (open squares) derived from HbV after administration to mice. DdY mice received a single injection of $^{125}$I-HbV or $^{3}$H-HbV from the tail vein at a dose of 1400 mg Hb /kg. Blood was collected from the inferior vena cava under ether anesthesia, and a plasma sample was obtained. The iron concentration was calculated from atomic emission spectrometric analysis.
using an absorbance of 415 nm. Each point represents the mean ± SD (n=3-6).

Fig. 4. The tissue-to-plasma partition coefficient (Kp) of $^{125}\text{I}$-HbV (open bar) and $^3\text{H}$-HbV (filled bar) 8 hr after administration to mice. Each bar represents the mean ± SD (n=6).

Fig. 5. Time course for radioactivity in liver (A) and spleen (B) after the administration of $^{125}\text{I}$-HbV (open circles) or $^3\text{H}$-HbV (filled circles) at a dose of 1400 mg Hb/kg to mice. DdY mice received a single injection of $^{125}\text{I}$-HbV or $^3\text{H}$-HbV from the tail vain at a dose of 1400 mg Hb/kg. Each point represents the mean ± SD (n=3-6).

Fig. 6. Time course for radioactivity in urine (A) and feces (B) after the administration of $^{125}\text{I}$-HbV (open circles) and $^3\text{H}$-HbV (filled circles) to mice. DdY mice received a single injection of $^{125}\text{I}$-HbV or $^3\text{H}$-HbV from the tail vain at a dose of 1400 mg Hb/kg. Urine and feces were collected at fixed intervals in a metabolic cage. Each point represents the mean ± SD (n=3-6).


TABLE 1

Dose-dependent pharmacokinetic parameters of HbV after administration of $^{125}\text{I}-\text{HbV}$ in mice

All mice received a single injection of $^{125}\text{I}-\text{HbV}$ (1, 10, 200 and 1400 mg Hb/kg) containing 5% rHSA. At each time (0.05, 0.5, 1, 2, 4, 6, 8, 12 and 24 hr) after the $^{125}\text{I}-\text{HbV}$ injection, blood samples were collected from the inferior vena cava, and a plasma sample was obtained. Each parameter was calculated by MULTI using a two-compartment model.

<table>
<thead>
<tr>
<th>dose (mg/kg)</th>
<th>1</th>
<th>10</th>
<th>200</th>
<th>1400</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{1/2\beta}$ (hr)</td>
<td>3.1±3.1</td>
<td>3.6±1.3</td>
<td>7.2±3.1</td>
<td>18.8±1.3</td>
</tr>
<tr>
<td>AUC (hr*% of dose/mL)</td>
<td>29.4±7.2</td>
<td>32.9±3.8</td>
<td>134±42</td>
<td>829±38</td>
</tr>
<tr>
<td>CL (mL/hr)</td>
<td>3.40±0.1</td>
<td>3.04±0.1</td>
<td>0.74±0.1</td>
<td>0.12±0.1</td>
</tr>
<tr>
<td>$V_1$ (mL)</td>
<td>2.35±0.2</td>
<td>2.43±0.3</td>
<td>2.24±0.2</td>
<td>1.75±0.6</td>
</tr>
</tbody>
</table>

The values are mean ± S.D. (n=3-6).
TABLE 2

Dose-dependent uptake clearance of HbV in liver, spleen, kidney, lung and heart after 125I-HbV administration in mice

All mice received a single injection of 125I-HbV (1, 10, 200 and 1400 mg Hb /kg) containing 5% rHSA. The uptake clearance for each organ was calculated by integration plot analysis at designated times from 1 min to 30 min after injection.

<table>
<thead>
<tr>
<th>dose (mg/kg)</th>
<th>CLuptake (μL/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>liver</td>
</tr>
<tr>
<td>1</td>
<td>2608±654</td>
</tr>
<tr>
<td>10</td>
<td>1473±440</td>
</tr>
<tr>
<td>200</td>
<td>452±114</td>
</tr>
<tr>
<td>1400</td>
<td>256±37</td>
</tr>
</tbody>
</table>

The values are mean ± S.D. (n=3-6).
TABLE 3

Pharmacokinetic parameters of HbV after administration of $^{125}$I- and $^{3}$H-HbV in mice and rats.

All mice and rats received a single injection of $^{125}$I-HbV or $^{3}$H-HbV at a dose of 1400 mg Hb/kg containing 5% rHSA. At each time after the injection of $^{125}$I-HbV or $^{3}$H-HbV, a blood sample was collected from the inferior vena cava (mice) or from the tail vein (rats), and plasma was obtained. Each parameter was calculated by MULTI using a two-compartment model.

<table>
<thead>
<tr>
<th></th>
<th>mice</th>
<th></th>
<th>rats</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{125}$I-HbV</td>
<td>$^{3}$H-HbV</td>
<td>$^{125}$I-HbV</td>
<td>$^{3}$H-HbV</td>
</tr>
<tr>
<td>$T_{1/2\beta}$ (hr)</td>
<td>18.8±1.3</td>
<td>19.9±0.9</td>
<td>30.6±4.0</td>
<td>30.9±4.7</td>
</tr>
<tr>
<td>AUC (hr*% of dose/mL)</td>
<td>829±28</td>
<td>899±44</td>
<td>210±23</td>
<td>247±22</td>
</tr>
<tr>
<td>CL (mL/hr)</td>
<td>0.12±0.04</td>
<td>0.11±0.03</td>
<td>0.46±0.04</td>
<td>0.41±0.02</td>
</tr>
<tr>
<td>$V_1$ (mL)</td>
<td>1.75±0.6</td>
<td>1.71±0.1</td>
<td>10.9±0.2</td>
<td>10.2±2.0</td>
</tr>
</tbody>
</table>

The values are mean ± S.D. (n=3-6).
Fig. 1

A

% of dose

\[ \frac{125 \text{ I-Hb}}{125 \text{ I-HbV}} \]

Time (hr)

B

% of dose

\[ \frac{125 \text{ I-Hb}}{125 \text{ I-HbV}} \]

kidney  liver  spleen  lung  heart
Fig. 2

A. Raw 264.7 cell

(a) uptake

(b) degradation

B. parenchymal cell

C. endothelial cell
Fig. 3

A graph showing the percentage of dose remaining over time for different substances. The y-axis represents the percentage of dose, ranging from 0 to 100. The x-axis represents time in hours, ranging from 0 to 24.

Four lines represent different substances:
- Open circles: $^{125}$I-HbV
- Solid circles: $^{3}$H-HbV
- Squares: Fe
- Solid line with dotted line: $^{3}$H-HbV

The graph indicates that the percentage of dose decreases over time for all substances, with $^{125}$I-HbV and $^{3}$H-HbV showing a slightly faster decay compared to Fe.
Fig. 4
**Fig. 5**

**A. liver**

![Graph showing the percentage of dose over time for liver](image)

**B. spleen**

![Graph showing the percentage of dose over time for spleen](image)
Fig. 6

A. urine

![Graph showing % of dose vs. Time (days) for urine.]

B. feces

![Graph showing % of dose vs. Time (days) for feces.]