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REVIEW ARTICLE

Pharmacokinetic properties of hemoglobin vesicles as a substitute for red blood cells

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Abstract

The development of artificial oxygen carriers has attracted considerable recent interest because of the increasing cost of collecting and processing blood, public concerns about the safety of blood products, complications from blood transfusions, military requirements for increased volumes of blood during military conflicts, and a decrease in the number of new donors. To overcome these problems, perfluorocarbon-based oxygen carriers as well as acellular- and cellular-type, hemoglobin-based oxygen carriers have been developed for use as artificial oxygen carriers. Despite their extensive evaluation, including formulation and pharmacology, they have not been extensively used in clinical settings. One of the reasons for this is that their pharmacokinetics have not been well characterized. Artificial oxygen carriers require not only an acceptable level of physicochemical activity, but also clinical efficacy, as reflected by their retention in the circulation, and the absence of measurable accumulation in the body, if unexpected adverse effects are to be avoided. In this review, the pharmacokinetic properties of artificial oxygen carriers are discussed, with a focus on recent developments of our research related to the pharmacokinetic properties a cellular type of hemoglobin-based oxygen carrier.

Keywords: Artificial oxygen carrier, disposition, liposome, mononuclear phagocyte system, hemorrhagic shock, hepatic chronic cirrhosis, accelerated blood clearance phenomenon

Introduction

In modern medical care, there is now little doubt that the transfusion of red blood cells (RBCs) is the gold standard for treatment of patients with massive hemorrhages and is currently in widespread use. Nevertheless, the potential for mismatching exists and infections by unrecognized pathogens, hepatitis, HIV, or West Nile virus, etc., are always a possibility. In addition, ensuring a steady supply of RBCs at a time of a disaster and during military conflicts could be difficult, because the lifetime of donated RBCs is limited to a short period. Further, a decrease in donors and an increase in recipients in some developed countries is also a problem. To overcome these problems, various artificial oxygen carriers have been under development worldwide. They can be divided into three major classes of materials, as follows: perfluorocarbon-based oxygen carriers, acellular-type, hemoglobin-based oxygen carriers (HBOCs), and cellular-type HBOCs (Figure 1). Despite the many efforts to develop artificial oxygen carriers during the past several decades, some of them were, unfortunately, rejected for use as the result of preclinical and clinical trials. It is noteworthy that perfluorocarbon-based oxygen carriers and acellular-type HBOCs were excluded as possible candidates for artificial oxygen carriers, even though they proceeded to the stage of clinical trials.

One of the reasons that induced these adverse effects was due to the insufficient characterization of pharmacokinetics of these artificial oxygen carriers under various situations. The desirable features of artificial oxygen carriers as a substitute for RBCs is not only a long retention in the circulation to sustain its pharmacological effects, but also no bioaccumulation, which could lead to adverse effects. Unlike other drugs, because the dosage volume of

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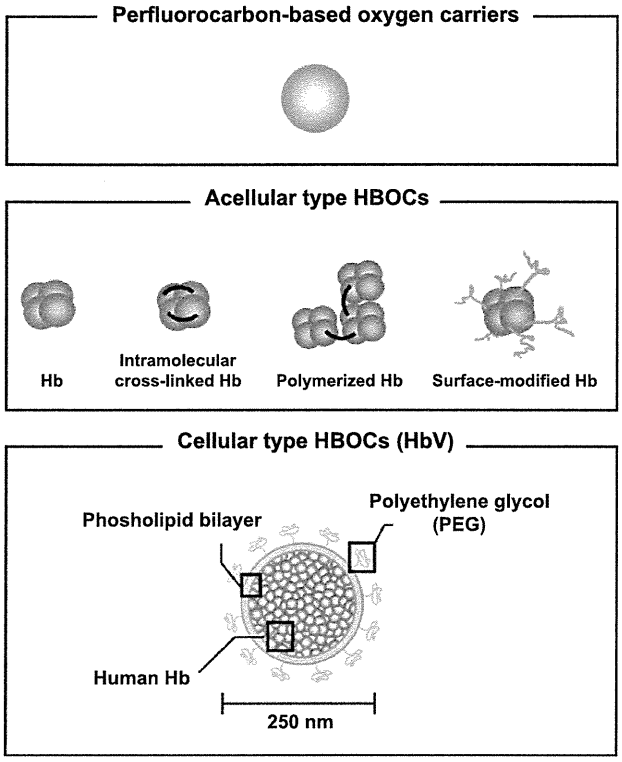

Figure 1. Schematic representation of perfluorocarbon-based oxygen carriers, acellular-type hemoglobin-based oxygen carriers (HBOCs), and cellular-type HBOCs (HbV). In the case of HbV, the surface is modified with polyethylene glycol (PEG) chains, and one HbV particle contains approximately 30,000 human Hb molecules obtained from outdated donated blood. The encapsulated Hb contains pyridoxal 5′-phosphate 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 DSPE-PEG_{5000} (0.3 mol%). The average particle diameter was regulated to approximately 250 nm.

an artificial oxygen carrier as an RBC substitute is more than a hundred times higher than that of other drugs, detailed information regarding the fate of an artificial oxygen carrier, including its constituent components, is needed, in order to predict unexpected adverse effects.

In this review, the pharmacokinetic properties of artificial oxygen carriers are discussed, with a focus on hemoglobin vesicles (HbVs), in which, among the current artificial oxygen carriers, its pharmacokinetic properties have been extensively characterized.

Perfluorocarbon-based oxygen carriers

The perfluorocarbon-based oxygen carriers are characterized by a high gas-dissolving capacity, low viscosity, and chemical and biological inertness (Spahn and Kocian, 2003). They are molecules that are constructed from cyclic or straight-chain hydrocarbons, in which the hydrogen atoms are replaced by halogens, and are virtually immiscible with water and, therefore, must be emulsified prior to their use in intravenous applications (Pape and Habler, 2007). When perfluorocarbon emulsion droplets are injected into an organism, they are rapidly taken up and slowly broken down by the mononuclear phagocyte system (MPS). After being degraded, the emulsion droplets are again taken up by the blood and transported to the lungs, where any unaltered molecules are excreted via exhalation (Spahn and Kocian, 2003; Jahr et al., 2007; Pape and Habler, 2007). However, perfluorocarbon-based oxygen carriers induced chronic pneumonitis due to their inefficient excretion from the body and their accumulation in the lung, a condition that persists for more than 1 year (Nose, 2004) (Table 1).

Acellular-type HBOCs

The stroma-free hemoglobin (Hb) was developed for use as artificial oxygen carriers, but their systemic half-lives were too short (~0.5–1.5 hours) for them to effectively function as an optimal oxygen carrier (Savitsky et al., 1978). In addition, the Hb tetramers dissociate into their component αβ dimers, which are then eliminated by the kidneys, and induce renal toxicity (Creteur and Vincent, 2003). In an attempt to increase their systemic half-life and stability, the following three groups of chemically modified acellular-type HBOCs were developed: surface-modified Hb (Smani, 2008), intramolecularly cross-linked Hb (Chen et al., 2009), and polymerized Hb (Jahr et al., 2008) (Figure 1). These acellular HBOCs have improved systemic half-lives, in the range of 18–24 hours, and show decreased renal failure (Stowell, 2005) (Table 1). The polymerized bovine-derived Hb has been approved for limited use in South Africa (Lok, 2001). However, it was recently reported that the use of some acellular-type HBOCs leads to the development of myocardial lesions, as the result of decreasing nitric-oxide levels 24–48 hours after a single topload infusion (Burhop et al., 2004), leading to an increase in mortality rates in humans (Natanson et al., 2008).

Hemoglobin vesicles

The hemoglobin vesicle (HbV) is a cellular-type HBOC that contains polyethylene glycol (PEG), in which phospholipid vesicles encapsulating highly concentrated human Hb are imbedded (Sakai et al., 2008) (Figure 1). The cellular structure of HbV (particle diameter: approximately 250 nm) most closely mimics the characteristics of a natural RBC, such as the cell-membrane function, which physically prevents the direct contact of Hb with the components of blood and the vasculature during its circulation. The characteristics of HbV are superior to donated RBCs in the following ways: the absence of viral contamination (Sakai et al., 1993; Abe et al., 2001), a long-term storage period of over 2 years at room temperature, and no blood-type antigens (Sakai et al., 2000; Sou et al., 2000) (Table 2). In addition, HbVs have the ability to transport oxygen equivalent to RBCs and also show improved survival in hemorrhagic shock animal models (Sakai et al., 2004b; Terajima et al., 2006; Sakai et al., 2009). Further, HbVs can control the release of oxygen by

adjusting the amount of allosteric effector and regulate rheological properties (e.g., viscosity and colloid osmotic pressure) to added human serum albumin (Sakai and Tsuchida, 2007). Therefore, HbV has attracted considerable attention as a possible new artificial oxygen carrier and has considerable promise for use in clinical settings.

We recently characterized the pharmacokinetic properties of HbV to clarify its efficacy and safety under conditions that mimic a clinical setting, as follows:

1. HbV was constructed from multiple components, including Hb, lipids, and iron from Hb. These components have potential risks for inducing harmful effects, when they accumulate at excessive levels in the body.
2. HbV is classified as a liposome preparation. It was previously reported that the pharmacokinetics of liposome-encapsulated amphotericin B differ between normal individuals and patients (Walsh et al., 1998; Bekersky et al., 2001).
3. The surface of HbV was modified by PEG to enhance the half-life in circulation and storage. It was recently reported that repeated injection of PEGylated liposomes influenced the pharmacokinetics of the second injected liposome (Dams et al., 2000; Ishida et al., 2003a).

Table 1. Pharmacokinetic properties of some artificial oxygen carriers.

	Perfluorocarbon-based oxygen carriers	Acellular-type HBOCs	Cellular-type HBOCs
Distribution	Liver, spleen	Liver	Liver, spleen
Metabolism	MPS	MPS	MPS
Excretion	Air	—	Internal Hb; urine outer membrane; feces
Half-life	~10 hours (rat)	~24 hours (rat)	30~40 hours (rat)
Existence in tissues	~1 year	—	~14 days

HBOCs, hemoglobin-based oxygen carriers; MPS, mononuclear phagocyte system.

Table 2. Physicochemical characteristics of HbV.

Parameter	
Particle diameter	ca. 250 nm
P_{50}	25–28 torr
Hb concentration	10 g/dL
MetHb	<3%
Colloid osmotic pressure	0 Torr
Intracellular Hb concentration	ca. 35 g/dL
Lipid composition[a]	DPPC/cholesterol/DHSG/ $DSPE\text{-}PEG_{5000}$
Stability for storage at room temperature	Over 2 years, purged with N_2

[a]DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DHSG, 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate; $DSPE\text{-}PEG_{5000}$, 1,2-distearoyl-*sn*-glycero-3-phosphatidyl-ethanolamine-*N*-PEG.

For these reasons, it becomes necessary to clarify the pharmacokinetic properties of HbV in various animal models and under conditions of repeated injection, if RBCs are to be used as a substitute in the future. For this purpose, 1) the disposition of HbVs was examined using isotope tracer techniques. In these experiments, ^{125}I-HbV, enclosed in HbVs, was radiolabeled with ^{125}I, and the lipid component vesicles of HbVs was radiolabeled with ^{3}H; 2) a pharmacokinetic study of HbVs in a rat model of hemorrhagic shock and hepatic chronic cirrhosis; 3) the repeated injection in normal and the hemorrhagic shock rat model; and 4) animal scale-up using an allometric equation, were conducted.

Some highlights of recent developments of our research related to the pharmacokinetic properties of HbV are discussed below.

The prior pharmacokinetic characteristics of HbV to stroma-free Hb

Two requirements need to be satisfied if HbV is to be accepted for use as an artificial oxygen carrier. For clinical applications, HbVs must have not only an acceptable physicochemical activity, but also must be safe for use in the clinic. In the latter case, the supply of oxygen tissues is one of the most important factors in sustaining the clinical effect of HbVs (Takaori, 2005). To fulfill these requirements, a prolonged half-life is a required property for HbVs.

We recently demonstrated that the half-life of HbV in mice was 30 times higher than that of stroma-free Hb at a dose rate of 1 mg Hb/kg (Table 3). Moreover, a dose-dependent study clearly showed that the plasma concentration curve and half-life of HbV in mice and rats increased with increasing doses of HbV (Figure 2, half-life; rats: 8.8±0.7, 11.5±0.3, and 30.6±4.0 hours at doses of 10, 200, and 1,400 mg Hb/kg, respectively; mice: 3.1±3.1, 3.6±1.3, 7.2±3.1, and 18.8±1.3 hours at doses of 1, 10, 200, and 1,400 mg Hb/kg, respectively) (Taguchi et al., 2009b).

These superior pharmacokinetic characteristic of HbV, compared to stroma-free Hb, could reflect their physicochemical differences, such as particle diameter, the absence or presence of a membrane structure, and PEG modification. In physiological conditions, free Hb that is released from ruptured RBC is rapidly bound to

Table 3. Pharmacokinetic parameters for HbV after the administration of ^{125}I-Hb and ^{125}I-HbV in mice at a dose of 1 mg Hb/kg.

	^{125}I-Hb	^{125}I-HbV	*p*
$t_{1/2}$ (hr)	0.1±0.1	3.1±1.0	<0.01
AUC (hr*% of dose/mL)	7.9±3.9	29.4±9.2	<0.001
CL (mL/hr)	12.7±2.1	3.4±0.1	<0.001
V (mL)	2.6±0.3	2.3±0.1	N.S.

$t_{1/2}$, half-life; AUC, are under the plasma-concentration versus time curve; CL, clearance; V, distributed volume; N.S., not significant.

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 removed by filtration through the kidney. Therefore, the reduction in HbV distribution in the liver and kidney could be due to the encapsulation of Hb by liposomes because this might not only suppress the binding of internal Hb to Hp, but also inhibit renal glomerular filtration. In fact, it was observed that the distribution of HbV in the liver and kidney was suppressed, compared with that of stroma-free Hb (Taguchi et al., 2009b). Moreover, the membrane surface modification by PEG also contributed to the increased half-life of HbV. In general, it is well-known that liposomes are scavenged and degraded by the MPS, such as Kupffer cells or macrophages in the spleen (Kiwada et al., 1998). PEGylation is a useful method for suppressing the capture of MPS, and the majority of the recently developed liposome formulations are modified with PEG (Noble et al., 2006; Sou et al., 2007; Okamura et al., 2009). Therefore, the modification of HbV with PEGylation is important to not only stabilize for a long-time storage, but also to maintain the good retention in the circulation. These balanced physicochemical activities result in a longer retention in the circulation, compared to stroma-free Hb and acellular-type HBOCs (Goins et al., 1995; Chang et al., 2003; Lee et al., 2006).

The disposition of HbV components

In clinical situations as a substitute of RBCs, massive amounts of HbV are typically given to patients. As a result, its associated components, including Hb, lipids from Hb, could result in undesirable consequences in the systemic circulation and organs during its metabolism and disposition. Such an extraordinary load of HbV components could result in the accumulation of components in the blood or organs, and has the potential to cause a variety of adverse effects, as follows: 1) high levels of lipid components, especially cholesterol, in the bloodstream, which are risk factors for kidney disease, arterial sclerosis, and hyperlipidemia (Grone and Grone, 2008); 2) Hb induces renal toxicity by dissociation of the tetramic Hb subunits into two dimers (Parry, 1988); and 3) free iron can trigger tissue damage induced by the Fenton reaction, which is mediated by heme (iron) (Balla et al., 2005). Therefore, it becomes necessary to clarify whether HbV and its components have favorable metabolic and excretion profiles. In order to investigate the disposition 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).

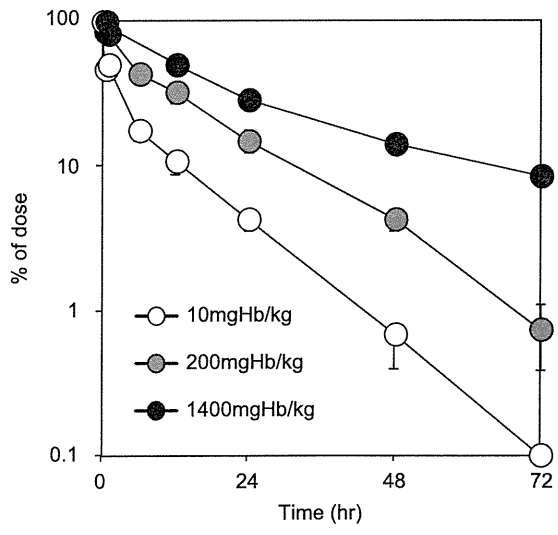

Figure 2. Dose-dependent plasma concentration curve of ^{125}I-HbV after administration of ^{125}I-HbV in rats. All rats received a single injection of ^{125}I-HbV at a dose of 10 (open squares), 200 (gray circles), and 1,400 mg Hb/kg (closed circles) containing 5% rHSA. At each time point (0.05, 0.5, 1, 6, 12, 24, 48, and 72 hours) after the ^{125}I-HbV injection, blood samples were collected from the tail vein, and a plasma sample was obtained. Each point represents the mean ± SD (n=3–5).

In the blood circulation, HbV typically maintains an intact structure for periods of up to 72 hours after injection, because similar plasma concentration curves for ^{125}I-HbV were observed for ^{3}H-HbV in rats (Figure 3A), and the pharmacokinetic parameters were also consistent between them (half-life: 30.6 ± 4.0, 30.9 ± 4.7 hours; clearance in plasma: 0.46 ± 0.04, 0.41 ± 0.02 mL/h, for ^{125}I- and ^{3}H-HbV, respectively). Moreover, ^{125}I-HbV and ^{3}H-HbV were mainly distributed in the liver and spleen (Figure 3B). Because HbV possesses a liposome structure, it would be predicted that it would be captured by the MPS in the liver and spleen (Kiwada et al., 1998). In fact, a previous *in vitro* study clearly demonstrated that HbV was specifically taken up and degraded in RAW 264.7 cells, which has been used as an alternative to Kupffer cells, but this was not the case for parenchymal and endothelial cells (Taguchi et al., 2009b). In addition, the uptake clearance (CL_{uptake}) in the liver and spleen were also similar between the two labeled preparations (liver: 1,141 ± 142, 1,098 ± 123; spleen: 619 ± 40, 518 ± 89 μL/h, for ^{125}I-HbV and ^{3}H-HbV, respectively). However, ^{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, but nearly disappeared after 14 days. These data indicate that HbV is mainly distributed to the liver and spleen in the form of intact HbV, and that it was degraded by the MPS. In order to identify the excretion pathway of HbV, the levels of radioactivity of ^{125}I and ^{3}H in the urine and feces were measured. The radioactive ^{125}I was excreted mainly in the urine, whereas the majority of the ^{3}H was excreted in the feces. Based on the above findings, 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 the MPS. Finally, the enclosed Hb and outer lipid components were mainly eliminated to the urine and feces, respectively, in the same manner as endogeneous substances (Figure 4). Similar results were also reported in mice and rabbits (Sou et al.,

2005; Taguchi et al., 2009b); these results indicate that HbV and its components have favorable metabolic and excretion profiles in mammalian species. In addition, the plasma concentration curve for heme (iron) derived from HbV was similar to that for ^{125}I-HbV and ^{3}H-HbV in mice (Taguchi et al., 2009b). Moreover, no significant differences in the ratio of the mercapt- (i.e., nonoxidized form) to the nonmercapt-form (i.e., oxidized form) of rat serum albumin, which serves as a marker of oxidative stress in the circulation system (Kadowaki et al., 2007; Shimoishi et al., 2007), were found between HbV and the saline administration groups for periods of up to 7 days after administration. These results suggest that excess free heme (iron) derived from HbV is not released in the plasma. However, the issue of the disposition of several HbV components, including PEG and phospholipid, was not clarified. It is also possible that these components in HbV are also metabolized and excreted in the same manner as endogenous substances, but further study will be needed to demonstrate this fact.

Pharmacokinetic properties of HbV under conditions of hemorrhagic shock

It is well known that clinical conditions can have an effect on the pharmacokinetics of numerous drugs (Abernethy et al., 1981; Turck et al., 1996). For example, it has demonstrated that the pharmacokinetics of liposome-encapsulated amphotericin B differ between normal individuals and patients in a clinical trial stage (Walsh et al., 1998; Bekersky et al., 2001). Consequently, it is possible that the pharmacokinetics of HbV would be also altered in the situation of a massive hemorrhage caused by injury, accidental blood loss, or a major surgery. To clarify this, we investigated the changes in HbV pharmacokinetics using a rat model of hemorrhagic shock induced by massive hemorrhage.

As shown in Figure 5, the retention of HbV in plasma under this condition was shorter, and the half-life of HbV was reduced significantly—by 0.66-fold—compared with the half-life of HbV in normal rats (30.6±4.0, 18.1±3.7 hours, for normal and hemorrhagic shock, respectively). At a glance, this appears to not be a desirable situation for the therapeutic use of HbV, because an important determinant of HbV efficacy is a long retention in the blood circulation. However, the distribution volume of the central compartment of HbV (V_1) was identical between normal and hemorrhagic shock rats, whereas the distribution volume of the peripheral compartment (V_2) in hemorrhagic shock rats was nearly 2-fold greater than that of normal rats (Figure 5, insert). Moreover, the time-course tissue distribution of HbV in the hemorrhagic shock rats was greater than normal rats. These findings indicate that the shorter half-life in hemorrhagic shock rats appears to be the result in an apparent reduction in HbV in the arteriovenous circulation. If this enhanced tissue distribution of HbV might be derived by an increased scavenging of HbV by the MPS, such as by Kupffer cells, red pulp zone splenocytes, and mesangial cells (Sakai et al., 2004a), it would not be expected to show significant pharmacological efficacy as an oxygen carrier, because HbV must maintain an intact structure to maintain its oxygen-carrying capacity. However, the pharmacological effect in the hemorrhagic shock model animal was significantly increased by the HbV treatment, similar to that for an RBC treatment (Sakai et al., 2004b; Terajima et al., 2006; Sakai et al., 2009). In addition, the amount of excretion into the urine, which is the major elimination pathway, did not differ between normal and hemorrhagic shock rats in our pharmacokinetic study. Therefore, HbV appears to be transferred from the arteriovenous blood to organ capillary beds as an intact structure, and is not excessively captured and metabolized by the MPS. These findings support the conclusion

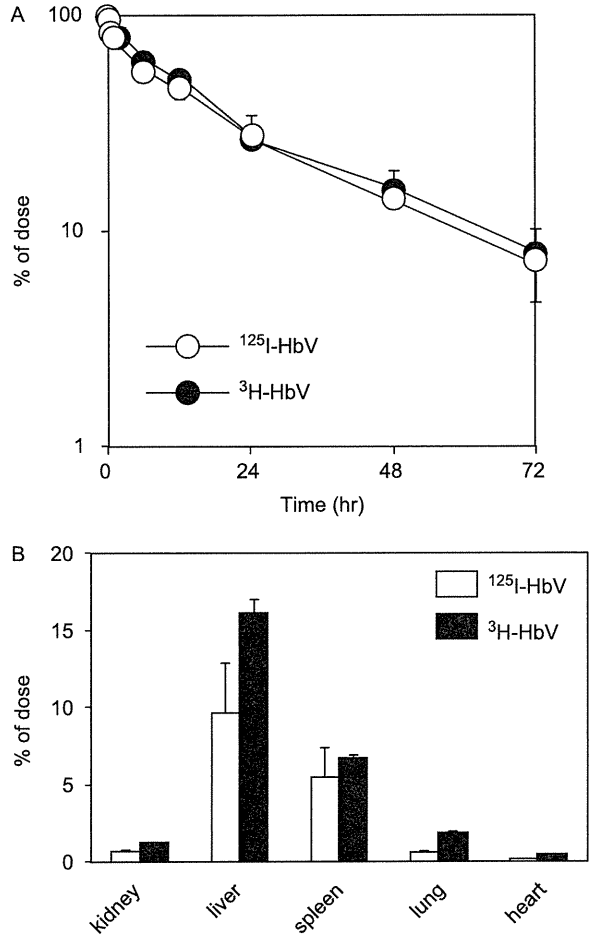

Figure 3. (**A**) Time course for the plasma level of ^{125}I-HbV (open circles) and ^{3}H-HbV (filled circles) after administration to rats. SD rats received a single injection of ^{125}I-HbV or ^{3}H-HbV to the tail vain at a dose of 1,400 mg Hb /kg. Blood was collected from the tail vein under ether anesthesia, and a plasma sample was obtained. Each point represents the mean ± SD (n=5). (**B**) Tissue distributions of ^{125}I-HbV (open bars) and ^{3}H-HbV (filled bars) at 24 hours after administration to mice. SD rats received a single injection of ^{125}I-HbV or ^{3}H-HbV from the tail vain at a dose of 1,400 mg Hb/kg. At 24 hours after injection, each organ was collected. Each bar represents the mean ± SD (n=5).

that HbV is pharmacologically efficacious in a rat model of HS induced by massive hemorrhage (Sakai et al., 2004b, 2009) is retained for a sufficiently long period to meet oxygen-delivery demands until autologous blood volume and oxygen-carrying capacity are restored.

Pharmacokinetic properties of HbV in the condition with chronic liver failure

As mentioned above, the liver is the determinant for the pharmacokinetic properties of HbV, because HbV is mainly degraded by Kupffer cells, and the lipid components of HbV, especially cholesterol, are excreted to the feces via biliary excretion (Sakai et al., 2001; Taguchi et al., 2009b). Consequently, HbV can be classified as a hepatically cleared and excreted drug. In the case of other hepatically cleared and excreted drugs, some are contraindicated for a person with a hepatic injury. Because hepatic impairment affects the pharmacokinetics of drugs, including their metabolism and excretion (Okumura et al., 2007), these changes have the potential to induce toxicity and accumulate in the body, subsequently causing unexpected adverse effects. Thus, if HbV and its components show the changes of pharmacokinetic properties under conditions of liver failure, it may also be contraindicated for a person with liver impairment under such conditions. Therefore, we investigated the pharmacokinetic properties of HbV using a chronic cirrhosis rat model with fibrosis induced by the administration of carbon tetracholoride, which is categorized as Child-Pugh grade B (Taguchi et al., 2011b).

After the administration of HbV to chronic cirrhosis rats, the plasma concentration of HbV varied widely among individuals, similar to their liver function. To clarify the effect of hepatic impairment on the plasma concentration of HbV, the clearance and the area under the concentration-time curve values for HbV, as calculated from the plasma concentration curve, were plotted against plasma aspartate aminotransferase (AST) levels. As a result, a good, negative correlation was found for the clearance of HbV with changes in plasma AST levels. In addition, the hepatic distribution of HbV was negatively

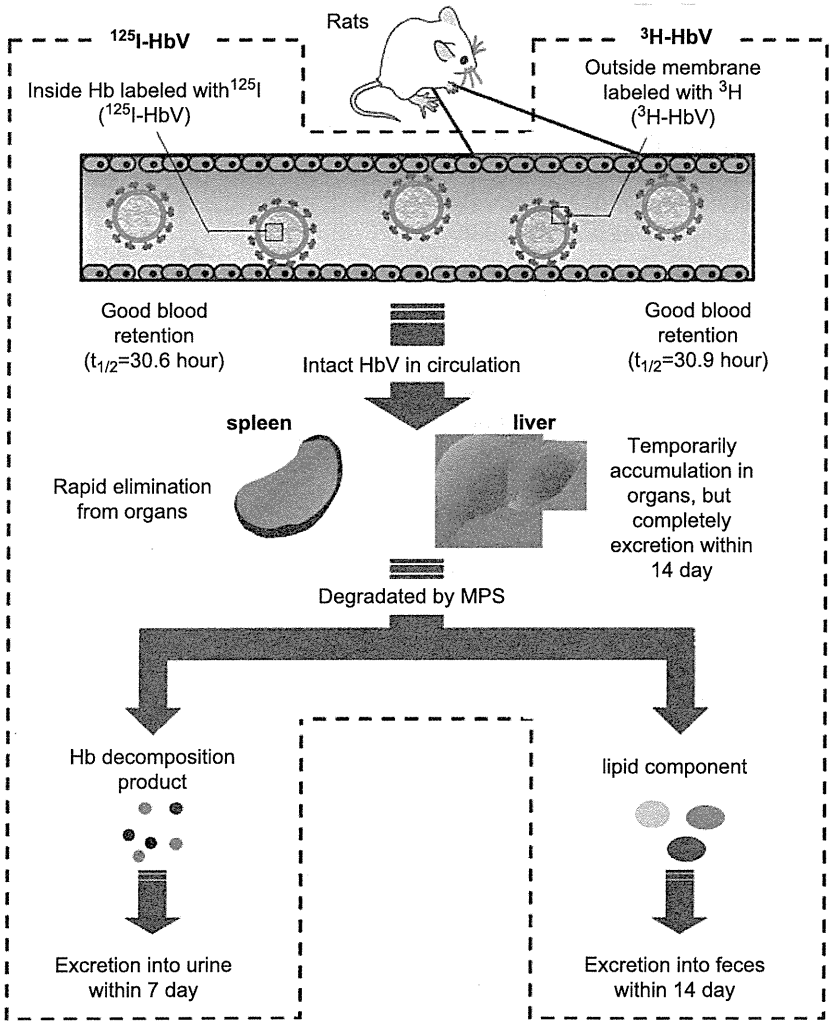

Figure 4. Representation of a sequence of HbV disposition, metabolism, and excretion from pharmacokinetic examinations, using ^{125}I-HbV and ^{3}H-HbV. After circulating in the form of stable HbV, it is distributed to the liver and spleen, where it is degraded by MPS. Finally, the enclosed Hb and outer lipid components are mainly eliminated to the urine and feces, respectively.

correlated with plasma AST levels, but this was not found for the spleen. Moreover, carbon clearance, which serves as a measure of phagocyte activity in Kupffer cells (Zweifach and Benacerraf, 1958), was also negatively correlated with plasma AST levels. Therefore, the changes in HbV pharmacokinetic properties were significantly influenced by a reduction in liver function and were especially dependent on a decrease in phagocyte activity by Kupffer cells in the chronic cirrhosis rat.

In addition, the excretion of lipid components (e.g., cholesterol) in feces was also negatively correlated with plasma AST levels. The cholesterol of the vesicles should reappear in the blood mainly as lipoprotein cholesterol after entrapment by Kupffer cells and should then be excreted in the bile after entrapment of the lipoprotein cholesterol by the hepatocytes (Kuipers et al., 1986). Therefore, the extent of damage to parenchymal cells also affects the pharmacokinetic properties of HbV components. Such a suppressed elimination of HbV components may have an impact on their tissue accumulation. However, the lipid components, especially cholesterol, nearly completely disappeared from organs after 7 days in the chronic cirrhosis rat. Further, our recent study showed that the plasma levels of other lipid components, such as phospholipids, was temporarily increased after the administration of HbV at a dose of 1,400 mg Hb/kg in the chronic cirrhosis rat, but recovered to baseline levels within 14 days (Taguchi et al., 2010). In addition, if the metabolic and excretion performance of HbV were reduced by chronic cirrhosis, tissue damage could be induced, resulting in a change in blood biochemical parameters. However, the morpholgical changes in organs were minimal (Figure 6), and only negligible changes in plasma biochemical parameters were observed after an HbV injection at a dose of 1,400 mg Hb/kg in the chronic cirrhosis rats. Based on these findings, it can be concluded that the pharmacokinetics of HbV were altered by hepatic impairment, and these changes can be attributed to a decrease in Kupffer-cell phagocyte activity (Figure 7). However, HbV and its components were completely metabolized and excreted within 14 days, and a temporary accumulation did not cause any obvious adverse effects.

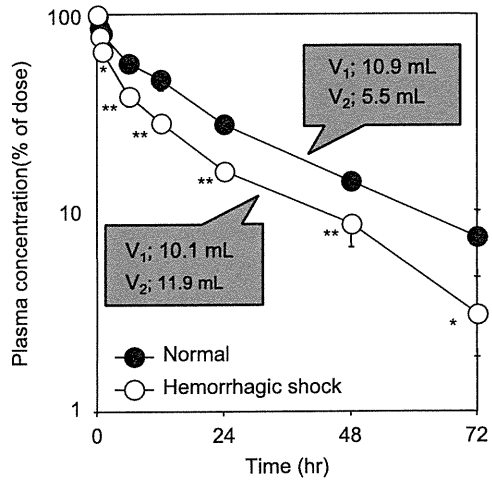

Figure 5. Relative plasma concentration of ^{125}I-HbV after administration of 1,400 mg Hb/kg via injection of normal (filled circles) or hemorrhagic shock rats (open circles). After inserting polyethylene catheters into the left femoral artery, SD rats received a single injection of ^{125}I-HbV to the left femoral artery at a dose of 1,400 mg Hb /kg. Blood was collected from the tail vein under ether anesthesia, and a plasma sample was obtained. Each point represents the mean ± SD (n=5).

Pharmacokinetic properties of HbV after repeated administration in mice

HbV is modified by PEG to prolong its half-life and prevent aggregation during long-term storage, etc., as well

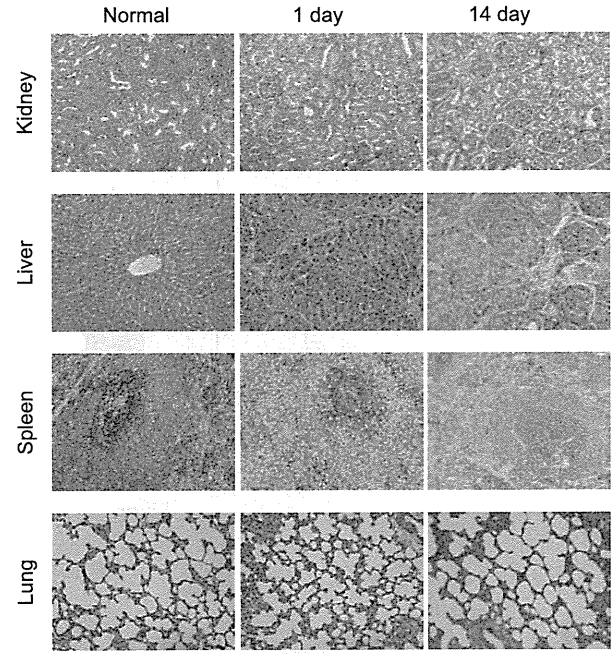

Figure 6. Light micrographs of kidney, liver, spleen, lung, and heart in CCl_4-treated rats after an HbV injection stained with hematoxylin and eosin (X100). Chronic cirrhosis model rats received a single injection of HbV at a dose of 1,400 mg Hb/kg. No noticeable changes were observed in all organs after HbV injection.

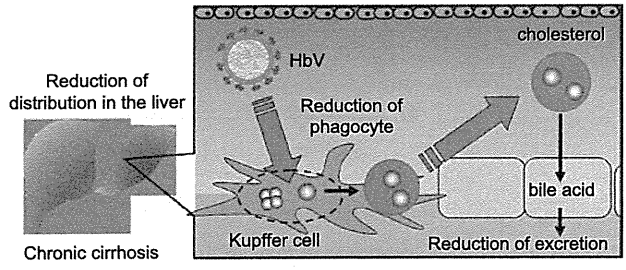

Figure 7. Representation of the pharmacokinetic properties of HbV in a rat model of chronic cirrhosis. Hepatic impairment altered the pharmacokinetic properties of HbV, such as blood retention, hepatic distribution, and fecal excretion, by a reduction in Kupffer cell phagocyte activity and damage to parenchymal cells.

as other liposome preparations. However, it was reported that repeated intravenous injection of PEGylated liposomes causes the second dose of liposomes to lose their long-circulating characteristics and accumulate extensively in the liver, when they are administrated at the same dose for the second time to the same animal within a several-day interval [referred to as the accelerated blood clearance (ABC) phenomenon] (Dams et al., 2000; Ishida et al., 2003a). The time frame between administration of the first and second dose for this to occur depends on the experimental animal, for example, 4–5□days for the rat and 7–10 days for the mouse. Repeated HbV injections of high doses would be routinely used in clinical practice for an RBC substitute. Therefore, the possibility remains that repeated injections of HbV could induce the ABC phenomenon in a clinical situation. If the ABC phenomenon were induced by repeated injections, then the pharmacological action of HbV could be influenced. Therefore, we investigated the issue of whether HbV induces the ABC phenomenon in mice at a low dose (0.1 mg Hb/kg), a dose that is generally known to induce the ABC phenomenon (Ishida et al., 2003a), or a high dose (1,400 mg Hb/kg), the putative dose for clinical use.

At 7 days, in which the ABC phenomenon in mice is typically observed the most strongly (Ishida et al., 2003b), after the first injection of nonlabeled HbV (0.1 or 1,400 mg Hb/kg), the mice received ^{125}I-HbV. At a low dose (0.1 mg Hb/kg), plasma HbV in the second injection was rapidly cleared, compared to that in the first injection. In contrast, at a high dose (1,400 mg Hb/kg), the pharmacokinetics of HbV were negligibly affected by repeated injections (Taguchi et al., 2009c). The liver and spleen are the major distribution organs for HbV (Taguchi et al., 2009b) and are related to the induction of

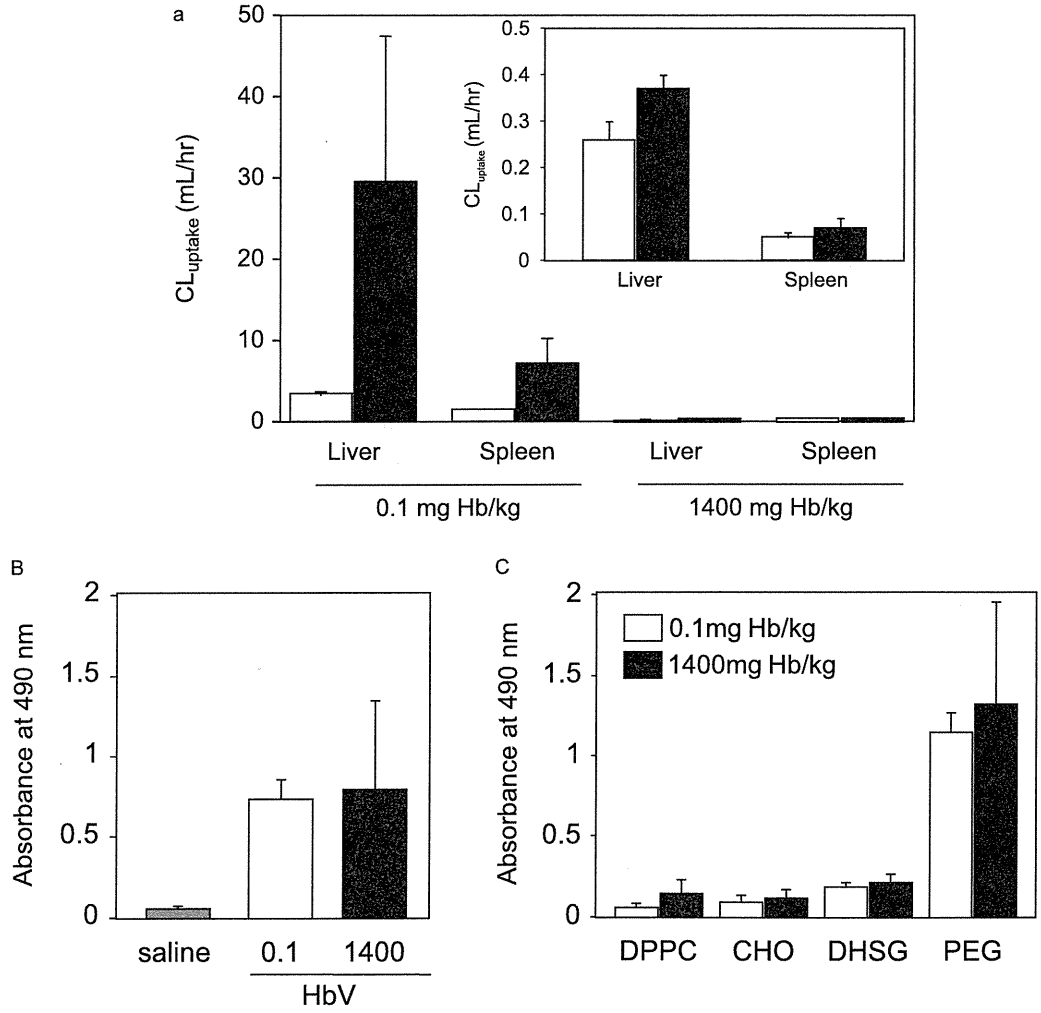

Figure 8. (**A**) Uptake clearance of HbV in the liver and spleen after 1 or 2 injections of ^{125}I-HbV. Uptake clearance for each organ was calculated by integration plot analysis at designated times from 1 to 30 minutes after injection. Each bar represents the mean ± SD (n=4). (**B**) Determination of IgM against HbV after a single intravenous injection of saline (gray bars), HbV at a dose of 0.1 mg Hb/kg (open bars), or 1,400 mg Hb/kg (closed bars) in mice. (C) Determination of the specific recognition site of IgM against HbV after a single intravenous injection of HbV at a dose of 0.1 mg Hb/kg (open bars) or 1,400 mg Hb/kg (closed bars) in mice. DdY mice were injected with saline or HbV (0.1 or 1,400 mg Hb/kg) containing 5% rHSA to the tail vein. At 7 days after an injection of saline or HbV, blood was collected from the inferior vena cava, and plasma was obtained. IgM against HbV and each lipid component were detected by ELISA. Each bar represents the mean ± SD (n=4).

the ABC phenomenon (Ishida et al., 2008). At a low dose, the hepatic and splenic CL_{uptake} for the second injection was 8.5 and 4.5 times higher than that for the first injection, respectively (Figure 8A), whereas at a high dose, the hepatic and splenic CL_{uptake} for the second injection was little changed, compared to that for the first injection (Figure 8A, insert). In addition, Ishida et al. proposed a mechanism for the ABC phenomenon as follows: Immunoglobulin M (IgM), produced in the spleen by the first injection with PEGylated liposomes, selectively binds to the PEG on the second injected PEGylated liposome, and subsequent complement activation by IgM results in an accelerated clearance and enhanced hepatic uptake of the second injected PEGylated liposome (Ishida et al., 2006a, 2006b). Therefore, we examined whether IgM against HbV is elicited by an initial injection of saline or HbV at a low or high dose. At 7 days after the HbV injection, IgM against HbV appeared at both the low and the high dose (Figure 8B). Moreover, the specific recognition site of IgM against HbV strongly bound to DSPE-PEG, and other lipid components (DPPC, cholesterol, and DHSG) were negligible at both the low and high dose (Figure 8C). These results indicate that repeated injections of HbV to mice at a dose of 1,400 mg Hb/kg did not appear to induce the ABC phenomenon, even though the plasma levels of IgM against HbV are elevated. Therefore, these data suggest that a clinical dose of HbV is not likely to induce the ABC phenomenon due to the saturation of phagocytic processing by the MPS.

Pharmacokinetic properties of HbV after repeated administration in hemorrhagic shock model rats

Because there are limited data available for the ABC phenomenon under various disease conditions, we also investigated whether the ABC phenomenon would be induced in the rat model of hemorrhagic shock induced by a massive hemorrhage, when HbV is injected at a dose of 1,400 mg Hb/kg at hourly intervals, typical conditions for transfusions of patients with massive hemorrhage.

The plasma concentration of HbV was prolonged in the second injection, compared with the first injection, and it was recovered to that in normal rats (Figure 9A). As mentioned above, Ishida et al. reported that a dosing interval of approximately 5 days induced the ABC phenomenon in rats, accompanied by the production of antiliposome IgM, which elicits a response by the spleen (Ishida et al., 2006b; Wang et al., 2007). Therefore, the inhibition of anti-HbV IgM production by short intervals appears to prevent induction of the ABC phenomenon. In fact, anti-HbV IgM was detected at 5 days after the administration of HbV to normal rats at a dose of 0.1 mg Hb/kg, but was not detected at 1 hour after HbV administration to hemorrhagic shock rats at a dose of 1,400 mg Hb/kg (Figure 9B). Therefore, it appears that the repeated administration of HbV under conditions of hemorrhagic shock has negligible effect on the pharmacokinetics of HbV, when short dosing intervals are involved. However, our recent study showed that the repeated injection of HbV induced the ABC phenomenon in the case of a longer dosing interval (4 and 7 days) accompanied by the production of antiliposome IgM and increased phagocyte activity (Taguchi et al., 2011a). Therefore, in a clinical setting, it would be necessary to consider the dosing regimen and interval for patients with hemorrhagic shock in the base where a longer dosing interval was used.

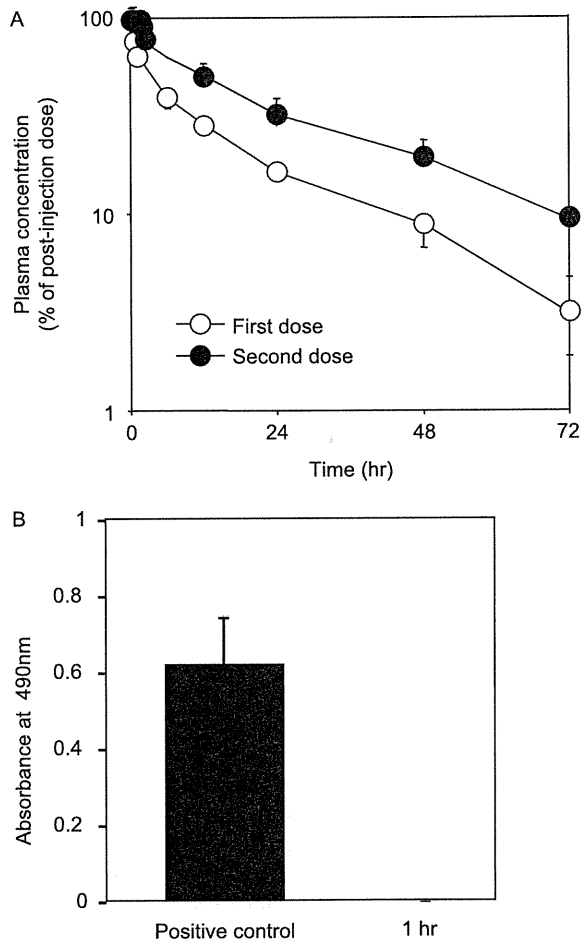

Figure 9. (**A**), Plasma concentration of ^{125}I-HbV as the percent of postinjection dose after the first (open symbol) or second dose (filled symbol) of ^{125}I-HbV to hemorrhagic shock rats at a dose of 1,400 mg Hb/kg for each injection. Each point represents the mean ± SD (n=5). Plasma concentration percentage profile for the first dose (○) was obtained from injection of a dose of ^{125}I-HbV administered after hemorrhagic shock. The profile for the second dose (•) was obtained from the injection of a dose of ^{125}I-HbV 1 hour after injection of the first dose of nonradiolabeled HbV administered after hemorrhagic shock. (**B**) Determination of IgM against HbV 5 days after a single intravenous injection of HbV to normal rats at a dose of 0.1 mg Hb/kg (closed bars) or 1 hour after a single intravenous injection of HbV to hemorrhagic shock rats at a dose of 1,400 mg Hb/kg (open bars) in mice. IgM against HbV were detected by ELISA. Each bar represents the mean ± SD (n=3-5).

Extrapolation to human subjects

From the viewpoint of future clinical applications, predictions of human pharmacokinetics based on data obtained from animal studies—so called, "animal scale-up"—is important for the determination of optimal doses and intervals (Izumi et al., 1996). Thus, we attempted to predict the half-life of HbV in humans using an allometric equation that is generally used in animal scale-up studies. Using the relationships observed for mice (Taguchi et al., 2009b), rats (Taguchi et al., 2009a), and rabbits (Sou et al., 2005), the half-life of HbV in healthy humans was predicted to be approximately 96 hours. In addition, based on half-life data and percent of injected dose values obtained from pharmacokinetic studies of HbV in rats and rabbits, Sou et al. also predicted that the half-life of HbV in healthy humans would be approximately 72 hours (Sou et al., 2005). Further, the half-life of liposomal preparations is empirically 2–3-fold greater in humans than in rats (Gabizon et al., 2003). In fact, the half-life of liposomal doxorubicin (Doxil formulation) in rats and humans is 35 and 56–90 hours, respectively (Gabizon et al., 2003). Therefore, the half-life of HbV in humans would be predicted to be 3–4 days (Sou et al., 2005). For HbV to function as an artificial oxygen carrier, it is desirable that intravascular persistence be at least equal to the time required to regenerate RBCs (Sehgal et al., 1984). Following a massive hemorrhage, the lost blood volume and oxygen-carrying capacity is replaced within approximately 5 days (Hughes et al., 1995; Awasthi et al., 2007). Because the half-life of HbV in humans was estimated to be approximately 3–4 days, HbV would function as a temporary oxygen carrier until a blood transfusion is available or until autologous blood is recovered after a massive hemorrhage.

Conclusion

Like other drugs, a pharmacokinetic evaluation is an important issue for the development of HbV as a substitute of RBC. In fact, though the perfluorocarbon-based oxygen carriers and acellular-type HBOCs were moved into the clinical trial stages, these artificial oxygen carriers dropped from further clinical development due to severe and unexpected side effects, which might have been predicted from pharmacokinetic analysis data. Therefore, it is also necessary to conduct an in-depth pharmacokinetic study of HbV before moving on to the clinical trial stage.

Our recent preclinical study of HbV clearly demonstrated five major findings on pharmacokinetic profiles. First, HbV and its components have favorable metabolic and excretion profiles in mammalian species, similar to endogeneous substances. Second, HbV is safe and useful under conditions of a massive hemorrhage. Third, HbV did not show any toxicity and accumulation in the body, even under conditions of hypometabolism and excretion (i.e., hepatic cirrhosis). Fourth, HbV has the potential to induce the ABC phenomenon, but the repeated use of HbV at a putative dose would not be expected to induce the ABC phenomenon in a clinical situation. Finally, HbV has a good retention in the blood circulation, and the half-life of HbV in humans was estimated to be approximately 3–4 days, which is sufficient for it to function as an oxygen carrier. These findings support previous views related to the pharmacological efficacy and safety of HbV in normal and hemorrhagic shock model rats from the view point of pharmacokinetics.

In addition to functioning as a substitute for RBCs, HbV would be expected to have a variety of other applications, based on its oxygen transport characteristics, such as in cardiopulmonary bypass priming solutions (Yamazaki et al., 2006), wound healing in critically ischemic skin (Plock et al., 2009), and as a radiation therapy agent (Yamamoto et al., 2009). Therefore, this issue deserves to be studied further, with further data collected in preclinical pharmacokinetic studies for future applications of HbV in the clinic.

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Declaration of interest

The authors declare no financial conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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人工血小板

半田　誠

要　旨：人工血小板は，凍結乾燥させたヒト血小板由来産物と生体適合性に優れたアルブミンなどの微粒子表面にヒトフィブリノゲンもしくはそのペプチドを結合させた人工物がある。人工血小板は残存した血小板の機能を補助することで，血小板輸血に代わって血小板減少症の止血や出血予防を行う。すでに，いくつかの試験物が初期臨床試験に供されているが未だ実用化には至っていない。
(J Jpn Coll Angiol, 2011, **51**: 333–338)

Key words: artificial platelet, activated platelet, fibrinogen, albumin particle, liposome

はじめに

血小板の量的・質的異常に起因した出血の予防や治療に使用される血小板濃厚液は，有効期限が短くしかも厳密な保存条件が必要なため，緊急時の輸血に対応することは極めて困難である。生きた細胞である血小板濃厚液の欠点を克服し，医療現場で常備できる人工血小板の開発が，主に軍事目的で，米国で始まってからすでに50年以上が経過した[1,2]。残念ながら，未だ実用化には至っていないが，いくつかの有望な試験物が報告され，一部は初期臨床試験に供され，将来の製剤化への取組みが継続されている(**Fig. 1**)[3]。血小板の止血機能と対比して，人工血小板の開発の現状を概説する。

人工血小板の種類

人工血小板(artificial platelets/ 広義)は二つに大別される(**Fig. 1**)。第一は血小板由来産物で，凍結乾燥処理した固定化ヒト血小板(fixed, lyophilized platelet, Stasix™)そのものや，あるいはすでに初期臨床研究に供された血小板膜断片(infusible platelet membrane; IPM, Cyplex™)がある。第二は狭義の人工血小板で，生体適合性のある担体(赤血球，アルブミン微粒子，リン脂質小胞体：リポソームなど)を用い，その表面に血小板受容体のリガンドを結合させたもの(リガンド結合微粒子)で，90年代初めにフィブリノゲンやその合成ペプチド(RGD)をコートした赤血球(thromboerythrocyte)が先駆けとなり，いくつかの微粒子が開発された[1]。まず最初に，ヒトフィブリノゲンを表面固定したアルブミン微粒子(Synthocyte™やFibrinoplate-S™)が初期臨床研究に供された。これらはフィブリノゲンやアルブミンなどの生物由来物質をそのまま使用した生物製剤(biological products)であり，それに続いて，いわば第二世代といえるすべてが人工物で構成された製剤(synthetic products)が開発されてきた。

人工血小板の機能

止血機構における血小板の機能は，止血部位への特異的な粘着，アデノシンニリン酸(ADP)などの刺激物質の放出と細胞の活性化反応，細胞同士の凝集反応による一次血栓の形成，活性化した細胞上での凝固反応の促進(凝固活性)に分けられる。しかし，血小板のすべての機能を代替することは不可能である。したがって，人工血小板は残存した血小板を介してこれらの機能を増強する。

1)血小板由来産物

期限切れとなったヒト血小板を破壊した後，加熱して凍結乾燥した血小板断片製剤(IPM)は止血局所を特異的に認識する血小板受容体GPIb/IXがインタクトに保存されていることに加えて，凝固活性を発現するフォスファチジルコリン等の陰性荷電リン脂質が豊富に表現されており，その血小板代替機能は主にフィブリン血栓の生成

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2011年5月2日受理

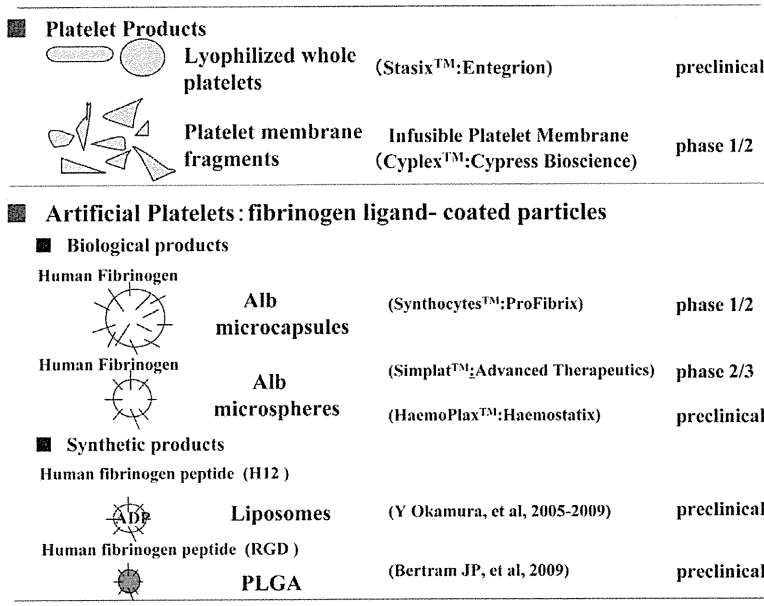

Figure 1 Artificial platelets.
Artificial platelets are classified into platelet products and artificial particles coated with fibrinogen or related peptides. The latter (artificial platelets by narrow definition) are produced using components derived from human or totally synthesized.

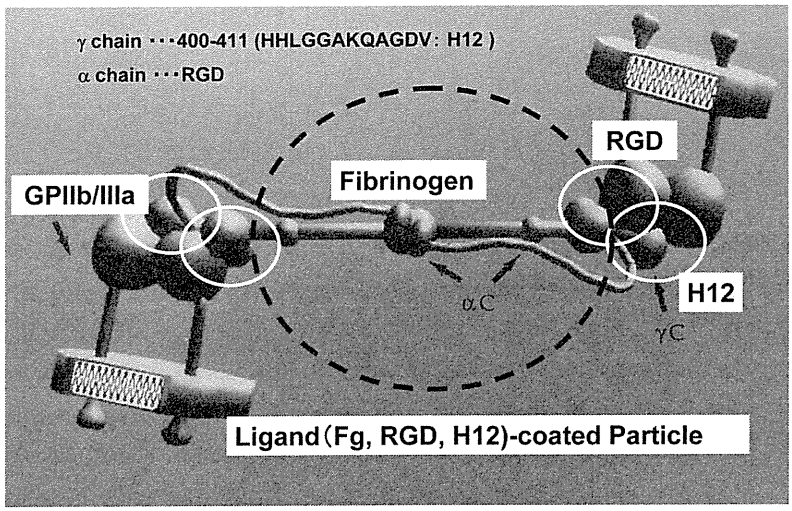

Figure 2 Platelet aggregation and artificial platelets.
Platelet aggregation is induced by bridging adjacent platelets with binding of plasma fibrinogen to activated GPIIb-IIIa complex on the cell surface.[5] The binding sites on the ligand for the receptor are localized at sequence of H12 or RGD. Fibrinogen or the synthetic peptide-coated particles reinforce platelet aggregation by receptor-ligand interactions being more multivalent than fibrinogen itself.

補助に起因するとされる[4]。一方，ヒト血小板をインタクトのままマイルドに固定化し，その後凍結乾燥して粉末状にし，使用時に再水溶化できる製剤(Stasix™)が開発されてきた[2]。本製剤は長い改良の歴史を持ち，特徴は止血局所集積性を体現するGPIb/IXとともにフィブリノゲンの受容体であるGPIIb/IIIaの機能も一部保持されており，IPMと類似の凝固活性を発揮することに加えて，血小板凝集を補助する作用を有すると報告されている。

2)人工血小板(狭義)

血小板凝集は，活性化した血小板膜GPIIb/IIIa複合体(受容体)への結合を介して，血漿中のフィブリノゲン(リガンド)が細胞同士の架橋となって引き起こされる(**Fig. 2**)[5]。フィブリノゲン分子のGPIIb/IIIa複合体との結合部位は，3カ所あり，そのうち2カ所はαサブユニット上のRGD配列に，後の1カ所はγサブユニットのカルボキシ末端を構成する12個のアミノ酸(^{400}HHLGGAKQAGDV^{411}：H12)に限定される。人工血小板は，フィブリノゲンもしくはその合成ペプチドを表面にコートさせた生体適合性を有する微粒子(アルブミン，リポソーム，乳酸・グリコール酸ポリマー)として，フィブリノゲンに代わり残存した血小板の凝集反応を増強するとされる。人工血小板は，その表面にリガンドを高密度に固相化することにより，血漿中のフィブリノゲンでは実現できない多点結合を介した血小板凝集を引き起こ

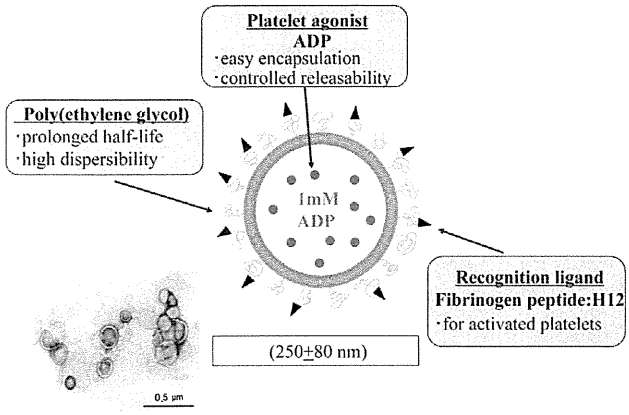

Figure 3　H12-(ADP) liposome.
H12-(ADP) liposome, one of the developing artificial platelets is surface-coated with polyethylene glycol to ensure its blood compatibility and dispersibility. H12 peptide is covalently conjugated to the tip of polyethylene glycol moiety as a target molecule of activated platelets. ADP, a physiologically important platelet agonist stored in platelet alpha granules is stably encapsulated into the inner space of the liposome.

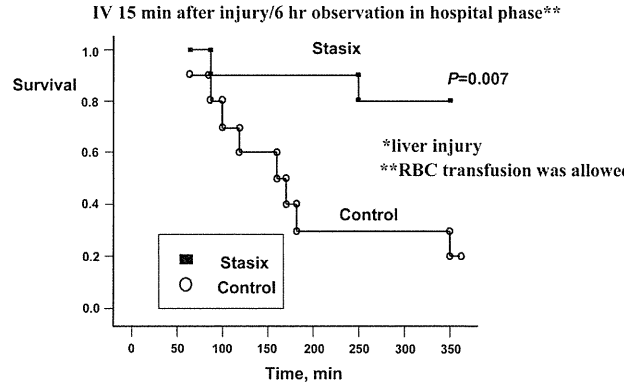

Figure 4　Evaluation of lyophilized fixed platelets (Stasix®) as an infusible hemostatic agent in experimental non-compressible hemorrhages* in swine.[8]
Using a non-compressible liver hemorrhage swine model, the effect of lyophilized fixed platelets (Stasix®) on survival was compared with normal saline. As a result, the survival rate in the Stasix group (80%) was significantly higher than that in the control (20%).

す。さらに，リポソーム製剤では，内水相に加えたADPの血小板凝集に伴う放出機能を付与させた(**Fig. 3**)。

人工血小板の開発状況

1)血小板由来産物

IPMは初期臨床試験に供され，1995年の米国でのフェーズ1臨床研究では，健常人22名への単回投与では急性毒性や免疫原性は認められず，8例の血小板減少患者(血小板数5万/mm³以下)のうち6例(75%)において，出血時間の有意な短縮効果が認められた[6]。1997年の出血症状を有する40例の血小板減少症患者を対象としたフェーズ2試験では，27例(65%)において症状の改善や止血効果等の有効性が示された[7]。しかしながら，その後，理由が明らかにされないまま開発は中止された。

一方，固定化凍結乾燥血小板であるStasixは，その強い凝固活性と極めて短い血中滞留性(半減期：約10分)を活かして，戦場や災害時の緊急避難的な止血剤としての利用が，米国国防省の援助のもと模索されている[8](**Fig. 4**)。

2)人工血小板/アルブミン微粒子

1995年に，平均径が1.2ミクロンのアルブミン・マイクロスフェア(Fibrinoplate-S™)が，1999年には，より大型の平均径3.5～4.5ミクロンのアルブミン・マイクロカプセル(Synthocyte™)が報告され，前者はフェーズIII，後者は少なくともフェーズIIまでの臨床試験が行われた。Fibrinoplate-Sは，63,214人の白血病や再生不良性貧血等の血小板減少患者(血小板数3万/mm³以下)への二重盲検比較対照試験で，出血時間の短縮効果が投与後24時間後でも有意に持続することが報告された(4th Asian Pacific Congress on Thrombosis & Haemostasis, 2006; Advanced Therapeutics社のホームページ)。一方，Synthocyteは，抗がん剤により惹起された血小板減少ウサギに投与することで耳介出血時間の短縮や腹部手術モデルでも術創からの出血量の減少効果が一定時間(3時間)持続し，止血局所への集積も形態的に証明され，有望な前臨床試験結果が公表された[9]。しかしながら，いずれもその後の経過の詳細は公表されておらず，未だ実用化に至っていない。さらに，第3の有望な人工物(HaemoPlax™)が開発されている。このHaemoPlax™は，アルブミン・マイクロスフェアの表面に，ヒトフィブリノゲンと高い親和性を有する合成ペプチドが固相化されたもので，血中に投与すると，その表面にフィブリノゲンが速やかに吸着されることで，上記の人工物と同様の血小板代替機能を発揮する。前臨床試験はすでに終了して，早期の臨床試験への移行を表明しているが，詳細は明らかでない(Haemostatix社ホームページ)。

3)人工血小板/リポソーム

フィブリノゲンのGPIIb/IIIaへの結合部位はそのγ鎖のカルボキシ末端を構成する12個のアミノ酸配列(^{400}HHLGGAKQAGDV^{411}：H12)である(**Fig. 2**)。そこ

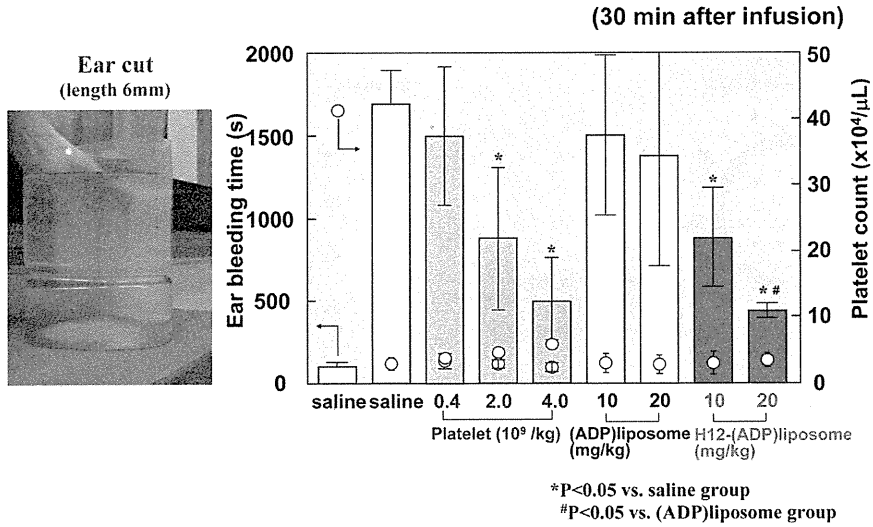

Figure 5 Comparison of hemostatic effects of H12-(ADP) liposomes with those of platelet-rich plasma in severely thrombocytopenic rabbits.[9]
The hemostatic effect of H12-(ADP) liposome was compared with that of platelet rich plasma in severely thrombocytopenic rabbit models induced by busulphan. Ear bleeding time of busulphan-induced thrombocytopenic rabbits was measured 30 min after infusion of platelet preparation or the liposome. Indeed, platelet transfusion effectively corrected the prolonged bleeding time in a dose-dependent fashion. Similar correcting effect was also observed with H12-(ADP)-liposomes in a dose-dependent fashion. The extent of correction by the liposome was just comparable to that obtained by platelet transfusion, indicating that H12-(ADP)-liposome exhibits in vivo hemostatic ability as efficiently as platelet transfusion.

で，表面結合リガンドとしてヒトフィブリノゲンの代わりにH12合成ペプチドを，担体として血液適合性に優れかつすでに臨床応用がなされているリポソームを使用した平均直径250ナノメータの(H12(ADP)リポソーム)が開発された。この微粒子の特徴は，止血作用を強化する目的でADPを内包化させたことである(**Fig. 3**)[10]。血小板凝集を増強するとともに，凝集依存性に内包化されたADPを放出することで，血小板に匹敵する止血効果(出血時間短縮効果)を発揮することが抗がん剤惹起ウサギ血小板減少症モデルで報告された(**Fig. 5**)。H12(ADP)リポソームの利点は，その表面をポリエチレングリコールで修飾することでその血中滞留時間を長く(平均6時間)できることである。したがって，血小板輸血を代替して出血の予防投与への適応が期待されている。

4)人工血小板/乳酸・グリコール酸ポリマー

H12(ADP)リポソームと同様に完全合成型の人工血小板である。フィブリノゲンのGPIIb/IIIaへの結合部位は，血小板に特異的なH12以外に，受容体ファミリーに非特異的に認識されるRGDがあるα鎖に局在する(**Fig. 2**)。この人工物は，生体吸収性に優れて多方面の医療材料に利用されている乳酸/グリコール酸共重合体(poly(lactic-co-glycolic acid)：PLGA)を担体として用い，その表面をPEGで修飾し，その先端にRGD配列を含んだペプチド(GRGDS)を結合させた，粒径およそ150ナノメータの微粒子である。本微粒子は，活性化した血小板にのみ結合して血小板の凝集を増強し，健常ラットの大腿動脈からの出血を陰性対照物に比して有意により短時間で止める機能がある(**Fig. 6**)[10]。しかし，投与量を多くすると血栓傾向が増強されるとされ，また血中半減期も短いことから，止血剤としての適応を目的としているようである。すくなくとも，緊急避難的に止血剤として欧米では標準的に使用されているリコンビナント活性化凝固第VII因子(Novoseven™)をはるかに凌駕する止血効果を示した。

人工血小板の課題

1)適応

人工血小板の適応は，内科的な出血予防を目的とした持続的な使用と外科的な止血を目的とした急性使用に分けられる。予防薬として必須の要件は，効果の持続性で

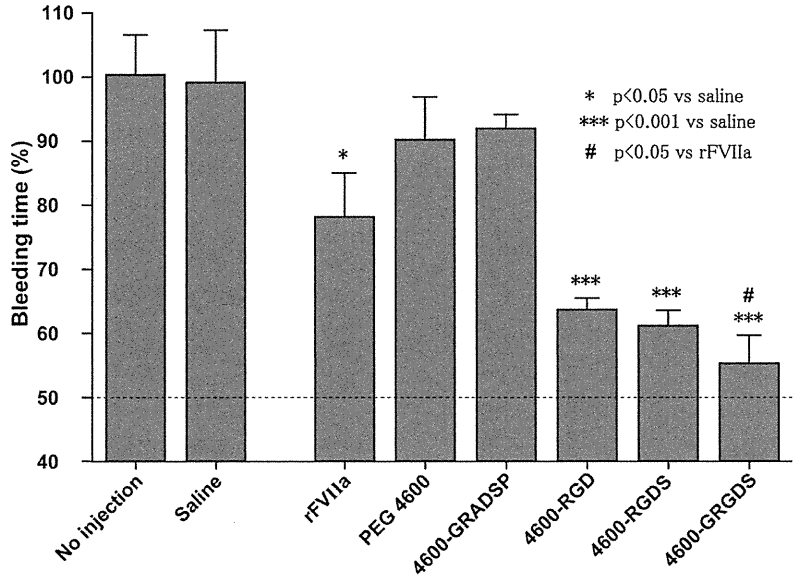

Figure 6　Effect of RGD-PLGA particles on bleeding time in rat femoral artery injury model.[10]
Using a rat femoral artery injury model, the effect of RGD peptide-coated PLGA particles (4600-RGD, 4600-RGDS and 4600-GRGDS) on bleeding time was evaluated by comparison with control treatments (Normal saline: Saline, recombinant coagulation factor VIIa: rFVIIa, RGD-uncoated particles; PEG 4600 and RGD reverse peptide-coated particles: 4600-GRADSP). The hemostatic ability of these RGD-PLGA particles was found to be significant as compared with saline control. In addition, the effect of RGD-PLGA particles was significantly higher than a clinically useful hemostaic agent rFVIIa.

ある。現在開発中の微粒子は血中半減期を延ばすためにその表面をポリエチレングリコールで処理している[9-11]。しかしながら，血小板に匹敵する体内寿命を獲得することは現在の技術では不可能であり，せいぜい一日2〜3回投与が限界である。さらに，生体の異物排除機構が働いて微粒子に対する抗体が惹起されることが危惧される。一方，緊急性の高い止血薬への適応は大いに有望である[8, 11]。血小板数の補正が生死を分ける状況下での緊急使用は意義の高いものである。そして，単回使用のため血中半減期は短くても構わないし，抗体の惹起は気にする必要がない。

2)副作用

人工血小板の働きは残存した血小板を介するものとそのものが血小板の代わりとなる場合がある。いずれの場合も，血栓症が最も危惧される副作用である。実際，Stasixを使用したブタモデルでの検討では心内膜や肺動脈の血栓が1例で観察されており，PLGA微粒子でも高用量の投与で血栓症と思われる心肺症状が高頻度に出現した[8, 11]。いずれの場合でも，血小板数の低下は明らかでなく，試験薬が血小板輸血の代替としての使用でないことを注意すべきである。あくまでも血小板減少症を対象とした人工血小板としての役割を評価すべきであろう。

おわりに

薬剤としての人工血小板は未だに実現されていない。長い歴史があるにもかかわらず，欧米を中心にその開発はベンチャー企業を主体に継続されているためデータがほとんど公表されていない。そのため，本稿では限られた情報に基づいた推測を多く取り混ぜた。実用化に向け，薬剤の開発目的を止血治療に限定するのか，あるいは適応範囲が広く採算性も高い出血予防に拡大するかが今後の鍵となろう。

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Artificial Platelets

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Key words: artificial platelet, activated platelet, fibrinogen, albumin particle, liposome

Artificial platelets are classified into lyophilized products derived from human platelets and fibrinogen or related peptide-coated microparticles made from blood-compatible materials such as albumin. The artificial platelets are substituted for platelets in transfusions for treatment or prevention of bleeding in patients with thrombocytopenia; they reinforce the hemostatic abilities of residual platelets. Although some of the products have been tested in early phase clinical trials, none of them have yet been approved for clinical use.

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INTRAOSSEOUS TRANSFUSION WITH LIPOSOME-ENCAPSULATED HEMOGLOBIN IMPROVES MOUSE SURVIVAL AFTER HYPOHEMOGLOBINEMIC SHOCK WITHOUT SCAVENGING NITRIC OXIDE

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ABSTRACT—Recently, we developed liposome-encapsulated hemoglobin (LEH), a novel cellular hemoglobin-based oxygen carrier. We hypothesized that the LEH effectively suppresses scavenging of nitrogen oxides by sequestering hemoglobin, thereby being useful for resuscitation from hemorrhagic shock, especially in prehospital settings where blood transfusion is not available. However, putting a catheter into the peripheral vessels is sometimes difficult in prehospital resuscitation, because these vessels collapse in patients with hemorrhagic shock. The intraosseous route does not collapse under such conditions. We here studied the resuscitation of severe hypohemoglobinemia following massive hemorrhage using intraosseous (intrafemur) transfusion with LEH in mice. First, we examined the effect of intravenous transfusion with LEH on the resuscitation of mice with fatal hypohemoglobinemia that was made with progressive hemodilution by blood exchanges. Despite a success in initial resuscitation without scavenging of NO_2^- or NO_3^-, LEH transfusion did not significantly improve mouse survival 72 h later as compared with red blood cell (RBC) transfusion. In other experiments, hypohemoglobinemic mice were also made with blood withdrawal and intraosseous infusion with 5% albumin. Thereafter, the mice were rescued with intraosseous transfusion of LEH or RBCs. Unlike intravenous transfusion, intraosseous transfusion with LEH (but not such transfusion with RBCs) significantly increased mouse survival without scavenging of NO_2^- or NO_3^-, presumably because LEH vesicles were much smaller than RBCs, thereby effectively flowing into the circulation from the femur. Thus, intraosseous transfusion with LEH may be a candidate strategy for efficient prehospital resuscitation from hemorrhagic shock.

KEYWORDS—Blood substitute, hemodilution, hemorrhage, resuscitation, NO_2^-, NO_3^-, TNF, erythropoietin

INTRODUCTION

Hemoglobin-based oxygen carriers (HBOCs) might be very useful for resuscitation of patients with fatal hypohemoglobinemic shock, especially in a prehospital setting where conventional blood transfusion is not available. Hemoglobin-based oxygen carriers have many advantages, such as long shelf life, not needing refrigeration and cross-matching, and reducing the risk of iatrogenic infection (1–3). Nevertheless, several clinical trials have reported that resuscitation from hemorrhagic shock using HBOCs does not seem to be very effective (4–7). We believe that one of the reasons for these unfavorable results is that hemoglobin (Hb) molecules of HBOCs might easily bind to NO when released into the vasculature, because the HBOCs used in the clinical trials had no lipid membrane, being termed *cell-free Hb* (8). Natanson et al. (7) have pointed out that this NO scavenging effect might result in systemic vasoconstriction, decreased blood flow, increased proinflammatory mediators and potent vasoconstrictors, and a loss of platelet inactivation, creating conditions that may lead to vascular thrombosis of the heart or other organs. Therefore, development of a novel cellular HBOC that prevents direct contact between Hb and NO is an attractive goal that would limit the effects of NO scavenging.

Liposome-encapsulated Hb (LEH) was developed at the Terumo Research and Development Center (Terumo Co, Tokyo, Japan) (9, 10). Unlike cell-free Hb, LEH has a unique structure, with Hb encapsulated within a lipid bilayer membrane (liposome)—mimicking human red blood cells (RBCs)—and thereby suppressing direct contact between the Hb and NO (11). Although LEH has a lipid membrane similar to that of natural RBCs, the vesicle size of LEH is 220 nm in diameter, which is smaller than a natural RBC (9). Recently, we demonstrated that LEH transfusion is capable of rescuing rats from lethal progressive hemodilution by improving tissue hypoxia (12). In that study, we also demonstrated that LEH transfusion does not decrease the plasma nitrogen oxide levels in rats, suggesting that LEH does not have a potent NO scavenging effect. However, we did not examine the plasma NO_2^- or NO_3^- levels precisely.

In the case of prehospital hemorrhagic shock patients, it is in practice difficult to put a catheter into the peripheral vessels, because these vessels are collapsed from massive blood loss and centralization of circulating blood. Alternative transfusion routes that do not collapse even in hemorrhagic shock are necessary for effective and prompt resuscitation. The intraosseous

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route may provide a useful means of rapidly establishing vascular access, because intraosseous infusion has been a rapid, reliable method of achieving vascular access under emergency condition in children (13–15). Therefore, we have developed the administration of a cellular-type HBOC, LEH, via the intraosseous route, and we here report that intraosseous infusion with the LEH can effectively rescue mice from hypohemoglobinemic shock without scavenging plasma NO_2^- or NO_3^-.

MATERIALS AND METHODS

Animal preparation

This study was conducted according to the guidelines of the Institutional Review Board for the Care of Animal Subjects at the National Defense Medical College, Japan. Male C57BL/6 mice (7 weeks old, 20–23 g; SLC Japan Inc, Hamamatsu, Japan) were studied.

Preparation of LEH, washed mouse RBCs, and 5% albumin solution

The LEH (TRM-645) was prepared at the Terumo Research and Development Center (Terumo Co, Tokyo, Japan). Briefly, purified human Hb solution was prepared from outdated human RBCs provided by the Japanese Red Cross, with inositol hexaphosphate as an allosteric effector, nicotinamide adenine dinucleotide as a coenzyme, and glucose, adenine, and inosine as substrates. After washing, human RBCs were hemolyzed with simultaneous virus inactivation. Subsequently, Hb was concentrated using a reverse osmosis membrane and sterilized. Thereafter, the purified Hb was adjusted to 40 to 50 mmHg of P_{50} (the oxygen partial pressure at which Hb is half saturated with oxygen) by adding inositol hexaphosphate. After adjusting the P_{50}, purified Hb was encapsulated with lipid ingredients through the use of high-speed emulsification. The surface of the encapsulating lipid membrane was then modified with 5-kd polyethylene glycol. The LEH was diluted with saline to achieve a final Hb concentration of 6 g/dL and then deoxygenated with N_2 bubbling for storage. The diameter of the LEH was approximately 220 nm. The total lipid concentration of the LEH solution was 3.9 g/dL, with the methemoglobin proportion at 6.3%. The content of lipopolysaccharide was less than 0.1 EU/mL (9, 10).

To prepare washed mouse RBCs, blood was taken from nontreated mice under ether anesthesia and added to citrate phosphate dextrose solution, which contained 2.63 g sodium citrate, 2.32 g glucose, 327 mg citric acid, and 251 mg sodium biphosphate per 100 mL.

The preservation time was less than 3 days. Before use, the preserved blood was centrifuged at 3,000*g* for 10 min at 4°C, washed three times with 0.9% saline, and diluted with saline to achieve a final concentration of 6 g/dL because RBC concentration has been determined to set equal oxygen transporting efficiencies for LEH and mouse RBCs (12).

Before use, human serum albumin (HSA; Kaketsuken, Kumamoto, Japan) was added to both the LEH and mouse RBC suspensions to achieve a final albumin concentration of 5 g/dL (colloid osmotic pressures, 20 mmHg) (16). Addition of HSA allows us to exclude osmotic effects during correction of hypovolemia, because precise evaluation is needed of the effects of the reagents on hypohemoglobinemic shock. The colloid infusions are usually more effective than crystalloids in restoring myocardial blood flow and oxygen transport after acute hemorrhage (17). A 5% HSA solution (5 g/dL) was also prepared.

Hypohemoglobinemic shock by blood exchange and intravenous fluid resuscitation

Under ether anesthesia, a plastic catheter was put into the mouse superior vena cava. Thereafter, 0.2 mL of blood was withdrawn, and immediately, isovolumetric 5% albumin was administered via catheter. This blood exchange was repeated until the mice died to establish a lethal hypohemoglobinemic shock model. No mice could survive beyond 8 such blood exchanges. Therefore, after 7 exchanges of blood, the mice received intravenous administration with 0.5 mL of LEH, 5% albumin solution, or washed RBCs.

The effect of additional intravenous LEH or RBC transfusion after first intravenous transfusion

The mice received intravenous administration with 0.5 mL of LEH (n = 20) or washed RBCs (n = 10) 6 h after blood exchange and the first intravenous transfusion.

Intraosseous or intravenous fluid resuscitation following massive hemorrhage

To establish an intraosseous infusion route in mice, the femur was punctured with a 25-gauge needle followed by laparotomy under deep ether anesthesia. Immediately after infusion with indocyanine green (ICG; Daiichi, Tokyo, Japan) via this intraosseous route, the inferior vena cava was stained with ICG (indicated by arrow, Fig. 1), suggesting a rapid flow into the systemic circulation.

To produce hemorrhagic shock in mice, a 27-gauge needle was put into the femoral vein, and 0.8 mL of blood was withdrawn. The mean arterial pressure (MAP) showed approximately 40 mmHg. Subsequently, the femur (opposite side) was punctured with a 25-gauge needle, and then, 1 mL of 5% albumin was administered intraosseously. This intraosseous administration with albumin effectively rescued the subject mice. Five minutes after initial resuscitation, 0.3 mL of blood was additionally withdrawn, and subsequently, 1 mL of LEH, 5% albumin, or washed RBCs was also administered via the intraosseous route or the intravenous route (femoral vein).

Measurement of Hb in mouse blood samples containing LEH and RBC

Hemoglobin concentration in LEH could not be accurately determined, because the liposome capsules interfered with the spectrophotometric measurement

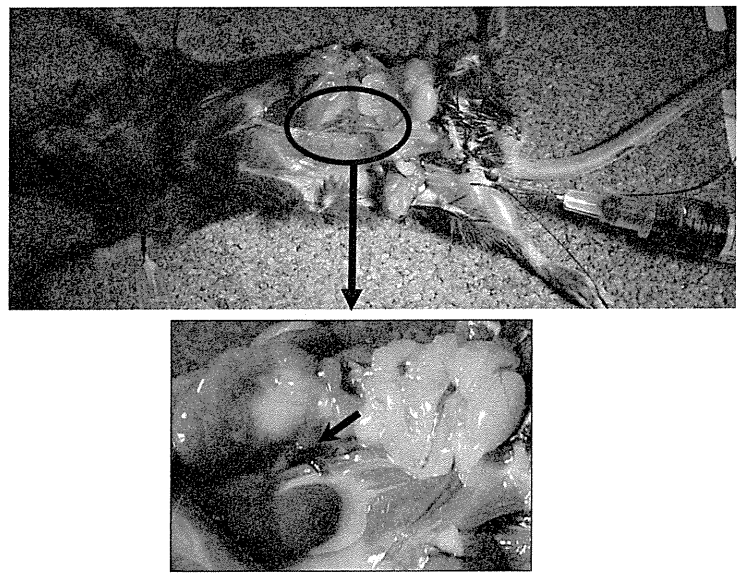

FIG. 1. **Indocyanine green was administered by intraosseous infusion via the right femur.** The circle shows the abdominal inferior vena cava, which was stained with ICG (arrow).