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Systemic Administration of Hemoglobin Vesicle Elevates Tumor Tissue Oxygen Tension and Modifies Tumor Response to Irradiation

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Background. **We have developed a phospholipid liposome vesicle encapsulating concentrated human hemoglobin (hemoglobin vesicle, HbV) as an artificial oxygen carrier, as an alternative to red cell transfusion. We have verified its oxygen transporting capability in a variety of preclinical models. Recent evidence suggests that artificial oxygen carriers may also be applicable for better oxygenation of ischemic or hypoxic tissues including tumors. To our knowledge, tumor oxygenation using a liposome-type artificial oxygen carrier has not been closely tested. In the present study, we tested whether systemic HbV administration changes tumor tissue oxygen tension, and if it modifies tumor response to irradiation.**

Materials and methods. **Lewis lung carcinoma was grown subcutaneously in the left hindleg of C57BL/6 mice. Experiments were initiated when the tumors reached approximately 8 mm. All experiments were done under room air. Tumor tissue oxygen tension was measured by phosphorescence quenching up to 45 min after systemic sample administration (saline: $n = 5$; HbV: $n = 5$; HbV containing methemoglobin (metHbV): $n = 4$; HbV with high oxygen affinity (lowP50HbV): $n = 8$) and compared between samples. To test the effects on irradiation response, samples (saline: $n = 7$; HbV: $n = 7$; metHbV: $n = 7$; lowP50HbV: $n = 7$) were administered prior to single 20-Gy irradiation, and tumor growth was compared.**

Results. **Tumor tissue oxygen tension transiently increased approximately 2-fold after HbV administration in comparison to other samples. Tumor growth was marginally delayed after irradiation by prior administration of HbV in comparison to other samples. HbV administration without irradiation did not affect significant tumor growth delay.**

Conclusions. **These results correlatively suggest that HbV augmented tumor growth delay following irradiation, at least in part, by affecting tumor tissue oxygen tension.** © 2009 Elsevier Inc. All rights reserved.

Key Words: **hemoglobin vesicle; artificial oxygen carrier; tumor oxygenation; radiosensitizer; liposome; HIF1alpha.**

INTRODUCTION

Artificial oxygen carriers are currently being actively developed for use as transfusion alternatives. Artificial oxygen carriers do not have blood types, are free of potential infectious pathogens, and can be stored much longer than red blood cells (RBCs) [1]. Several preclinical studies indicate that they can be effectively applied as temporal resuscitative fluids, and some are undergoing clinical trials [2, 3].

Hemoglobin (Hb)-based oxygen carriers are classified into acellular chemically modified Hbs and encapsulated Hbs [4, 5]. We have developed a phospholipid liposome vesicle encapsulating concentrated human hemoglobin (hemoglobin vesicle, HbV) as an artificial oxygen carrier [1]. The cellular structure of HbV has characteristics that

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resemble those of RBCs. It has lipid bilayer membranes that prevent the direct contact of hemoglobin with blood components and the endothelial lining, thus shielding the side effects of molecular hemoglobin [6, 7]. HbV particles are eventually captured by the phagocytes in the reticuloendothelial system and are metabolized through existing physiological pathways [8–10]. We have studied the oxygen transporting capabilities of HbV using several exchange transfusions, and hemorrhagic shock animal models [11–15]. In these studies, we have shown that HbV effectively restores the systemic circulation similar to red cell transfusion. Recently, evidence is accumulating that artificial oxygen carriers may be potentially applicable as so-called oxygen therapeutics, which enable oxygenation of ischemic tissues [16, 17]. Increasing tumor tissue oxygen tension is one such possibility. In the present study, we show that systemic administration of HbV transiently increases tumor tissue oxygen tension and modifies tumor response to irradiation.

MATERIALS AND METHODS

Preparation of Hemoglobin Vesicles

Preparation of poly(ethylene glycol) modified HbV was performed at Waseda University under sterile conditions as previously reported [18–20]. Hemoglobin was purified from outdated donated blood provided by Japanese Red Cross Society (Tokyo, Japan). The encapsulated hemoglobin (38 g/dL) contained 14.7 mmol/L of pyridoxal 5′-phosphate (PLP; Sigma, St. Louis, MO) as an allosteric effector at a molar ratio of Hb/PLP = 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 Chemicals, Osaka, Japan) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) (NOF Corp., Tokyo, Japan; 0.3 mol% of the total lipid). HbV was suspended in saline at the Hb concentration of 10 g/dL. The physicochemical parameters of the HbV were as follows: particle diameter, 251 ± 80 nm; methemoglobin concentration, <1%; HbCO concentration, <2%; and oxygen affinity (P_{50}), 29 Torr.

Preparation of Methemoglobin Vesicles

Methemoglobin vesicles (metHbV) were formed by oxidation of hemoglobin contained within HbV (methemoglobin formation), using the oxidative properties of nitrosylhemoglobin [21]. Nitric oxide gas was infused into the deoxygenated HbV suspension to transform hemoglobin to nitrosylhemoglobin. After infusion of nitrogen gas to expel the excess nitric oxide, oxygen was infused to convert nitrosyl-hemoglobin to methemoglobin, yielding metHbV. Vesicle properties were considered to be equivalent to HbV except for its lack of oxygen transporting ability.

Preparation of Low P50 Hemoglobin Vesicles

HbV with P_{50} of approximately 8 Torr (lowP50HbV) was prepared in the same way as HbV except that pyridoxal 5′-phosphate was not added. Particle diameter was controlled to 250 ± 64 nm. Vesicle properties were considered to be equivalent to HbV except for its P_{50}.

Animal and Tumor

Male C57BL/6 mice, approximately 9 wk old, weighing 21 to 25 g (Oriental Yeast Co., Tokyo, Japan) were used for the experiment. The animals were housed 5 per cage in a specific pathogen-free, temperature-controlled, 12-h light/dark-cycled room with free access to food and water. For the experiments, the animals were anesthetized with intramuscular injection of a cocktail of 90 mg ketamine hydrochloride (Parke-Davis, Morris Plains, NJ) and 9 mg xylazine (Fermentia, Kansas City, MO) per kilogram body weight.

Lewis lung carcinoma cell line (Dainippon Sumitomo Pharma Co., Tokyo, Japan) was used for this study. In a separate group of donor mice, the tumors were grown subcutaneously and passaged. Under anesthesia, an approximately 1-mm-diameter tumor fragment was taken from the donor mouse and was placed subcutaneously in the left hindleg of mice in the experiment groups. Experiments were initiated 10 d after tumor implantation, at which time the tumors reached approximately 8 mm in diameter.

For material administration, the tail vein was cannulated under anesthesia by a 30-gauge needle and the needle was fixed by a cyanoacrylate adhesive. All experiments were carried out under room air.

All experimental protocols were reviewed by the Committee on the Ethics of Animal Experiments at School of Medicine, Keio University, and were carried out in accordance with Guidelines for Animal Experiments issued by the School of Medicine Keio University Experimental Animal Center and The Law (No. 105) and Notification (No. 6) issued by the Japanese Government. These guidelines meet the guidelines for animal handling issued by the National Institutes of Health (NIH).

Tumor Tissue Oxygen Tension Measurements

The tumor tissue oxygen tension was measured by the oxygen-dependent quenching of phosphorescence using Oxyspot phosphorimeter systems (Medical Systems Corp., Greenvale, NY). The skin over the tumor was carefully removed, and a glass coverslip was placed over the tumor. The skin on the back was also carefully removed and the back muscle was exposed followed by coverslip placement. Saline was injected under the coverslip to sustain moisture. The porphyrin probe (Oxygen Probe; Harvard Apparatus, Holliston, MA), 5,10,15,20-tetrakis(4-carboxylphenyl)porphinatoparadium, was provided as a lyophilized powder containing 9% probe, 80% bovine serum albumin (clinical grade, low fatty acid), 3% Trisma buffer, and 8% NaCl. It was dissolved in distilled water (pH 7.4) and administered through the tail vein (0.9 mL/kg). Approximately 5 min after probe administration, oxygen tension measurement was initiated. The light from the flash-lamp was filtered through an interference filter with 545-nm center wavelength and half-bandwidth of 45 nm. It was conducted through the light guide and focused on an approximately 8-mm-diameter area of the tumor tissue, approximately 4 mm from the tumor surface. The phosphorescence collected by the lens was introduced into the light guide and was filtered through a long pass filter at 645 nm, which was then led into the photomultiplier. The phosphorescence decay was fitted to a single exponential to determine the lifetime and oxygen partial pressure using the Stern–Volmer equation [22].

Measurements were made 3 times and averaged for each time point. Back skeletal muscle tissue oxygen tension was measured before and after tumor tissue oxygen tension measurements as control values. Tumor tissue oxygen tension was measured before (time 0), 1, 2, and 3 min after sample administration, and every 3 min thereafter until 45 min. Samples (saline: saline group, $n = 5$; HbV: HbV group, $n = 5$; metHbV: metHbV group, $n = 4$; lowP50HbV: lowP50HbV group, $n = 8$) were administered through the tail vein,12 mL/kg, in approximately 1 min.

Tumor Growth Measurements

Tumor growth measurements were made after sample administration followed by irradiation (saline: salinerad group, $n = 7$; HbV: HbVrad group, $n = 7$; metHbV: metHbVrad group, $n = 7$; lowP50HbV: lowP50HbVrad group, $n = 7$) or without irradiation (saline: salinenon-

rad group, $n = 5$; HbV: HbVnonrad group, $n = 4$). Single-dose irradiation (20 Gy) was delivered to the tumor-bearing mice using a device designed for mouse bone marrow irradiation (Hitachi Medicotechnology, MBR-1520R-3, Hitachi, Japan). To irradiate only the tumor-bearing left hindlimb, a cage that shields the whole body except the tumor area from irradiation was used (Hitachi Medicotechnology, MBR-1520R-3). Power output of Xray-irradiation was 150 Kv, 20 mAmp. A 0.5-mm aluminum filter was used to filtrate forward-scattered radiation. Before mouse placement, a dose measurement probe was placed inside the shield cage at the assigned tumor site, and test irradiation was done to determine the dose rate at the tumor location. Next, the probe was placed in an allocated position outside the cage, and test irradiation was done. From these 2 test dose rates, the dosage at the tumor site was automatically calculated in proportion to the dosage at the measurement probe. The dose rate at the tumor site was approximately 2.2 to 2.4 Gy per min. It took approximately 8 min to complete 20-Gy irradiation using this device. Based on the measurements in tumor tissue oxygen tension following HbV administration, samples were administered approximately 10 min before the start of irradiation (salinerad, HbVrad, metHbVrad, lowP50HbVrad groups). Additionally, saline and HbV were administered without irradiation (salinenonrad, HbVnonrad groups). After sample administration with or without irradiation, the tail vein needle was removed, and the animals were allowed to recover from anesthesia. Tumors were measured using a venier caliper every 2 d up to 28 d after tumor implantation, after which point some animals started to show tumor-related distress. Estimated tumor weight was calculated as previously described [23]:

$$\begin{aligned} &\text{Estimated tumor weight (mg)} \\ &= \text{longer tumor diameter (mm)} \\ &\times [\text{shorter tumor diameter (mm)}]^2/2. \end{aligned}$$

Histological Studies and Hypoxia-Inducible Factor-1-alpha Analysis

In a separate group of animals, tumors were resected 20 min after intravenous administration of HbV ($n = 4$) or metHbV ($n = 4$). Half the tumor was fixed in 10% formalin for histology. The other half was immediately snap frozen in liquid nitrogen and stored at −80°C.

Paraffin sections were prepared from fixed tumor specimens and stained with hematoxylin and eosin for morphology. To locate HbV in tumor tissue, the human hemoglobin contained in the HbV was stained as previously described [8], with a rabbit polyclonal antibody against human hemoglobin (DAKO A/S, Copenhagen, Denmark) as the primary antibody. This antibody did not cross-react with mouse hemoglobin, which was evident from the fact that mouse RBCs were not stained. Reaction with the secondary antibody and color development were performed with the Ventana alkaline phosphatase RED detection kit using the Ventana NX system (Ventana Med. System, Inc., Tucson, AZ).

Western analysis for HIF1alpha protein was performed with the standard method. Briefly, cells were lysed with a denaturing buffer, and the lysate was centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was mixed with Laemmli buffer and applied to SDS-PAGE gels. The proteins were separated by 10% SDS-PAGE and then transferred to PVDF membrane for 90 min at 90 V using Novex Tris-Glycine system (Invitrogen, Carlsbad, CA). The primary antibody for HIF1alpha (clone 54; BD Bioscience, Franklin Lakes, NJ) was incubated with the blot overnight. The secondary anti-mouse IgG was incubated with the blots for 1 h. Bands were detected by enhanced chemiluminescence using Super Signal substrate (Pierce, Rockford, IL). Band densitometry was quantified using Image J (NIH, Bethesda, MA).

Data Analysis

Data are shown as mean ± standard deviation. Changes in tumor tissue oxygen tension and tumor growth measurements with or without irradiation were compared by analysis of variance followed by Scheffe's post-hoc test (StatView; Abacus, Berkeley, CA). Differences between groups at particular time points were compared by Mann–Whitney t-test (StatView; Abacus). Band densitometry was compared by Mann–Whitney t-test (StatView; Abacus). P values smaller than 5% were considered significant.

RESULTS

Sample administration and irradiation were well tolerated in all of the animals with no apparent changes in behavior or feeding.

Tumor Tissue Oxygen Tension Measurements

The tissue oxygen tension of back muscle before (saline: 14.4 ± 1.2; HbV: 14.2 ± 1.6; metHbV: 15.3 ± 1.2; lowP50HbV: 14.8 ± 1.2 (Torr)) and after (saline: 14.5 ± 1.1; HbV: 14.3 ± 1.4; metHbV: 15.3 ± 1.0; lowP50HbV: 14.6 ± 1.3 (Torr)) sample administration did not change significantly within or between groups. There were no significant differences in tumor tissue oxygen tension before sample administration (saline: 4.1 ± 1.1; HbV: 4.3 ± 0.8; metHbV: 4.3 ± 1.1; lowP50HbV: 4.3 ± 1.1 (Torr)). After sample administration, tumor tissue oxygen tension increased transiently in the HbV group during the observation period. Differences became significant from 15 to 30 min after sample administration in comparison to other groups. There was also a slight transient increase in the tumor tissue oxygen tension in the lowP50HbV group. Difference was significant between the saline group at 12 and 15 min after sample administration. Multiple comparisons using analysis of variance showed significant differences between HbV group curve and other groups. There was also a significant difference between lowP50HbV group curve and saline group curve (Fig. 1).

Tumor Growth Measurements

There were no significant differences in estimated tumor weight between groups at d 10 before sample administration, with or without irradiation (salinerad: 252 ± 27; HbVrad: 248 ± 38; metHbVrad: 239 ± 43; lowP50HbVrad: 248 ± 38; salinenonrad: 250 ± 33; HbVnonrad: 249 ± 38 (mg)). HbV administration without irradiation (HbVnonrad group) did not affect significant tumor growth delay in comparison to saline administration without irradiation (salinenonrad group). In both of these nonirradiated groups, tumor growth was significantly faster in comparison to any of the irradiated groups. Tumor growth delay after irradiation was marginally greater in the HbVrad group in comparison to other groups. Differences reached significance in the HbVrad group after irradiation in comparison to all other groups, from 16 to 28 d, except at d 24, at which point the difference was not significant between the metHbVrad group. Multiple comparisons using analysis of variance

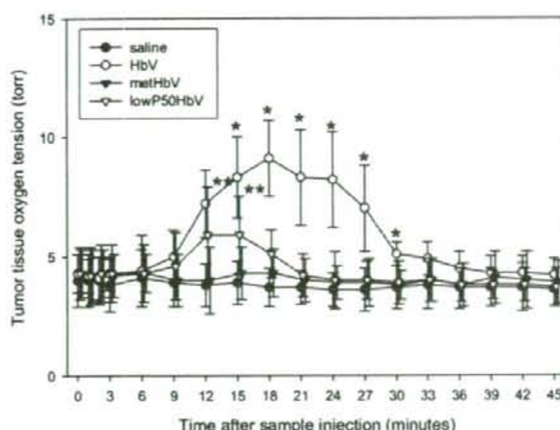

FIG. 1. Tumor tissue oxygen tension increased transiently in the HbV group. Differences became significant from 15 to 30 min after sample administration (* $P < 0.05$ *versus* all other groups, Mann–Whitney *t*-test). There was also a transient increase in the tumor tissue oxygen tension in the lowP50HbV group. Difference was significant between the saline group at 12 and 15 min after sample administration (** $P < 0.05$ *versus* saline group, Mann–Whitney *t*-test). Multiple comparisons using analysis of variance showed significant differences between the curves of HbV group and other groups. There was also a significant difference between the curves of lowP50HbV group, and saline group.

showed significant differences between HbVrad group curve and all other groups (Fig. 2).

On hematoxylin and eosin staining, the tumors at d 10 were morphologically composed primarily of tumor cells, and tumor vessels, with very little extracellular matrix. Human hemoglobin staining revealed the presence of stained material not only within the tumor vessel (Fig. 3A) but also in the extracellular matrix (Fig. 3B), which were presumably extravasated HbV. On Western blot analysis, HIF1alpha level was significantly decreased in the HbV group in comparison to the metHbV group (Fig. 4).

DISCUSSION

One of the primary causes of tumor resistance to irradiation may be tumor tissue hypoxia. Although still far from conclusive in the clinic, this notion is generally accepted [24]. *In vitro* studies show that the presence of oxygen increases the cytotoxicity of irradiation, resulting in roughly a 3-fold difference in radiosensitivity between hypoxic and aerobic cells [25, 26]. This phenomenon is widely attributed to the oxygen's ability to chemically modify radiation-induced DNA damage, creating adducts that are not repaired by cells.

A correlation between tumor tissue oxygen tension status and therapeutic response after treatment with irradiation or chemotherapeutic agents has been observed in many preclinical studies. There is also accumulating clinical evidence that therapeutically significant tissue hypoxia frequently exists in human tumors. As a result, there has been a longstanding active research into novel methods of improving tumor tissue oxygenation, targeting hypoxic tumor cells, and/or modulating the effect hypoxia has on how tumors respond to treatment. Several methods of overcoming tumor tissue hypoxia are currently under investigation. These include hyperbaric oxygen, hyperthermia, and the use of potential radiosensitizers such as nitroimidazole-based substances, and pentoxifylline [27]. Artificial oxygen carriers, such as perfluorochemicals, and modified hemoglobins have also been evaluated for this purpose in several preclinical studies [28–34]. We have previously reported the effect of a totally synthetic-heme-based artificial oxygen carrier in oxygenating tumor tissue [35]. To our knowledge, tumor oxygenation using a liposome-type artificial oxygen carrier has not been closely tested.

The dose of HbV administered in this study, 12 mL/kg, is rather high in the context of a therapeutic drug. But since HbV is being developed primarily as a transfusion alternative, it should be possible to administer this dosage without complications in order for HbV to be clinically applicable. No adverse effects directly attributable to material administration were apparent with any of the administered samples. Oxygen tension of back muscle tissue did not change significantly with sample administration, but the measurements were

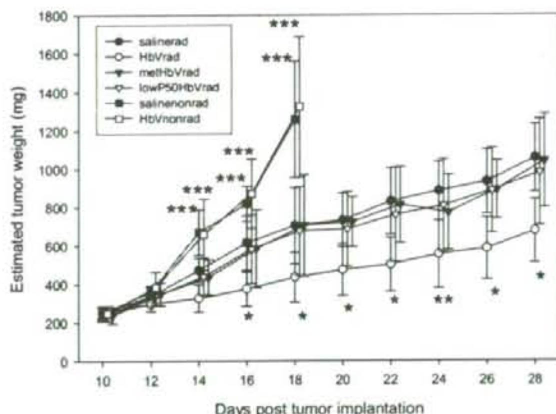

FIG. 2. Sample administration, saline, HbV, metHbV, lowP50HbV, with irradiation (salinerad, HbVrad, metHbVrad, lowP50HbVrad, groups respectively) or without irradiation (salinenonrad, HbVnonrad groups) were carried out on d 10. In the salinenonrad and HbVnonrad groups, tumor growth was significantly faster in comparison to any of the irradiated groups (*** $P < 0.05$ *versus* groups that received irradiation, Mann–Whitney *t*-test). Tumor growth delay after irradiation was significantly increased in the HbVrad group in comparison to other groups (* $P < 0.05$ *versus* all other groups, Mann–Whitney *t*-test), except at d 24, at which point the difference was not significant between the metHbVrad group (** $P < 0.05$ *versus* all other groups except metHbV group, Mann–Whitney *t*-test). Multiple comparisons using analysis of variance showed a significant difference in the growth curves between HbVrad group, and all other groups.

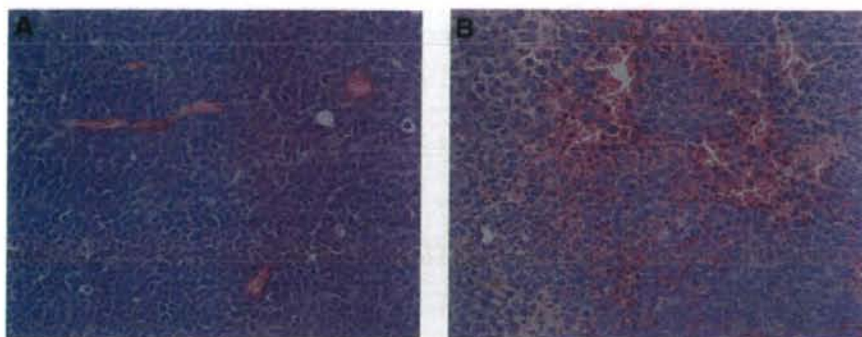

FIG. 3. Human hemoglobin staining could be seen not only within the tumor vessels (A) but also in the extracellular matrix (B) (human hemoglobin staining, ×20). (Color version of figure is available online.)

made before and after the completion of tumor tissue oxygen tension measurements primarily to verify the integrity of light guide and lens. Therefore, a transient rise in muscle tissue oxygen tension as well as other normal tissues, possibly leading to toxicity, cannot be denied. Further studies are needed in this area.

In the present study, systemic administration of HbV transiently reduced tumor hypoxia, and moderately augmented tumor growth delay in response to irradiation in comparison to groups administered saline, metHbV, or lowP50HbV. HIF1alpha is a transcription factor activated by hypoxia, controlling many pathways in response to hypoxia in both normal and tumor cells. In the present study, tumor HIF1alpha protein level was significantly decreased at 20 min after HbV administration compared with metHbV administration. These results correlatively suggest that HbV augmented tumor growth delay following irradiation, at least in part, by affecting tumor tissue oxygen tension. However, the growth delay in the HbV group, although statistically significant, was marginal. A study using different irradiation dosages to determine TCD50 is necessary to further verify this effect. The involvement of other mechanisms is also possible. In the present study, metHbV administration did not have a significant effect on tumor tissue oxygen tension or irradiation response. Nevertheless, the effect of heme itself needs further investigation, because the heme within HbV could be the primary source of oxygen radical species formation following irradiation rather than the oxygen it delivers. It is also known that changes in HIF1alpha level itself modifies irradiation

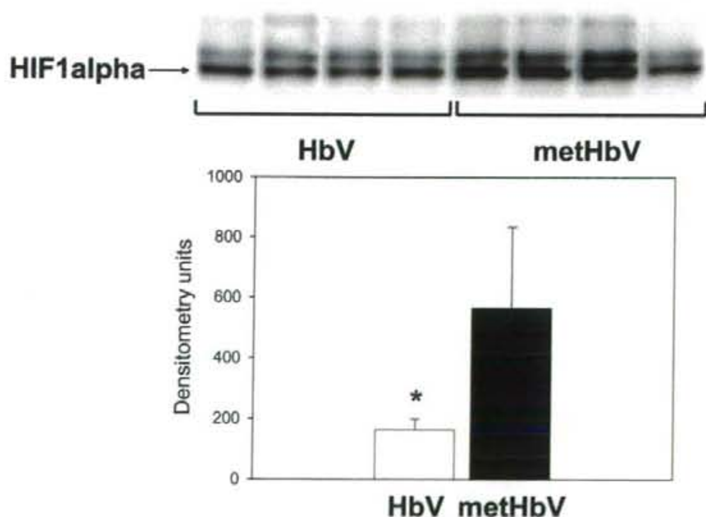

FIG. 4. HIF1alpha level was significantly decreased in the HbV group in comparison to the metHbV group. $^{*}P = 0.021$ Mann–Whitney U-Test. (Color version of figure is available online.)

response, but both radiosensitization and radioprotection by changes in HIF1alpha level have been observed [36]. Further studies, including measurements at longer time points and with administration of other samples, are required to find out if the change in HIF1alpha level, with respect to or irrespective of change in tissue oxygen tension, is involved in this particular animal model.

The timing of tumor tissue oxygen tension elevation after artificial oxygen carrier administration differs among previous reports from approximately 4 min to 2 h [9, 28–35]. We do not have a clear answer as to why the increase in tumor tissue oxygen tension was transient in the present study. If the increase in tumor tissue oxygen tension was due to circulating HbV, the effect should have appeared over a longer duration because HbV is known to have a circulating half-life of approximately 48 h [37]. Therefore, we speculated that the increase in tumor tissue oxygen tension may have been affected primarily by extravasated HbV rather than circulating HbV and was hence transient. Tumor vessels are known to be morphologically heterogeneous and has highly permeable sites in comparison to normal vessels. Liposomes ranging in diameter from approximately 100 to 400 nm have been shown to extravasate into the tumor extracellular matrix presumably through these sites [38, 39]. In the present study, human hemoglobin staining revealed the presence of stained material not only within the tumor vasculature but also in the extracellular matrix. We know from previous experiments that hemoglobin is encapsulated within HbV until it is metabolized, which does not occur in this timeframe of 20 min after systemic administration [40]. So we consider that these were extravasated HbV. Lewis lung carcinoma used in this study formed tumors composed primarily of tumor cells and tumor vessels, with very little extracellular matrix. Because of this, the extravasated HbV mostly existed in the vicinity of tumor cells, and hence, may have modulated the tumor response to irradiation. Obviously these results may not be directly applicable to tumors encountered in the clinic, which contain significantly greater amounts of extracellular matrix.

Oxyspot used in this study only measures tissue oxygen tension close to the tissue surface. But since the Lewis lung carcinoma cell line used in this study formed a histologically homogeneous tumor composed primarily from tumor cells and vessels, with little extracellular matrix, we assume that the measurements made in this study sufficiently reflected tissue oxygen tension changes within the whole tumor. To this end, Lewis lung carcinoma line was suitable for this particular study, but its clinical relevance may be limited.

It has been reported that increase in hemoglobin affinity is beneficial for oxygen delivery to normal tissue particularly when oxygen supply is severely compromised [41, 42]. In the present study, there was only a minimal transient increase in tumor tissue oxygen tension after lowP50HbV administration, and benefit was not so apparent. Physiology of oxygen extraction may have been different between different tissues or tumor lines. Blood flow in tumor vessels is known to be highly variable, and regulatory mechanisms may not have functioned as in normal tissue. P_{50} of 8 Torr in lowP50HbV used in this study may have been too low for significant oxygen release in this particular model. Additionally, in the present study, the increase in tumor tissue oxygen tension may have been due to extravasated rather than circulating HbV, in which case the findings from previous studies which examine materials with low P_{50} supplying oxygen from within the circulation may not be directly applicable. Further studies are needed to find out if optimization of P_{50} significantly contributes to oxygenating tumor tissue.

Many types of liposomes are currently being developed to improve selective drug delivery to tumor tissue. HbV used in this study was developed as a transfusion alternative, but it may be possible to increase relative tumor distribution and further optimize HbV's irradiation augmentation effect by modifying its liposomal components as well as its P_{50}. These improvements may lead to similar or better outcome, with less dosage. The timing of irradiation also needs further study, including the use of fractionation.

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Review of Hemoglobin-Vesicles as Artificial Oxygen Carriers

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Abstract: Blood transfusion systems have greatly benefited human health and welfare. Nevertheless, some problems remain: infection, blood type mismatching, immunological response, short shelf life, and screening test costs. Blood substitutes have been under development for decades to overcome such problems. Plasma component substitutes have already been established: plasma expanders, electrolytes, and recombinant coagulant factors. Herein, we focus on the development of red blood cell (RBC) substitutes. Side effects hindered early development of cell-free hemoglobin (Hb)-based oxygen carriers (HBOCs) and underscored the physiological importance of the cellular structure of RBCs. Well-designed artificial oxygen carriers that meet requisite criteria are expected to be realized eventually. Encapsulation of Hb is one idea to shield the toxicities of molecular Hbs. However, intrinsic issues of encapsulated Hbs must be resolved: difficulties related to regulating the molecular assembly, and management of its physicochemical and biochemical properties. Hb-vesicles (HbV) are a cellular type of HBOC that overcome these issues. The in vivo safety and efficacy of HbV have been studied extensively. The results illustrate the potential of HbV as a transfusion alternative and promise its use for other clinical applications that remain unattainable using RBC transfusion. **Key Words:** Blood substitutes—Hemoglobin—Liposome—Transfusion—Perfusion.

PROBLEMS OF TRANSFUSION SYSTEM AND EXPECTATIONS FOR ARTIFICIAL O_2 CARRIERS

Allogeneic blood transfusion was developed early in the last century as a routine clinical practice; it has contributed immensely to human health and welfare. Infectious diseases such as hepatitis and HIV are social problems associated with transfusion, but strict virus testing using the nucleic acid amplification test (NAT) is effective to reduce the risk of infection. Even so, NAT poses problems such as detection limits during the window period and limited species of viruses for testing. The continuing emergence of new viruses and a new type of pathogen, the prion, both threaten humans. In Japan, the storage period of donated red blood cells (RBCs) is limited to 3 weeks. Platelets can be preserved for only a few days. Immunological responses (such as anaphylaxis and graft vs. host disease) and contingencies of blood type incompatibility further limit the usefulness of blood products. To obviate or minimize homologous transfusion, the transfusion trigger has been reconsidered, and roughly reduced to 6 g/dL. Bloodless surgery and preoperational enhancement of erythropoiesis for storing autologous blood have become common. However, these treatments are not always practical for all patients. Some developed countries with aging populations must confront a decreasing number of young donors and an increasing number of aged recipients. On the other hand, in some developing countries, establishment of a safe blood donation system is difficult. Under such circumstances, research to develop blood substitutes has gathered great attention and has continued to develop

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worldwide. In Japan, for example, the government has given strong support to a spectrum of projects for development of blood substitutes in the wake of two tragedies: the infection, with AIDS, of hemophiliac patients who had received nonpasteurized plasma products; and the Great Hanshin Earthquake disaster.

Among blood components, some substitutes for plasma fractions are established, such as electrolyte solutions, plasma expanders, and recombinant coagulation factors. Especially, the commercialization of recombinant human serum albumin (rHSA) was finally approved in Japan in 2008. On the other hand, substitutes for cellular components—platelets and RBCs—are challenging. This review of the literature specifically examines hemoglobin (Hb)-based oxygen carriers (HBOCs). The optimal molecular structures and the physicochemical properties of HBOCs are selected based on the sciences of molecular assemblies of biopolymers, bio-conjugation, metal complexes, and nanotechnology, along with important results of physiological, and pharmacological studies (1,2).

THE CONCEPT OF CELLULAR HB-BASED O_2 CARRIERS AND THEIR DEVELOPMENT

Historically, the first attempt to use an Hb-based O_2 carrier was made in the 1930s. Stroma-free Hb was used because Hb in RBCs binds and releases O_2. However, several problems became apparent: impurity of stroma-free Hb, dissociation into dimers, a short circulation time, renal toxicity, high oncotic pressure, and high O_2 affinity. Since the 1970s, various approaches have been developed to overcome these problems, especially in the USA. In some cases, chemically modified Hbs (intramolecularly crosslinked Hb, recombinant Hb, polymerized Hb that were contaminated with nonpolymerized Hb) caused side effects such as vasoconstriction and abnormal esophageal function (3). Those effects are presumably attributable to the specific affinity of Hb to endogenous gas molecules, NO and CO, which are important messenger molecules for vasorelaxation (4). Although many companies have developed chemically modified Hb solutions as transfusion alternatives for elective surgery and trauma, some have suspended clinical trials because of vasoactive properties. The fact that myocardial lesions are caused by intramolecular crosslinked and polymerized Hbs has deterred further development of these HBOCs (5–7). The side effects of molecular Hbs described above imply the importance of the cellular structure and the larger particle dimension of HBOCs.

For those reasons, the optimal structure of HBOCs might be to mimic the RBC cellular structure. Pioneering work of Hb encapsulation to mimic the cellular structure of RBCs was performed by Chang in 1957, who prepared microcapsules (5 μm) made of nylon, collodion, and other materials. In Japan, Toyoda and the Kambara-Kimoto group investigated encapsulation of Hbs with gelatin, gum arabic, silicone, etc. in the 1960s. Nevertheless, results underscored the extreme difficulty in regulating the particle size to be appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility simultaneously. After discovery of liposomes in 1964, it seemed reasonable to use such vesicles for Hb encapsulation. Djordjevich and Miller in 1977 prepared liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acids, etc. Hunt et al. prepared neohemocytes (7). The US Naval Research Laboratories showed remarkable progress in the use of LEH (8). Terumo Corp. (Tokyo) was supported by the New Energy and Industrial Technology Development Organization (2003–2005) for industrialization in its development of different LEH, so-called Neo Red Cells or TRM-645 (9,10) (Table 1).

In fact, Hb encapsulation is expected to shield toxic effects of molecular Hbs. However, the intrinsic issues of encapsulated Hbs have emerged that are related to the nature of molecular assembly and particle dispersion, such as particle size distribution, protein purity, high Hb content, blood compatibility, and stability for long-term storage, and prompt degradation (2,9). To overcome such difficulties, the Waseda–Keio group has extensively studied the physicochemical properties, safety, and efficacy of Hb-vesicles (HbV) that mimic the cellular structure of RBC with the support of Japan's Ministry of Health, Labour and Welfare (1997–2008) (2) (Fig. 1, Tables 1 and 2). The HbV characteristics are as follows: (i) human Hb is purified via pasteurization at 60°C and ultrafiltration to eliminate pathogens; (ii) a concentrated Hb solution, nearly 35–40 g/dL, is encapsulated within a thin bilayer membrane; the Hb concentration of the resulting HbV suspension is 10 g/dL; (iii) a new synthetic lipid is used to prevent platelet activation and complement activation (11). (iv) Subsequently, polyethylene glycol-modification guarantees long-term storage of more than 2 years at room temperature (12), with blood compatibility, and extended circulation half-life. (v) The resultant cellular structure shields all side effects of molecular Hb (13). (vi) The particle size (250 nm) is appropriate for homogeneous distribution in the plasma phase to flow through narrower vessels, where large RBCs

TABLE 1. *Representative liposome-encapsulated Hbs (LEH) studied extensively for future commercialization*

Product Name	Group	Characteristics	References	Current status
Hb-vesicles (HbV)	Waseda Univ. & Keio Univ.	1. Pasteurization of HBOC at 60°C for virus inactivation, and high purity and concentration of encapsulated Hb (35–40 g/dL) 2. Lipid composition to improve blood compatibility 3. PEG modification and deoxygenation for 2 years storage 4. [Hb] = 10 g/dL (and others, see Table 2)	(2, 4, 11, 12)	Preclinical
Neo Red Cells (NRC) TRM-645	Terumo Corp.	1. Inositol hexaphosphate to regulate P_{50} (= 51.4 torr) 2. Lipids: soybean hydrogenated phosphatidylcholine / cholesterol / stearic acid / PEG-DSPE 3. Storage in a refrigerator for 6 months 4. Preservation of enzymes for metHb reduction 5. [Hb] = 6.2 g/dL	(9, 10)	Preclinical
Artificial Red Cells (ARC)	NOF Corp. & Waseda Univ.	1. Polymerized lipids (DODPC) for stabilization 2. Storage in powdered or frozen state 3. Difficulty in degradation in RES	See Refs. in (1, 2)	Suspended
Liposome-encapsulated Hb (LEH)	US Naval Research Laboratory	1. Freeze-dried powder with trehalose 2. Low Hb encapsulation efficiency 3. Thrombocytopenia, complement activation	(8)	Suspended
Neohemocyte (NHC)	Univ. of California, San Francisco	1. Wide particle distribution (0.1–1.5 μm) 2. Lipids: egg yolk phosphatidylcholine / dipalmitoyl-phosphatidic acid / cholesterol / alpha tocopherol 3. [Hb] = 15.1 g/dL 4. P_{50} = 24 torr	(7)	Suspended
Synthetic Erythrocytes (SE)	Rush–Presbyterian–St. Luke's Medical Center, Univ. Illinois	1. First attempt of LEH	See Refs. in (1, 2)	Suspended

cannot flow. (vii) Finally, HbV are captured by the reticuloendothelial system and are degraded promptly without decomposition (hemolysis) during blood circulation. Phospholipid vesicles for encapsulation of Hb are expected to be beneficial for heme detoxification through their preferential delivery to the reticuloendothelial system, a physiological compartment for degradation of senescent RBCs, even at

FIG. 1. (Left) Schematic representation of HbV. One particle contains about 30 000 Hb molecules. The surface of one particle is modified using polyethylene glycol (PEG) chains, which ensures the dispersion stability of HbV during storage and during circulation in the bloodstream. The average particle diameter is about 250 nm. (Middle) The packed HbV suspension appears turbid, like a mixture of milk and red wine, because of light-scattering of the particle suspension. (Right) Reoxygenation of HbV by a membrane oxygenator (artificial lung), clearly displaying the change of color from purple to red. HbV would be useful as a priming fluid for extracorporeal membrane oxygenators and for perfusion of organs for transplantation.

TABLE 2. *Characteristics of Hb-vesicles (HbV) developed at Waseda University*

Parameter	
Particle diameter	250–280 nm
P_{50}*	25–28 torr
Hb concentration	10 g/dL
Suspending medium	Physiologic saline solution (0.9% NaCl)
MetHb	<3%
Rate of MetHb formation† (at 37°C, Po_2 = 40 torr)	4%/h
Colloid osmotic pressure	0 torr
Intracellular Hb concentration	ca. 35–40 g/dL
Lipid composition‡	DPPC / cholesterol / DHSG / DSPE-PEG_{5000}
Weight ratio of Hb to lipids	1.6–1.9 (w/w)
Stability for storage at room temperature	2 years
Circulation half-life	32 h (rats)

* P_{50} is regulated with co-encapsulated pyridoxal 5′-phosphate.
† The rate of autoxidation is dependent on Po_2, and accelerated at a reduced Po_2. Because of the pasteurization of Hb solution (60°C) to obtain purified Hb and guarantee the utmost safety from infection, the resulting HbV contains no enzymes. Therefore, autoxidation of Hb is unavoidable. Recently, it was clarified that co-encapsulation of L-tyrosine significantly retards autoxidation because of the catalase-like activity provided by metHb and L-tyrosine, which eliminates H_2O_2, a by-product of Hb autoxidation.
‡ DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DHSG, 1,5-*O*-dihexhadecyl-*N*-succinyl-L-glutamate.

doses greater than putative clinical doses (2). Results of animal tests have clarified the efficacy of HbV as a transfusion alternative and their possible use for other clinical applications. The results of ongoing HbV research make us confident in advancing further development of HbV, with the expectation of its eventual clinical application.

HB-VESICLES AS A TRANSFUSION ALTERNATIVE

Advantages of the HBOCs are the absence of blood-type antigens and infectious viruses, and their stability for long-term storage: those advantages overwhelm those of present RBC transfusion. The shorter half lives of HBOCs in the bloodstream (2–3 days) limit their use, but they are applicable in shorter periods for use as (i) a resuscitative fluid for hemorrhagic shock in emergency situations for temporary benefits or bridging until packed RBCs are available, or (ii) as a fluid for preoperative hemodilution or perioperative O_2 supply fluid for a hemorrhage in an elective surgery to avoid or delay allogeneic transfusion, or (iii) as a priming solution for the circuit of an extacorporeal membrane oxygenator.

One particle of HbV (~250 nm diameter) contains about 30 000 Hb molecules. Because HbV acts as a particle in the blood, not as a solute, the colloid osmotic pressure of the HbV suspension is nearly zero. It necessitates the addition of a plasma expander for a large substitution of blood to maintain blood volume. The candidates for use as plasma expanders are HSA, hydroxyethyl starch, dextran, and gelatin, depending on the clinical setting, cost, country, and clinician. rHSA is becoming an alternative in Japan. The absence of any infectious disease from humans is the greatest advantage of rHSA. Moreover, it presents none of the immunological and hematological abnormalities that are often associated with using dextran. Aiming at application of HbV suspended in a plasma expander to the above indications, HbV was tested in rodent models for resuscitation from hemorrhagic shock and extreme hemodilution. Physiological and pharmacological studies of HbV have clarified that HbV can maintain the fundamental parameters of respiration, hemodynamics, and tissue oxygenation that are comparable to results of RBC transfusion. Realization of an HBOC will contribute considerably to the renovation of present clinical practices, especially in emergency medicine and surgery, where the HBOC can be injected instantaneously without concern of blood type mismatch, infection, or other blood-borne disease.

Hb-vesicles used as a perioperative infusion fluid or as a priming fluid for extracorporeal circuit

Extensive in vivo studies of HbV suspended in plasma-derived HSA or rHSA revealed sufficient O_2 transporting efficiency that is apparently comparable to RBCs in extreme blood exchange experiments with up to 90% blood exchange (14). All systemic parameters and tissue oxygen tensions were well preserved, although the blood exchange with HSA showed considerable hypotension, acidosis, and death within 30 min after completion of blood exchange. As confirmed in rat models after 40% blood exchange with HbV in clinically relevant conditions, hematopoietic activity was preserved and the decreased hematocrit reverted to its original level within 1–2 weeks, whereas HbV captured in RES disappeared completely (15), indicating that HbV is useful for preoperative blood exchange or perioperative infusion in the event of hemorrhage to prevent or minimize homologous blood transfusion.

Although miniaturization of the cardiopulmonary bypass (CPB) circuit has reduced the priming volume, it remains insufficiently low to achieve an acceptable level of hemodilution in small patients.

Homologous blood use is considered the gold standard for CPB priming in infants despite exposure of patients to potential cellular and humoral antigens. A recent experimental study of HbV suspended in rHSA as a priming solution for CPB in a rat model demonstrated that HbV protects neurocognitive function by transporting O_2 to brain tissues, even when the hematocrit is markedly reduced (16). The results indicate that the use of HbV for CPB priming might prevent neurocognitive decline in infants caused by considerable hemodilution.

Hb-vesicles as a resuscitative fluid for hemorrhagic shock

The first attempt using an HbV suspension to restore systemic conditions after hemorrhagic shock was conducted with anesthetized Wistar rats (17). Shock was induced by 50% blood withdrawal. The rats exhibited hypotension and considerable metabolic acidosis and hyperventilation. After 15 min, they received isovolemic HbV suspended in rHSA (HbV/rHSA, [Hb] = 8.6 g/dL), shed autologous blood (SAB), or rHSA alone. The HbV/rHSA group restored mean arterial pressure, similarly to the SAB group, which was significantly higher than the rHSA group. No remarkable difference was observed in the blood gas variables between the resuscitated groups. However, two of eight rats in the rHSA group died before 6 h.

After removing the catheters and awakening, the rats were housed in cages for up to 14 days. The HbV/rHSA group gained body weight; the reduced hematocrit reverted to the original level in 7 days, indicating elevated hematopoiesis. Both groups showed AST and ALT elevation at 1 day because of systemic ischemia reperfusion injury. Splenomegaly was significant in the HbV/rHSA group at 3 days because of HbV accumulation, but it had subsided by 14 days. Histopathological observation revealed that a substantial amount of HbV accumulated in the spleen macrophages, although it had completely disappeared by 14 days. In conclusion, HbV showed a sufficient resuscitative effect that was comparable to that achieved with transfusion. The injected HbV were phagocytized in the reticuloendothelial system by 14 days. The elevated hematopoietic activity caused the complete recovery of hematocrit by 7 days.

These results reflect that HbV is useful as a resuscitative fluid for hemorrhagic shock. Its performance is comparable to that of SAB. We have progressed to similar experiments using beagles. Those results confirm the long-term survival of more than one year after resuscitation with HbV.

OTHER POTENTIAL CLINICAL APPLICATIONS OF HB-VESICLES

One advantage of HbV is that the physicochemical properties of HbV are adjustable, such as oxygen affinity (P_{50}, oxygen partial pressure at which Hb is half saturated with oxygen) and rheological properties. Historically, it has been widely believed that the O_2 affinity should be regulated similarly to RBC, at about 25–30 torr, using an allosteric effector or by a direct chemical modification of the Hb molecules. Theoretically, this enables sufficient O_2 unloading during blood microcirculation, as can be inferred from the arterio-venous difference in the levels of O_2 saturation in accordance to an O_2 equilibrium curve. It has been expected that decreasing the O_2 affinity (increasing P_{50}) increases O_2 unloading. Regarding the blood viscosity, lowered viscosity is believed to increase cardiac output and facilitate peripheral blood flow. However, these beliefs are being reviewed and revised in the field of blood substitute research. The suspension of HbV can provide unique opportunities to modify these physicochemical properties easily and to observe their physiological impacts.

Oxygenation of ischemic tissues using HbV

In ischemic tissues, blood flow is extremely reduced. As a result, O_2 tension is very low, for example, 5 torr. Normal RBCs are expected to have already released O_2 before they reach the ischemic tissue. The left-shifted curve (lower P_{50}) indicates that Hb does not release O_2, even in the venous side in a normal condition. However, RBC or an HBOC with a P_{50} lower than usual can carry O_2 to an ischemic tissue (18). Dr. Erni and colleagues at Inselspital Hospital of the University of Berne developed a hamster skin flap model in which the blood flow of one branch is blocked completely; the tissue becomes completely ischemic. Exchange transfusion was performed using low and high P_{50}-HbV, which revealed improved oxygenation of the ischemic part, especially with the low P_{50}-HbV (19,20). Collateral blood flow is expected to occur even to the ischemic part and the HbV conveys O_2 to the ischemic part via the collateral arteries. This is the first example demonstrating the effectiveness of HbV for an ischemic tissue, implying its applicability for other ischemic diseases. In addition to the lower P_{50}, the viscosity of the HbV suspension is expected to contribute to improvement of microcirculation. The combination of HbV and dextran solution or hydroxyethyl starch solution induces flocculation of HbV, thereby rendering the suspension non-Newtonian and viscous (21).

The higher viscosity of the circulation fluid would increase shear stress on the vascular wall, thereby inducing vasorelaxation. A viscous fluid also homogeneously pressurizes the capillaries to improve the functional capillary density. Recent studies have demonstrated the effectiveness of HBOCs with a lower P_{50} (higher O_2 affinity) as a means of implementing O_2 delivery targeted to ischemic tissues (22). Our experimental data support those earlier observations and ensure the possible utilization of HBOCs for remedying ischemic conditions (23).

Oxygenation of organs for transplantation and regenerated tissues in the future

Even though the organ transplant law went into effect in 1997 in Japan, organ transplants are expected to occur far less frequently than in the USA or Europe, probably because of the ethical and religious controversy related to the criteria for diagnosis of brain death. However, it is expected to be accepted eventually in Japan with the prevailing view of the need for transplantation and carrying an organ donor card. In a clinical setting of transplantation, its success is dependent in part on the prevention of ischemia-reperfusion injury after transplantation by an improved preservation condition. The representative organ preservation fluid is the University of Wisconsin (UW) solution, which comprises not only crystalloids, but also a plasma expander. The two-layer method is to dip the dissected organ at the interface of the UW solution and a perfluorocarbon (PFC) solution. Oxygen diffuses from PFC to the organ, and the transport of nutrients and metabolites takes place through contact with the UW solution. It is speculated that there should be a limitation of the distance for diffusion of oxygen, carbon dioxide, and small molecules to keep the tissues alive. One idea is to use HbV as an intra-arterial perfusion fluid to carry oxygen, nutrients, and metabolites. Actually, we tested perfusion of the liver, heart, and intestine with HbV (24,25). We confirmed the preservation of organ functions for a few hours. Our next step will be to prolong the perfusion period to the greatest extent possible. In fact, HbV can be reoxygenated easily by perfusion through an oxygenating "artificial lung" (Fig. 1). We must design the composition of HbV suspension to provide not only oxygen but also nutrients and homogeneous fluid distribution to all capillaries, which would presumably require a certain level of viscosity.

Tissue reconstruction and tissue regeneration have become popular since Vacanti et al. reported the formation of an auricular-shaped cartilage on the back of a mouse by cell culturing onto a polymer scaffold. In Japan, an innovation occurred with the discovery of thermoresponsive polymer gel, poly-(isopropylacrylamide), for cell culturing by Okano et al. This material is hydrophobic at 37°C during cell culturing in a Petri dish; it becomes hydrophilic at 32°C. Moreover, it can be removed easily from the cells to provide a cell sheet. The resultant two-dimensional cell sheet is applicable to many clinical indications. Cell culture requires not only a supply of oxygen and nutrition but also the removal of metabolites, which can be achieved by replacing the culturing media periodically in the case of a two-dimensional cell culturing. However, in the case of constructing a three-dimensional bulky tissue, it would require perfusion with a fluid that can serve the functions of blood in addition to angiogenesis in a regenerating tissue on a scaffold. Such functionality would necessitate the design of the composition of HbV described above. Consequently, HbV can provide unique opportunities to manipulate physicochemical properties that cannot be provided by RBCs.

CONCLUSION

The circulation half-life of HBOCs including HbV after intravenous administration is much shorter than that of RBC. The total amount of the membrane component of HbV is 2–3 times more than that of RBCs. HbV degraded promptly and completely in the reticuloendothelial system. However, a massive dosage of HbV transiently induces splenohepatomagaly, reduces phagocytosis, and modifies immunological responses (2). On the other hand, HBOCs are superior to conventional transfusion in terms of their absence of blood type and pathogens, and their long storage period. The outstanding difference between HbV and other HBOCs such as chemically modified Hb solutions is that the physicochemical properties of HbV, not only P_{50}, but also rheological properties, are adjustable. Accordingly, the use of HbV provides unique opportunities for versatile therapeutic approaches that cannot be attained using a conventional transfusion system.

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Histopathological changes of rat brain after direct injection of Hb-vesicles (artificial oxygen carriers) and neurological impact in an intracerebral hemorrhage model

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Abstract: For use as artificial oxygen carriers during transfusion, the safety and efficacy of Hb-vesicles (HbV, 250 nm), have been investigated extensively. Nevertheless, their neurotoxicity remains unknown. We explored potential adverse effects of HbV in the brain using a rat intracerebral hemorrhage model. Male Wistar rats were anesthetized with sevoflurane and fixed on a stereotaxic frame. Then HbV or homologous RBC suspension ([Hb] = 8.6 g/dL, 20 μL) was injected into the right caudate nucleus. All animals survived, gained weight, and maintained their well-being until the time of sacrifice; except during the first few days after surgery. However, both groups showed slight weakness in hind leg retraction, occasional ataxia/gait, and piloerection. Neutrophils accumulated at the onset of injury in perihematomal tissues in both groups at 1st day, but had disappeared by 3 days. Infiltration of small HbV in the perihematomal tissue was prominent at 1st day; phagocytized HbV were detected in macrophages. Hemeoxygenase-1 and hemosiderin deposition appeared after 3 days, reflecting the degradation of both HbV and RBC. The HbVs were detectable even after 28 days in the HbV group, but no residual RBCs were detected in the RBC group. Both groups showed proliferation of astrocytes, named gliosis, for tissue reconstruction after 3 days. This study revealed no notable differences in adverse effects between the intra-cerebral injection of HbV and the RBC control on behavioral functions and brain tissue responses. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 00A: 000–000, 2008

Key words: artificial oxygen carriers; blood substitutes; liposome; intracerebral hemorrhage; neurotoxicity

Author Proof

INTRODUCTION

For possible use as a transfusion alternative, Hb-vesicles (HbV), or liposome-encapsulated Hb, were developed as artificial oxygen carriers.[1–5] The HbV safety and efficacy have been studied extensively in our laboratory. HbV is void of blood-type antigens and blood-borne pathogens, and can be stored for at least 2 years at room temperature.[6–8] The cellular structure and HbV particles are sufficiently large to prevent extravasation across the hepatic fenestrated endothelium, so as not to modulate the reaction with endogenous NO and CO as vasorelaxation factors.[9–11] The fate of HbV after oxygen transport is accumulation and prompt degradation in the reticuloendothelial system, primarily by spleen macrophages and liver Kupffer cells, and similar to that of senescent RBCs. The circulation time, however, was shorter for HbV than for RBC.[5,12–14]

It is known from clinical information that hemorrhagic shock and resuscitation often produce brain damage from the opening of blood brain barrier (BBB), and infiltration of blood components into the brain tissue, leading to tissue inflammation and neuronal cell death.[15] However, HbV would not infiltrate through the BBB because of its vesicular size, which is 250 nm on average, is considerably larger than that of any other plasma proteins. We further investigated possible HbV neurotoxic effects by direct injection of HbV into the rat brain compartment. When the BBB is damaged by a traumatic injury or an intracerebral hemorrhage (ICH) and when blood is infiltrated into the brain, the process

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induces local inflammation and migration of immuno-protective cells such as microglia. In patients suffering from brain hemorrhage or stroke, the released Hb by breakdown of RBC and its byproducts such as iron are known to produce neuronal damage.[16–18] HbV encapsulates purified Hb solution in phospholipid vesicles. Therefore, an equivalent neurotoxicity of RBC might be expected from the released Hb and its byproducts when the HbVs break down. However, because the chemical composition and the stability of HbV lipid membrane differ from those of RBCs, the HbV breakdown process might produce quantitatively different neurotoxicity. In 2004, the FDA listed eight unsolved safety-related problems of Hb-based oxygen carriers. One was neurotoxicity because it is anticipated that Hb, entering the brain from the vasculature, can cause considerable tissue damage, engendering disability or morbidity.[19,20]

Given this background, the current study tested the possible neurotoxic effect of HbV comparative to that of RBC, when it is injected directly into the brain compartment. For the purpose, HbV was injected into basal ganglia on one side and the neurotoxic effects were examined using pathological procedures and using histochemical and immunohistochemical methods. Their consequent behavior responses were also studied.

MATERIALS AND METHODS

Preparation of Hb-vesicles

HbVs were prepared by Oxygenix Co. (Tokyo, Japan) as reported previously.[6,7,21,22] The Hb was purified from outdated and donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dL) contained 14.7 m*M* pyridoxal 5′-phosphate (PLP; Sigma-Aldrich Corp., St. Louis, MO) as an allosteric effector at a molar ratio of PLP/Hb = 2.5. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co., Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidyethanolamine-*N*-PEG_{5000} (NOF Corp., Tokyo, Japan) at a molar composition of 5/5/1/0.033. The lipopolysaccharide content, measured using a modified *Limulus* amebocyte lysate test, was <0.2 EU/mL.[23] The physicochemical characteristics of HbVs are P_{50}, 25 Torr, 251 ± 81 nm particle diameter, and < 3% MetHb. Prior to use, HbV suspended in saline (0.9% sodium chloride; [Hb] = 10 g/dL, 8.6 mL) was mixed with recombinant human serum albumin (rHSA, 25 g/dL, 1.4 mL; Nipro Corp., Osaka, Japan), making the final rHSA concentration in the suspending medium 5 g/dL; this caused the final Hb concentration to 8.6 g/dL.[4,5] At this condition, the colloid osmotic pressure (COP) and the viscosity (300 s^{-1}, 37°C) of the HbV/rHSA were, respectively, 20 mmHg and 2.9 cP.

Preparation of washed rat RBC

Blood was withdrawn from the anesthetized donor Wistar rats via the caudal vena cava into a heparinized syringe. The blood was centrifuged for 10 min at 4000 × *g*; the supernatant and the buffy coat were removed. The sedimented RBCs were re-suspended in saline and centrifuged. This procedure was repeated twice. The RBCs were suspended finally in a 5 g/dL rHSA solution. The final Hb concentration in the suspension was made to 8.6 g/dL and to the same Hb concentration as that in the HbV suspension.

Injection of the sample solution into brain tissue

The experimental protocol was fully approved by the Institute of Laboratory Animal Care and Use Committee, Keio University School of Medicine. Relevant NIH guidelines (NIH Publication no. 85-23 Rev. 1985) were observed. All survival surgical procedures were performed under the Institutional Animal and Use Committee guideline. In all, 69 male Wistar rats were used for this study (Sankyo Labo Service Co., Tokyo, Japan). Each animal was placed in a gas-tight glass jar; the anesthesia was induced with ether. The animal was then mounted to a small animal standard stereotaxic frame (Kent Scientific Corp., Torrington, CT). The surgical anesthesia was maintained with 1.3–1.5% sevoflurane (Maruishi Pharm., Co., Osaka, Japan) delivered through a flow regulated gas anesthetic machine (Model TK-4 Biomachinery; Kimura Medical Instrument Co., Tokyo, Japan) and through a nose cone attached to the stereotaxic frame and to the animal. Body temperatures of the animals were maintained at 37°C throughout the surgery using a heating pad. Each animal's head was shaven; the skin was disinfected with 70% ethanol and Isozin, and a local anesthetic, bupivacaine hydrochloride (0.25%; Marcain[R] injection; AstraZeneca, Osaka, Japan) before making an incision. The skull was exposed by midline incision; underlying muscle layers were retracted to expose the bone surface. Lambda was identified. The injection site was at the basal ganglia area, marked according to the rat stereotaxic map coordinate of 2.4 mm anterior to lambda, 4 mm lateral to midline, and 5 mm ventral from the surface. A hole was drilled through the skull bone on right side.

The testing solution (either one of HbV or RBC), 20 μL, was injected at a rate of 4 μL/min through the 28-G needle attached to Hamilton micro-syringe; the needle was held in place for 3 min after the completion of injection (HbV, n = 34; RBC, n = 28). The needle site was plugged with Gelform (Pharmacia and Upjohn, Kalamazoo, MI) to prevent cerebrospinal fluid leak; the hole of the skull was sealed with dental cement (Ionotite; Tokuyama Dental Co., Tokyo, Japan). The skin was closed with either wound clips or an absorbable suture. An antibiotic, Cefmetazole sodium, 20 mg/kg SC (Sankyo Pharmaceutical, Tokyo), and an opioid analgesic, Buprenorphine hydrochloride (Lepetan injection; Otsuka Pharm., Tokyo, Japan), 0.05 mg/kg, SC, were given after the surgery. The animal was kept on a temperature-controlled bed in a cage and observed until

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full recovery from the anesthesia. The animals were then housed in cages and provided with food and water *ad libitum* in a temperature-controlled room with a 12-h dark/light cycle. The animals were observed at 6-, 12-, and 24-h post-surgery for any sign of bleeding from the site of incision, as well as pain and discomfort and infection. The well-being of animals was followed daily throughout the experiment. Buprenorphine injections were repeated every 8 h if there was any sign of pain or discomfort.

Behavioral testing

Body weights of animals and their well-being were observed at 1, 2, 3, 5, 14, and 28 days after the surgery and prior to the time of sacrifice for histopathological studies. Similarly, behavioral testing, which indicated motor dysfunction, was performed on the days presented in Table I. Data of the normal rat group (no treatment, $n = 7$) were also collected.

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Histopathological examination

At 1, 3, 7, and 28 days after the surgery, seven animals in each group were euthanized with ether in a gas chamber. The brain was isolated; its wet weight was measured. The brain was then fixed in a 10% formalin-phosphate buffer solution (Wako Pure Chemical Industries, Tokyo, Japan). The fixed brain was sliced to 1-mm thickness, anterior and posterior, vertical to the injection needle mark.

The sliced brain was examined histochemically using hematoxylin and eosin (HE) staining for any pathological changes, and Berlin blue staining to confirm the presence of hemosiderin. Immunohistochemical stains were performed for glial fibrillary acidic protein (GFAP) of astrocytes, inducible hemoxygenase-1 (HO-1), human Hb of HbV, and apoptotic cells.

For immunohistochemistry to observe astrocytes, the paraffin sections (4-μm thick) were mounted on 3-aminoprophyl triethoxysilane-coated glasses. After deparaffinization, the sections were subjected to microwave treatment for 10 min with 10 m*M* citrate buffer (pH 6.0) for antigen retrieval. They were incubated with methanol containing 0.3% hydrogen peroxide (H_2O_2) for 30 min at room temperature, and subsequently with 2.5% normal horse serum for 15 min at room temperature. The tissues were then reacted with mouse monoclonal antibody against GFAP (1/200 dilution; Dako, Glostrup, Denmark) for 3 h at room temperature. After washing in PBS, they were incubated in ImmPress Reagent Peroxidase anti-mouse Ig (ImmPress Reagent Kit; Vector Laboratories, CA) for 1 h at room temperature. The color was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB, 0.2 mg/mL; Dojindo Laboratories, Kumamoto, Japan) in 0.05 *M* Tris-HCl (pH 7.6) containing 0.003% H_2O_2; the tissues were then counterstained with hematoxylin.

The staining methods of anti-rat HO-1 and anti-human Hb antibody have been described previously.[13,14] Briefly, the sections were treated with proteinase K (Dako, Glostup, Denmark) for antigen retrieval. After blocking the nonspecific binding with DAKO Antibody Diluent Solu-

TABLE I
Daily Behavioral Testing After Direct Injection of the Sample Solutions into the Brain

Days	Circling Behavior[a] (%)			Piloerection[b] (%)			Hind Leg Weakness[c] (%)			Absence of Startle Response[d] (%)			Hanging Time[e] (s)		
	Normal Rat	HbV	RBC	Normal Rat	HbV	RBC	Normal Rat	HbV	RBC	Normal Rat	HbV	RBC	Normal Rat	HbV	RBC
1	0	0	0	71	41	25	14	71	78	0	23	13	18 ± 3	10 ± 1*	9 ± 1*
2	0	0	0	29	30	43	14	63	86	0	11	15	15 ± 4	12 ± 1	10 ± 1
3	0	0	0	29	61	53	29	56	62	0	26	23	14 ± 3	15 ± 2	11 ± 1
5	0	0	0	43	60	70	14	44	36	0	7	20	16 ± 4	17 ± 2	11 ± 2
7	0	0	0	29	33	70	29	33	21	0	13	0	13 ± 2	16 ± 3	12 ± 3
14	0	0	0	14	36	57	14	50	57	0	0	0	13 ± 4	13 ± 2	8 ± 2
28	0	0	0	43	50	86	29	75	100	0	0	0	5 ± 1	9 ± 2	9 ± 2

The number of animals (n) at each day: HbV group, 1d (34), 2d (27), 3d (23), 5d(15), 7d (15), 14d (11), and 28d (8); RBC group, 1d (28), 2d (21), 3d (17), 5d (10), 7d (10), 14d (7), and 28d (7); the normal rat group (no treatment), 1d – 28d (7).
[a]Spontaneous body circling.
[b]Ruffling of the body hair.
[c]Hesitant to walk on the beam (1.5 cm wide), and misses steps often; drags the foot as a rat walks on the beam.
[d]Startles and jumps to the blow of air to the face.
[e]Length of time spent hanging in second on a round plastic rod, diameter 4 mm. Mean ± SE.
*$p < 0.05$ vs. the normal rat group.

tion, they were incubated with mouse monoclonal antibody against rat HO-1 (GTS-3; Takara, Tokyo, Japan). They were then incubated with the secondary antibody (Simple Stain Rat MAX-PO[M]); Nichirei Corp., Tokyo, Japan). The color was developed using DAB, and the sections were counterstained with hematoxylin. A comparative staining procedure was performed with non-immune mouse IgG to confirm that the staining was specific to HO-1 and not derived from the color of hemosiderin deposition (DAKO).

For detection of human Hb derived from HbV, the sections were treated with rabbit polyclonal antibodies against human Hb (Dako). They were further incubated with alkaline phosphatase-conjugated swine antibodies against rabbit immunoglobulins (Dako)). The color was then developed using a New Fuchsin Substrate Kit (Nichirei Corp., Tokyo, Japan); the sections were counterstained with hematoxylin.

Apoptotic cells were stained with terminal deoxynucleotidyl transferase dUTP Nic-end labeling (TUNEL) with ApopTag^{R} Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA). The detailed protocol is described in the kit operating instruction. After pretreatments for antigen retrieval and quenching of endogenous peroxidase, the sections were incubated with a terminal deoxynucleotidyl transferase (TdT) enzyme solution, followed by a solution of anti-digoxigenin conjugate. The color was developed with peroxidase substrate and counterstained with hematoxylin.

Transmission electron microscopic observation (TEM) was performed to visualize the presence of the HbV particles in the brain tissue (PCL Japan, Tokyo, Japan). One brain of the HbV group at 1, 3, 7, and 28 days was fixed with 2.5% glutaraldehyde solution, cut in ~2-mm^3 blocks, and stored in 8% sucrose solution (0.1 mol/L phosphate buffer, pH 7.4). The fixed blocks were then washed with 0.1 mL/L phosphate buffer and stained with 2% osmic acid solution at 4°C for 2 h. Next, the blocks were dehydrated with ethanol solution by a stepwise increase in ethanol concentrations (50, 60, 70, 80, 90, 95, and 100%), 10 min for each step, washed with propylene oxide; then polymerized using Quetol 812 at 60°C for 28 h. The obtained blocks were sliced into 60–70 nm thickness using an Ultracut S microtome. The sliced tissues were stained with 3% uranyl acetate solution for 16–20 min, then treated with Satoh's lead solution (lead acetate, lead nitrate, and lead citrate) in citrate for 5 min, washed, and dried. The sliced brain tissues were examined under a transmission electron microscope (TEM, JEM-100CX; JEOL, Tokyo, Japan) and photographed.

Data analysis

The *in vivo* data are presented as mean ± SE with the indicated number of animals tested. Unpaired *t*-tests were used to compare the HbV and RBC groups.

In vitro stability of Hb-vesicles and RBC

Degradation rate of HbV would be influenced by the stability of HbV vesicles withstanding physical stimuli and enzymatic attack. To collect some information related to stability, the following three sets of *in vitro* experiments were performed:

1. Hypotonic hemolysis was induced by mixing HbV or RBC suspensions with distilled water at a 1:4 volume ratio ([Hb] = 1.72 g/dL). The mixture was then centrifuged and the supernatant Hb concentration was measured using a cyanomethemoglobin method[24] to determine the percentage of hemolysis.
2. Resistance of hemolysis to freeze-thawing was tested by diluting the sample of HbV or RBC 10 times with saline solution ([Hb] = 1.72 g/dL). One milliliter of the sample was put in a plastic tube and dipped in liquid-nitrogen for a few minutes. It was then thawed at room temperature. The samples were centrifuged and the supernatant Hb concentration was determined by the same method.
3. Hemolysis was induced by the enzymatic attack with phospholipase A_2 (PLA_2) to membrane phospholipids.[25] Four hundreds microliters of sample solutions of HbV or RBC in PBS ([Hb] = 1.6 g/L, pH 7.2) was added to a 400 μL stock solution of 10 m*M* $CaCl_2$ and 3 μg/mL PLA_2 (from Naja mossambica mossambica; Sigma, MO). The mixture was incubated for 0.5 and 2 h at 37°C; EDTA was then added to make a final concentration of 2 m*M*; the tube was cooled in ice for 5 min. The testing solutions were centrifuged or ultracentrifuged, and the supernatant was determined for Hb concentration to calculate the degree of hemolysis. The data are expressed as mean ± SE.

RESULTS

Body weight and behavioral testing

All animals in both HbV/rHSA and RBC/rHSA groups survived. After a slight weight reduction during the first 3 days, their body weights increased steadily to 327 ± 3 g at 28 days after the treatment (Fig. 1). Because of the initial loss, both groups showed lighter body weights in comparison to the normal rat group (untreated). The wet brain weight for both groups increased along with the increasing body weight (on the average HbV group, from 1.84 ± 0.04 to 1.94 ± 0.02 g; RBC group, from 1.86 ± 0.02 to 1.97 ± 0.02 g). No significant difference was apparent between the HbV and the RBC treated groups in brain weights.

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The results of behavioral testing (Table I) indicate that animals in both HbV and RBC treated groups displayed no overt circling movement. Piloerection and startle responses were apparent for both groups; however, the startle responses reverted to normal by 7 days in both groups. The testing of the hind leg weakness on the beam showed signs of disability, that is, miss-steps and dragging of the left foot, in both treated groups; they were observed more often than in the untreated rat group. The testing of the

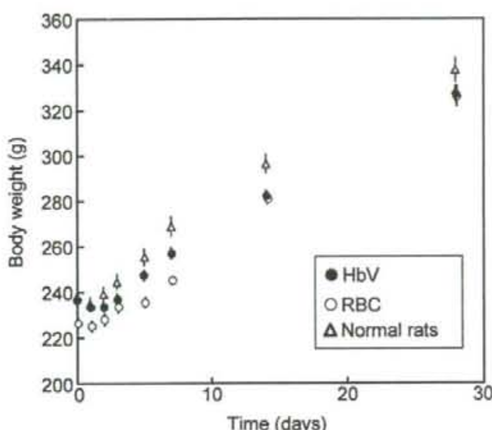

Figure 1. Changes in body weight after intracerebral injection of HbV or RBC. A slight reduction of body weight was apparent for 3 days at the beginning in both groups. However, the rats grew and gained weight rapidly to about 330 g at 28 days. Mean ± SE. Data of normal rats (no treatment) were also plotted as a reference.

ability to hang on a bar with front paws, which was the most sensitive test to detect the lack of motor coordination in our behavioral testing measures, showed some reduction in hanging time (HbV, 10 ± 1 s vs. RBC, 9 ± 1 s), with a significant difference from the untreated group (18 ± 3 s); however, no significant difference was found after 2 days until 28 days.

Histopathological examination of brain

The tip of the needle was positioned to the area of basal ganglia (Fig. 2). One day after the injection, HE staining showed the presence of hematoma in both groups. The HbV group showed a spread of red coloration in the perihematomal region, indicating that small HbV infiltrated into the surrounding parenchyma tissue. In the HbV group, the hematoma was composed of injected HbV and autologous blood because of bleeding caused by the needle insertion. A magnified photograph showed the presence of neutrophils surrounding the hematoma in both HbV and RBC groups because of the inflammatory reaction. The quantities of neutrophils were highest 1 day after injection in both groups; the numbers decreased significantly by 3 days. No notable difference in cellular responses was found between the two groups.

Staining with anti-human Hb antibody also supported the fact that HbV were diffused into the parenchyma. It was inferred from the micrographs taken 1, 3, and 28 days after the HbV injection (Fig. 3) that HbV was phagocytized by macrophages. The number of such macrophages decreased markedly by 28 days. However, the micrograph clearly showed the presence of human Hb in HbV, even after 28 days. Correspondingly, TEM clearly showed the presence of HbV in the phagosomes of macrophages at 1 and 3 days (Fig. 4). A macrophage phagocytizing both HbV and RBC is apparent as shown in the HbV group.

Staining with anti HO-1 antibody showed the induction of HO-1 at 3 and 7 days after injection for both HbV and RBC groups, particularly at the rim of the hematoma region (Fig. 5). However, the macrophages stained with HO-1 in the HbV group appeared more widely distributed in the perihematoma region compared with the RBC group at 28 days (data not shown). The DAB staining procedure with non-immune IgG instead of anti HO-1 antibody showed no brown staining (data not shown). It is concluded, therefore, that the brown stains are specific to HO-1 and not derived from hemosiderin.

Berlin blue method confirmed the hemosiderin deposition from 3 days after injection. A large amount of hemosiderin was deposited at a nearby hematoma site in both groups at 7 days (Fig. 6). The tissue area containing the HbV, however, showed less hemosiderin deposition. At 28 days, the hemosiderin remained in both groups.

Regarding immunochemical studies with GFAP, the reactive proliferation of astrocytes and gliosis was detectable at 3 days in both groups (Fig. 7). The hypertrophic and hyperplastic astrocytes were distributed in the area of the hematoma. The reconstruction processes were seen to occur by scar tissue formation, namely gliosis. Astrocytic cell reaction progressed. The glial fibers became stouter and more numerous, showing fibrillary gliosis at 28 days in both groups.

Furthermore, TUNEL-staining clarified the presence of apoptotic cells surrounding the hematoma in both groups. The HbV group showed 4.3 ± 3.6 cells (1 day), 2.0 ± 0.4 cells (3 days), 8.0 ± 1.9 cells (7 days), and 13.2 ± 4.1 cells (28 days). The RBC group showed 11.9 ± 7.2 cells (1 day), 1.5 ± 0.7 cells (3 days), 20.4 ± 9.8 cells (7 days), and 19.0 ± 6.1 cells (28 days). Both groups showed considerable variation in number of apoptotic cells. However, the RBC group displayed a greater number of apoptotic cells overall than in the HbV group, especially at 7 days.

In vitro stability of Hb-vesicles and RBC

Hypotonic challenge induced 94% hemolysis for rat RBC, although HbV showed essentially no hemolysis (Table II). Freezing of water, crystallization of bulk water molecules, facilitates dehydration of the surface of lipid membranes, and destroys the cellular structure. Freeze-thawing induced 78% hemolysis for

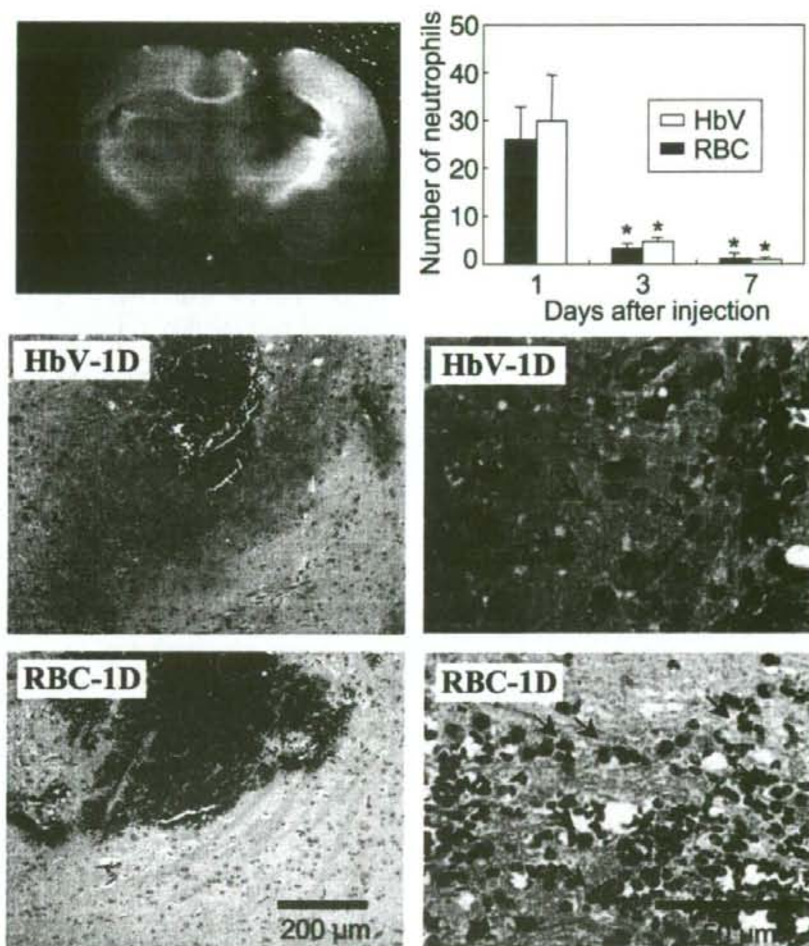

Figure 2. (Left Top) The needle tip for the intracerebral injection reached to the caudate nucleus. (Left middle, bottom) Hematoxylin/eosin staining of rat brain showing the presence of hematoma 1 day after injection. The HbV group showed the red area in the parenchymal region, indicating the presence of dispersed HbV. The RBC group showed that most of the RBCs remained in the hematoma. (Right, middle, and bottom) Hematoxylin/eosin staining of rat brain showing the presence of neutrophils (indicated with arrows) surrounding the hematoma in both HbV and RBC groups 1 day after injection because of the inflammatory reactions. (Right top) The quantities of neutrophils (in 0.26 mm^2) were greatest 1 day after injection. Then they decreased significantly 3 days after injection (* $p < 0.05$ vs. 1 day). However, no differences were found between any of the HbV with the RBC groups. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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RBC whereas HbV showed only 20% hemolysis. HbV was resistant to the attack of PLA_2. Hemolysis was almost not induced in the HbV sample, whereas RBC showed 18% hemolysis.

DISCUSSION

The main finding of this study is that the intracerebral injection of 20 µL HbV showed normal progression of pathological responses to ICH. No difference was evident between HbV and RBC injections in the conventional rat ICH model, except that HbV distributed more widely in the perihematomal tissue and that a slight amount of HbV remained even after 28 days.

A clinical application of Hb-based oxygen carriers (HBOCs) can be a resuscitative fluid, which can be used for traumatic hemorrhaged patients.[26] However, most conventional experimental animal models have tested the effectiveness of HBOCs on artificially created hemorrhagic conditions that do not involve