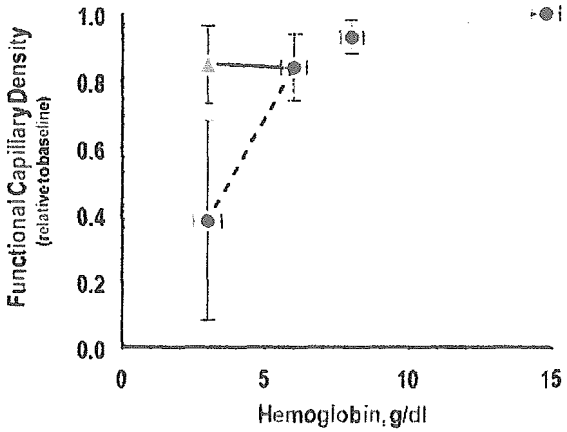

Figure 2 Changes of FCD following isovolemic hemodilution with low- and high-viscosity plasma expanders. ● Hemodilution with dextran 70 kDa maintains FCD up to a hematocrit (hemoglobin) that is 40 percent of normal. Further hemodilution with the same low-viscosity diluent causes the fall of FCD to pathologically low levels. ▲ Continuation of hemodilution with dextran 500 kDa after reaching 40 percent of normal hemoglobin with hemodilution with dextran 70. FCD is maintained to normal levels by the increased plasma viscosity. Redrawn from Tsai et al. [2].

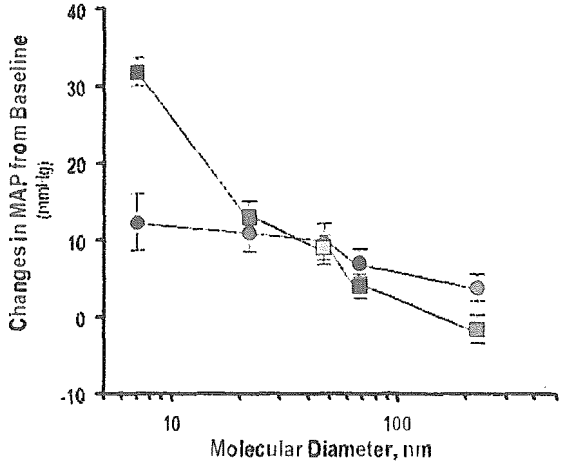

Figure 3 Changes in mean arterial blood pressure after a 5 percent by volume (5 Hb g/dL) topload infusion of free hemoglobin solutions of different molecular diameters and vesicle encapsulated hemoglobin. ■ Pressor effect after infusion. ● Pressor effect 3 hours after infusion. Redrawn from Sakai et al. [5]. (see color insert)

vasoactive properties of the hemoglobin molecule because they cause a significant decrease in blood viscosity after reaching the transfusion trigger. An additional factor attendant to the restoration of blood volume upon reaching the transfusion trigger with a plasma-like viscosity fluid is that this process brings the organism to near extreme hemodilution conditions, characterized by decreased shear stress on the endothelium, lowering the production of endothelial-derived vasodilators. Increasing plasma viscosity to about 2.0 to 2.5 cP increases shear stress and the production of vasodilators, which breaks up the vicious circle caused by extreme hemodilution, compensatory vasoconstriction and low viscosity.

Experimental results shown in Figure 2 show that the maintenance of FCD is not directly linked to oxygen delivery, but to mechanical factors related to the viscosity of the perfusion fluid and the production of vasodilators by mechanotransduction in the endothelium. Therefore an acellular oxygen carrier should maintain plasma viscosity above a specific threshold, while ensuring that overall blood viscosity does not exceed normal values.

Low blood viscosity can be compensated for by hemoglobin solutions with high viscosity. This can be achieved by mixing the hemoglobin molecule with a viscogenic material such as hydroxyethyl starch (HES) at suitable concentrations, or by modifying the hemoglobin molecule to produce an intrinsically viscous solution by increasing its molecular dimension. This latter process can be implemented by polymerization or conjugation with various molecules such as starch and polyethylene glycol, as described by Sakai et al. [5], who showed that the pressor effect is inversely related to molecular size (Figure 3). Hemoglobin molecules with these and several other beneficial features are polyethylene glycol conjugated hemoglobin molecules [6].

The Vasoconstrictive Effect of Hemoglobin

Natural hemoglobin molecules are presumed to be vasoconstrictive because of their ability to scavenge NO. However, recent experimental evidence shows that whereas NO binding is virtually identical for most hemoglobin molecules [7], the vasoconstrictive effect is not, being essentially absent in polyethylene glycol modified hemoglobins and in some very large hemoglobin polymers.

NO is produced by the endothelium as a result of shear stress and other processes. The affinity of the hemoglobin molecule for NO is due to the physical similarity between NO and O_2. Thus in general, hemoglobins with high affinity for O_2 generally also have a high affinity for NO, and vice versa. The production of genetically modified hemoglobins that appear to have little affinity for NO, while maintaining a normal affinity for O_2, may challenge this generalization; however, the fact remains that interfering with NO production with administration of L-arginine methyl ester hydrochloride (L-NAME) or scavenging NO with cell free unmodified hemoglobin causes the constriction of aortic rings, and an increase in blood pressure in experimental subjects.

The concept that hemoglobin extravasation and its location between the endothelium and smooth muscle is the principal factor causing hypertension and vasoconstriction is also questionable because the extravasated molecule will eventually saturate. In fact the presence of a NO-avid mole-

cule in plasma is sufficient to distort the diffusion field of NO from the endothelium, whereby hemoglobin does not need to extravasate to be vasoconstrictive.

NO scavenging does not provide a consistent explanation for the pressor effect of free hemoglobin in the circulation that is applicable to the different hemoglobin modifications. The lack of correlation between pressor responses and NO scavenging characteristic of hemoglobin molecules led McCarthy et al. [8] to propose that hypertension following the introduction of molecular hemoglobin in the circulation is caused by a mechanism related to the process of facilitated diffusion of oxyhemoglobin. According to this hypothesis the presence of molecular hemoglobin causes an additional flux of oxygen in the plasma layer due to the diffusion of oxyhemoglobin. Although the diffusion constant of hemoglobin is low, the amount of oxygen carried is large because hemoglobin binds a large amount of oxygen. The net result of this process is that a comparatively small concentration of molecular hemoglobin augments oxygen transfer to the vessel wall, leading to a hyperoxia signal, and consequently a vasoconstrictive response.

In vivo, peripheral vascular resistance is autoregulated at the level of the arterioles by a mechanism that senses oxygen tension, producing vasodilatory signals when blood and tissue pO_2 is low, and vice versa. This conceptualization is supported by the finding that large hemoglobin molecules are not vasoactive, although they carry oxygen. As an example, poly (ethylene glycol) (PEG) surface decorated hemoglobins (PEG-Hb) have consistently been shown to be vasoinactive. These molecules have a large volume because of the water bound by PEG. Since the diffusion constant is inversely proportional to molecular radius, it can be shown that PEG-Hb has a diffusion constant that is about one fifth that of the native hemoglobin.

Experimentation with different levels of hemoglobin surface decoration show that vasoinactivity may be partially related to the degree to which the surface of the hemoglobin molecule is shielded by the water–PEG combination [6]. This phenomenon suggests that free hemoglobin may also cause a pharmacological effect mediated at the surface of the endothelium, and that conjugation of hemoglobin with PEG may produce a shield that prevents this process.

The vasoconstrictive effects of molecular hemoglobin may have several components that sometimes reinforce each other. When blood viscosity becomes too low, there is a reflex vasoconstriction that attempts to maintain perfusion pressure, a phenomenon independent of blood oxygen-carrying capacity. Oxygen regulation plays a crucial role since the arteriolar walls and the tissue sense both the rate of oxygen delivery from the red blood cell column and local pO_2. When molecular hemoglobin is present in plasma, there is a significant additional flux of oxygen to the arteriolar wall by facilitated diffusion, a process enhanced with right-shifted oxygen dissociation hemoglobin molecules. NO scavenging can also be a factor that may be balanced by increased NO (and/or prostacyclin) production resulting from elevated shear stress caused by high-viscosity hemoglobin molecules. Furthermore, considering that modest amounts of small molecular hemoglobin can elicit a pressor response, a pharmacological effect due to "naked" small hemoglobin molecules in the circulation may also be present.

Vasoconstriction limits perfusion and decreases FCD. Although healthy organisms could probably compensate for moderate hypertensive episodes leading to corresponding decreases in FCD, these same episodes may place the organism in jeopardy if they are superposed to other vasoconstrictive stimuli, such as those inherent to hemorrhagic shock. Conversely, high plasma viscosity is critical in resuscitation, as an OCPE is administered in conditions of extreme hemodilution because there is no need for using these products prior to reaching the transfusion trigger.

Optimal Oxygen Disassociation Properties

The development of oxygen carriers has implicitly assumed that the oxygen dissociation curve should be right shifted, thus facilitating the release of oxygen. This approach does not consider the longitudinal gradient of oxygen tension in the circulation, whereby a right-shifted dissociation curve favors oxygen unloading from small arteries and arterioles. Hemodilution with hemoglobin-filled vesicles of different p50 in the hamster window chamber model has shown that improved tissue oxygenation is obtained when this parameter is 16 mmHg, instead of 34 mmHg (Department of Polymer Chemistry, Waseda University, Tokyo, Japan). PEG-conjugated hemoglobin (Hemospan, 4% Mal-PEG hemoglobin) produced by Sangart (San Diego, CA), with a p50 of 5 mmHg, used at low concentration in hemodilution maintains FCD and positive acid–base balance.

This apparent paradox may be understood by analyzing the distribution of oxygen in the microcirculation as shown in Figure 4, where oxygen tension in the microcirculation in normal conditions has a baseline tissue pO_2 level of 22 to 24 mmHg (which appears to be common for most tissues). It is notable that although oxygen is regulated to achieve this partial pressure in the tissue, anaerobic metabolism occurs when tissue pO_2 is below 2.4 to 2.9 mmHg.

A possible rationale for the high pO_2 tissue regulation is that the organism has excess oxygen-carrying capacity, not only as a requirement for extreme efforts, but also for compensation of oxygen delivery inhomogeneity in the microcirculation. The effect of this inhomogeneity becomes apparent in considering the variability of oxygen partial pressure distribution in the hamster window chamber, which is of the order of $\pm$ 4 mmHg. This variability is a consequence of the quasirandom distribution of the transport properties of the microcirculation, and therefore intrinsic to any level of tissue oxygenation. In conditions of extreme hemodilution tissue pO_2 decreases to 3 to 5 mmHg; thus, if

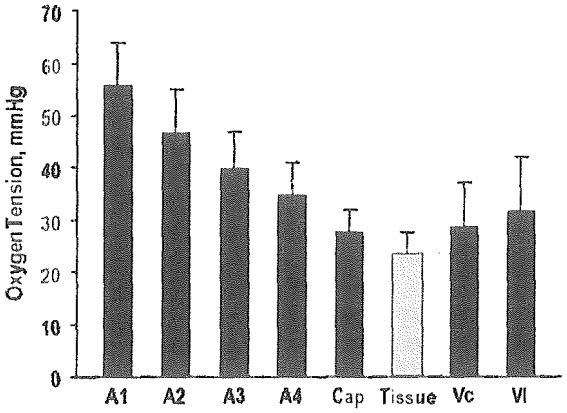

Figure 4 Distribution of pO_2 in the microvessel of the microcirculation of the hamster window preparation as a function of the arteriolar order of branching (As) and the venular order of branching (Vs). It is apparent that tissue pO_2 is narrowly regulated to a value in the range of 22 to 24 mmHg, which is significantly higher than the level associated with anaerobic metabolism.

the same variability is present, there is a significant amount of tissue that is anoxic. In the presence of a fraction of the oxygen-carrying capacity that can only be released at very low pO_2s, a form of targeted oxygen delivery, the effects of this variability will be nullified, ensuring that all the tissue, even at low pO_2 is oxygenated above the anaerobic threshold.

Tissue pO_2 levels that may be considered harmful could, in fact, be quite safe if it were possible to eliminate the inherent variability of oxygen delivery shown by the variability of tissue pO_2. A small quantity of a low-p50 hemoglobin oxygen carrier in the circulation accomplishes this because it delivers oxygen only to portions of the tissue where the anoxic threshold is passed, while the presence of even significant amounts of right-shifted hemoglobin would have no effect since most of the bound oxygen would be unloaded in oxygenated regions.

Cross-linked or polymerized hemoglobins developed so far have a high p50, presumed to be beneficial since it facilitates oxygen unloading. However, pO_2 in the microcirculation is regulated so that there is a significant decrease in oxygen tension from the systemic circulation to the capillaries, which typically have a pO_2 of about 30 mmHg. At this p50 half of the blood oxygen is delivered by arterioles in normal conditions; however, if the p50 of the OCPEs is above this value, as in the case of Oxyglobin (p50 = 54 mmHg), most of the oxygen in the blood should be delivered by the arterioles if this material were to replace blood. These vessels extract a significant amount of oxygen from the circulation while consuming a major portion of this oxygen flux, thus increasing their oxygen supply increases tissue oxygen inhomogeneity, which is further aggravated by the vasoconstrictor autoregulatory response already discussed.

Oxygen-Carrying Capacity

Measurements of pO_2 in the microcirculation utilizing the technique of phosphorescence oxygen quenching show that when hemodilution carried out to a total hemoglobin content in red blood cells of 5.6 g/dL, then tissue oxygen is somewhat higher than normal but not statistically significant. The required oxygen-carrying capacity can also be obtained by a simple calculation that relates the whole-body oxygen consumption and cardiac output, which yields a nearly identical number for the organism at rest. Therefore, in principle, the oxygen-carrying capacity of an OCPE does not need to reproduce the value for normal blood and can be significantly lower.

Colloid Osmotic Pressure

It is generally assumed that a blood substitute should have a colloid osmotic pressure similar to that of blood and in the range of 20 to 25 mmHg; however, several plasma expanders have zero colloid osmotic pressure (saline, Ringer's lactate) and small-volume resuscitation utilizes fluids with very high osmotic properties. To date there is no definitive answer on what is the osmotic and/or oncotic property that is most appropriate, and in all probability this is a variable that depends on the type of blood loss to be corrected. Resuscitation with noncolloidal fluids leads to tissue edema. Conversely fluids with high colloidal and osmotic pressures cause tissue fluid to come into the vascular compartment, thus decreasing the amount of fluid to be administered. Most conditions of hemorrhage are associated with endothelial edema, which has been demonstrated to be rapidly reversed upon the introduction of hyperosmotic and hyperoncotic fluids. Volume expansion fluids such as hydroxyethyl starch have relative high colloid osmotic pressures, typically in the range of 30 to 50 mmHg depending on formulation. Small molecule hemoglobin–based OCPEs have their oncotic pressure adjusted to be that of plasma, but PEG-hemoglobin modified OCPEs tend to have higher oncotic pressures.

Synthesis of an Effective Oxygen-Carrying Plasma Expander

An OCPE based on the preceding concepts is a fluid with properties fundamentally different from those of blood, since it has low oxygen-carrying capacity, p50 is low and in the neighborhood of 5 mmHg, viscogenic properties are such that when introduced into the circulation plasma viscosity should be of the order of 2.0 to 2.5 cP, and colloidal osmotic pressure can be high. A fluid with these properties can be obtained by conjugating hemoglobin with PEG, and various formulations have been tested in both animal experiments and human trials with excellent results. Notably this

formulation is vasoinactive, and its NO-scavenging characteristics do not appear to be relevant since these fluids have the same NO binding constant as other vasoactive formulations that are vasoactive [7].

These fluids are in some cases more effective than blood because they are designed to maintain FCD, which is as necessary as restoring tissue oxygenation for the recovery from blood losses. Because in the foreseeable future OCPEs will use human hemoglobin, these fluids are practical: Their hemoglobin content is low, and more than two units of blood equivalent unit of resuscitation fluid can be obtained from one unit of blood. Finally, this low oxygen-carrying capacity is practical and safe because it yields a significant improvement of microvascular function.

Experimental Evidence

The effectiveness of different resuscitation modalities was tested experimentally in studies of extreme hemodilution and hemorrhagic shock in the microcirculation of the hamster chamber window model, which allows microcirculatory monitoring in the awake condition for a period up to 1 week, after the effects of the surgical intervention have subsided. Extreme hemodilution was chosen because in most instances, lowering systemic hematocrit to 50 percent of baseline with a suitable plasma expander does not alter microvascular hemodynamics and transport in our experimental model. Animals were hemodiluted to 60 percent of normal with dextran 70 kDa, and further hemodiluted to a final hematocrit of 11 percent using the different products simulating blood losses initially remedied with conventional plasma expanders, which upon passing the transfusion trigger are corrected with an oxygen-carrying blood substitute.

A compendium of findings in extreme hemodilution to 50 percent of normal with dextran 70 kDa and further hemodilution to a final hematocrit of 11 percent with the different products is shown in Figure 5, including results obtained with PEG-Hb vesicles developed at Waseda University, Tokyo, using a somewhat different protocol where extreme hemodilution was achieved with a continuous exchange of a hemoglobin vesicle suspension. FCD is shown as a function of blood base excess, which represents systemic conditions and suggests the definition of *critical functional capillary density* as the value for this parameter at which base excess is no longer sustained and drops following modest reductions of total blood hemoglobin, that is, in the neighborhood of a 50 percent FCD reduction. The most important result is that normal base excess is obtained with total blood hemoglobin of 5 percent, if 1 percent of this is Mal-PEG-Hb—a result not found with other OCPEs.

Extreme hemodilution is not a clinically relevant procedure and serves only to study basic mechanisms. A clinically relevant test is to rescue a subject in hemorrhagic shock. Studies were therefore conducted to determine the effects of resuscitation with blood, starch, and Mal-PEG-Hb in a conventional 50 percent bleed shock protocol. The animals were resuscitated after 1 hour without any additional volume manipulation using shed blood, HES, and Mal-PEG-Hb with 25 percent of the blood volume. The results, shown in Figure 6, indicate that Mal-PEG-Hb is superior to both HES and blood in reestablishing microvascular function. Concurrently it was found that base excess was higher in the Mal-PEG hemoglobin-resuscitated animals than in the blood-resuscitated animals. An explanation for these findings is that low p50 hemoglobin targets oxygen delivery of oxygen to only the anoxic tissue.

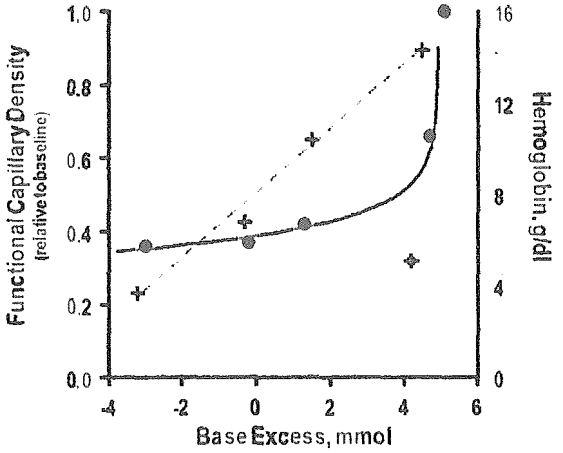

Figure 5 Relationship between total circulating hemoglobin and base excess, and FCD and base excess, for different hemoglobin modifications and concentrations, including hemoglobin vesicles, in normovolemic hemodilution experiments. The data marked ● shows the relationship between FCD and base excess, showing that MAL-Peg-Hb (▼) yields high FCD and base excess at low hemoglobin concentrations. It is apparent that base excess is a direct function of hemoglobin concentration (+) with the exception of MAL-Peg-Hb (✥), which presents normal base excess at a very low total hemoglobin content. (see color insert)

An extreme hemorrhage study was performed with Mal-PEG-Hb in which rats were 50 percent exchange transfused before hemorrhage with either αα-cross-linked hemoglobin, or 4 percent Mal-PEG hemoglobin (Figure 7). These animals were then subjected to a continuous exponential bleed (1 hour, 60 percent of blood volume) whereby at the end of the second hour after the start of bleeding 50 percent of the control animals succumbed. In these experiments it was found that at the end of one hour all animals that received Mal-PEG hemoglobin before hemorrhage survived, while all of those receiving αα-cross-linked hemoglobin did not survive.

Summary and Conclusions

The revision of microvascular physiology related to modifying basic transport properties of blood such as plasma viscosity, p50, and hemoglobin concentration shows

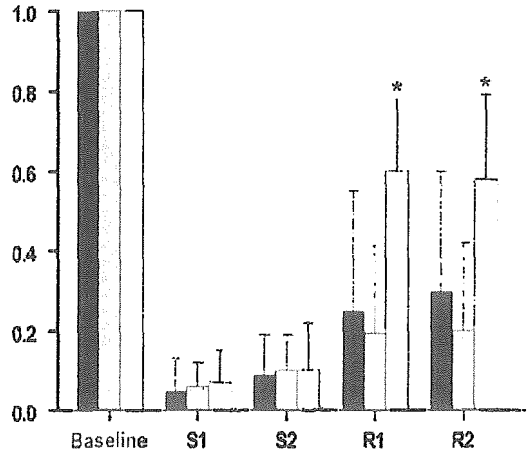

Figure 6 Recovery of FCD during resuscitation from 1 hour hemorrhagic shock with identical volumes of shed blood (*black bars*), 5 percent HES (*shaded bars*), and 4 percent MAL-Peg-Hb (*white bars*). S1 and S2 initial and final conditions during the shock period. R1, Recovery immediately after resuscitation; R2, 1 hour after resuscitation. $p < 0.05$ relative to shed blood and HES.

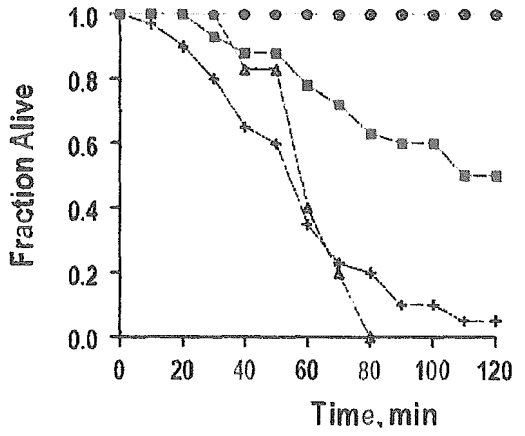

Figure 7 Controlled bleeding in rats that are 50 percent exchange transfused with MAL-Peg-Hb (●), αα-cross-linked hemoglobin (▲), and a polymerized hemoglobin (+), versus controls (■) with no treatment. The study was designed so that 50 percent of the untreated (not transfused controls) would survive 120 minutes.

that blood or a bloodlike fluid may not be the optimal oxygen-carrying volume-restoring fluid. A critical parameter for either oxygen-carrying or noncarrying blood replacements is their viscosity, which is a factor in maintaining capillary flow.

Analysis of the microvascular consequences of changing blood rheological conditions and particularly plasma shows that low plasma viscosity is not of universal benefit. Patients following trauma, peripheral arterial occlusive disease, and acute myocardial infarction have elevated plasma viscosity, a condition presumed to be pathological. However, there are situations where increased viscosity may be a protective or beneficial mechanism.

Plasma expanders are not used after reaching the transfusion trigger because the reduction of blood oxygen-carrying capacity beyond this point is assumed to jeopardize tissue oxygenation, according to the systemic evaluation of the organism portrayed by blood gases. Conditions in the microcirculation and local microscopic tissue environment when the reduction of red blood cells is extended beyond the transfusion trigger have not been consistently explored and presently show that oxygen-carrying capacity is not the major factor in determining tissue survival.

Studies show that the transfusion trigger is also the limit for the organism to adapt to low blood viscosity in acute conditions; thus *the conventional transfusion trigger is also a viscosity trigger*. Since the administration of a molecular oxygen carrier is physically similar to continuing fluid therapy after reaching the transfusion trigger, the maintenance of FCD requires the increase of plasma viscosity which through shear stress–dependent mechanisms operating in the endothelium ensures the maintenance of optimal microvascular function. Oxygen-carrying capacity is exhausted upon red blood cell (or hemoglobin) losses that are significantly greater than those represented by the transfusion trigger. However, these losses of oxygen-carrying capacity do not need to be compensated on a one-to-one basis, if microvascular function (i.e., FCD) is maintained and an oxygen carrier is introduced only to deliver oxygen to anoxic tissue regions. This approach ensures a uniform maintenance of the whole organism above the anaerobic threshold, while limiting the amount of oxygen carrier needed to maintain metabolism. Thus the combination of maintenance of microvascular function and targeted oxygen delivery is the primary determinant of an efficacious human hemoglobin-based blood substitute that is more effective than blood in acute conditions and that also expands the available blood supply, since a unit of blood yields more than two units of surrogate blood.

Glossary

Functional capillary density: Number of capillaries in a unit volume of tissue that presents the passage of red blood cells. This parameter is experimentally determined by measuring the length of red blood cell–perfused capillaries in a microscopic field of view.

Microvascular function: A combination of parameter including flow, number of open capillaries, intact vascular permeability, and level of vessels tone that allows for the proper interaction between blood and tissue at the microscopic level.

Oxygen-carrying capacity: The amount of oxygen in milliliters at standard atmospheric conditions and temperature contained in a fluid.

p50: Partial pressure of oxygen at which hemoglobin is 50 percent saturated with oxygen.

Plasma expander: A fluid used to restore circulatory volume when oxygen-carrying capacity is adequate.

Transfusion trigger: Level of blood hemoglobin at which the decision is made to introduce red blood cells into the circulation in order to restore oxygen-carrying capacity.

Vasoactivity: Inherent property of compounds that cause vasoconstriction and the elevation of systemic blood pressure.

Acknowledgments

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Biographies

Amy G. Tsai obtained her Ph.D. in bioengineering at the University of California, San Diego, where she is currently a Senior Research Scientist. She is widely recognized for her findings on oxygen consumption by the microvasculature and the development of high-viscosity plasma expanders. She is an expert in mathematical modeling, measuring methods for the in vivo study of the microcirculation and small animal experimentation.

Dr. Pedro Cabrales received his Ph.D. from the Universidad de los Andes, Bogotá, Colombia, studying the microvascular effects of extreme hemodilution with perfluorocarbons. He specializes in hemodynamic transport phenomena, having developed techniques for the analysis of tissue oxygenation at the microscopic level. He is presently at the Laboratory of Microhemodynamics of the University if California, San Diego.

Dr. Hiromi Sakai received his Ph.D. in polymer chemistry from Waseda University, Tokyo, Japan, where he is now Associate Professor. He specialized in the synthesis and characterization of oxygen carriers from the viewpoint of molecular assembly. For several years he was a visiting scholar at the University of California, San Diego, where he developed expertise in microhemodynamics. He is currently working on the optimization of oxygen carriers using in vivo methods in order to determine their safety and efficacy.

Prof. Marcos Intaglietta received his Ph.D. in applied mechanics from the California Institute of Technology in Pasadena and developed his academic career at the University of California, San Diego, where he is one of the founders of the bioengineering program and department. His specialty is the study of transport phenomena in the microcirculation and the development of blood substitutes. He has developed and implemented most of the methods presently used for the study of the microcirculation.

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Performances of PEG-modified hemoglobin-vesicles as artificial oxygen carriers in microcirculation

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Abstract. Hemoglobin-Vesicles (HbV; diameter, 250 nm) are artificial O_2 carriers encapsulating purified and concentrated human Hb solution in phospholipid vesicles (liposomes), and their safety and efficacy, as a transfusion alternative, have been studied. In this paper, we summarized the characteristics of HbV that have been clarified by the microcirculatory observations.
Keywords: Blood substitutes, liposome, microcirculation, EDRF, oxygenation

1. Introduction

Hemoglobin (Hb)-based O_2 carriers (HBOCs) have been developed for use as a transfusion alternative and some of them are now in the process of clinical trials [1]. The advantages of the HBOCs are the absence of blood-type antigenicity and infectious pathogens, and stability for long-term storage when compared with the RBC transfusion [2–4]. A phospholipid vesicle or liposome encapsulating concentrated human Hb (Hb-vesicle, HbV) has been developed as an O_2 carrier [2,5–9]. The cellular structure of the HbV (particle diameter, ca. 250 nm) has characteristics similar to those of natural RBCs, since both have lipid bilayer membranes that prevent the direct contact of Hb with the components of blood and the endothelial lining [10]. The reasons for the Hb encapsulation in RBCs should be: (1) a decrease in the high viscosity of Hb and a high colloidal osmotic pressure; (2) prevention of the removal of hemoglobin from the blood circulation; and (3) preservation of the chemical environment in the cells such as the concentration of phosphates (2,3-DPG, ATP, etc.) and other electrolytes. Moreover, during the long history of the development of HBOCs, many side effects of molecular Hb have become apparent. These side effects of molecular Hb would imply the importance of the cellular structure.

Our *in vivo* studies of HbV have revealed the sufficient O_2 transporting efficiency comparable to RBCs [11–14], the safety in terms of blood compatibility [15], and prompt degradation in the reticuloendothelial system [16–19], all of which make us confident about advancing to the further development of HbV.

In this paper, we focus on the performances of our polyethylene-glycol (PEG)-modified HbV from the viewpoint of hemorheology and microcirculation.

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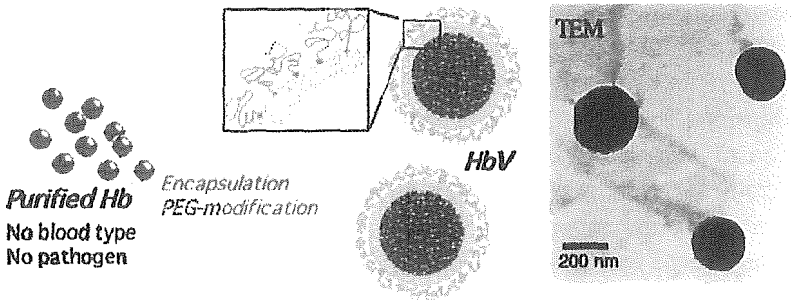

Fig. 1. Hemoglobin-vesicles (HbV) encapsulate the ultrapurified and concentrated human Hb solution (35 g/dl) with phospholipid bilayer membrane, and the surface is modified with polyethylene glycol chains. The well-regulated particle size (about 250 nm) was confirmed by TEM. One particle contains about 30,000 Hb molecules and about 1500 PEG chains were fixed on the surface.

2. Impact of PEG-modification of HbV

The rheological property of an HBOC is important because the infusion amount should be significantly large and that may affect the blood viscosity and hemodynamics. One HbV contains about 30,000 Hb molecules so that the suspension of HbV does not have colloid osmotic pressure (COP) (Fig. 1). The HbV suspended in 5 g/dl human serum albumin (HSA) at [Hb] = 10 g/dl shows comparable COP and viscosity to the blood.

We tested he function of PEG-modified and unmodified HbV as a blood replacement in the subcutaneous microvasculature of awake hamsters during severe hemodilution in which 80% of the red blood cell mass (70 ml/kg) was substituted with suspensions of the vesicles in 5% HSA solution [20,21]. Both materials yielded normal mean arterial pressure, heart rate, and blood gas parameters, which could not be achieved with albumin alone. Subcutaneous microvascular studies showed that PEG-modified HbV/HSA significantly improved microhemodynamic conditions (flow rate, functional capillary density, vessel diameter, and O_2 tension) relative to unmodified HbV/HSA. PEG-modified HbV was homogeneously dispersed in the plasma phase while the unmodified HbV showed aggregation in venules and capillaries. Even though it was confirmed *in vitro* that the aggregates dissociated reversibly at higher shear rates, it is unlikely that they would dissociate in vessels where the flow rate or shear stress was low. Aggregation and decreased flow rate may constitute a vicious circle that reinforces negative effects on blood flow. PEG reduced vesicular aggregation and viscosity, improving microvascular perfusion relative to the unmodified type. From this result, PEG modification is important for HbV in microvascular blood flow.

3. Interaction with NO and CO

As clinical trials of the chemically modified Hbs are extended to include larger numbers of individuals, it becomes apparent that the principal side effect consistently reported in the administration of acellular Hb solutions is hypertension presumably because of vasoconstriction. Hypertension, a well-defined reaction of the acellular intramolecularly cross-linked Hb (XLHb), was proposed to be beneficial in the treatment of hypotension concomitant to hemorrhagic shock [22]. However, vasoconstriction reduces blood flow, lowering functional capillary density, and therefore affecting tissue perfusion and oxygenation [23,24]. Nitric oxide (NO) scavenging by Hb due to intrinsic high affinity of NO to Hb is the mechanism presumed to cause vasoconstriction and hypertension [25,26].

We analyzed the relationship between the constriction of resistance vessel and hypertension after administration of acellular Hb and the extent to which the effect is dependent on the size of acellular Hb molecules modified by polymerization, polymer conjugation, and cellular liposome encapsulation [8,27]. Conscious Syrian golden hamsters with dorsal skinfold preparation were used. After the top load infusion of Hb products (7 ml/kg) into arterial catheter into jugular vein, mean arterial pressure, and heart rate were monitored through jugular arterial catheter, and microvascular responses were monitored by an intravital microscopy. The Hb products included intra-molecularly crosslinked Hb (XLHb), PEG-conjugated pyridoxalated Hb (PEG-PLP-Hb), hydroxyethylstarch-conjugated XLHb (HES-XLHb), glutaraldehyde-polymerized XLHb (Poly-XLHb) and HbV. Their molecular diameters were 7, 22, 68 and 224 nm, respectively. The top load infusion of 7 ml/kg of XLHb (5 g/dl) caused the immediate increase of MAP, which was 34 $\pm$ 13 mmHg higher 3 hrs after infusion. There was a simultaneous decrease in diameter of A_0 vessels (79 $\pm$ 8% of basal value), which caused blood flow to decrease throughout the microvascular network. The diameter of smaller arterioles did not change significantly. Infusion of HBOCs of greater molecular size resulted in lesser vasoconstriction and hypertension with HbV showing the smallest changes. Infusion of HSA was used as control and produced no microvascular or systemic effects. Constriction of resistance arteries was found to be correlated to the level of hypertension, and the responses proportional to the molecular dimensions of HBOCs. Since the results correlate with molecular size it is likely that the effects are related to the diffusion properties of the different hemoglobin molecules.

The liver is a major organ that detoxifies excess amount of heme by the action of heme oxygenase (HO). HO decomposes protoheme IX to generate biliberdin-IXa and CO. Under normal conditions, liver contains at least two OH isozymes for physiologic degradation of the heme: HO-1 and HO-2. One of the important roles of the HO reaction is to generate CO that serves as an endogenous regulator that is necessary for maintaining microvascular blood flow [28]. Since Hb strongly binds with CO (about 200 times stronger than O_2), it is necessary to confirm the effects of HbV in hepatic microcirculation in comparison with stroma free Hb solution. Suematsu et al. studied the perfusion of a rat liver with an acellular Hb solution and HbV, and found out that the Hb solution increased vascular resistance by 30% [29]. The smaller acellular Hb molecules (7 nm) extravasate across the fenestrated endothelium with a pore size of about 100 nm, and reach to the space of Disse. Heme is excessively metabolized by hemeoxygenase-2 to produce CO and bilirubin. Even though CO acts as a vasorelaxation factor in the liver, the excess amount of Hb in the space of Disse rapidly binds CO, resulting in the vasoconstriction and the increase in vascular resistance. On the other hand, Hb-vesicle (250 nm) is large enough to maintain in the sinusoid, and the vascular resistance is maintained.

These results indicate the importance of the size of the oxygen carriers, and the size of HbV is appropriate for the maintenance of microvascular blood flow.

4. Oxygen releasing behavior of HbV and oxygen therapeutics

We measured the O_2 release from HbV perfused through an O_2 permeable fluorinated ethylenepropylene copolymer tube (inner diameter, 28 μm), that was exposed to a deoxygenated environment [30] (Fig. 2). The addition of HbV to RBC did not influence on the O_2-releasing rate. On the other hand, the addition of 50-vol% acellular Hb solution to RBC significantly enhanced the rate of deoxygenation. This outstanding difference in the rate of the O_2 release between the HbV suspension and the acellular Hb solution should mainly be due to the difference in the particle size (250 vs. 7 nm) that affects their

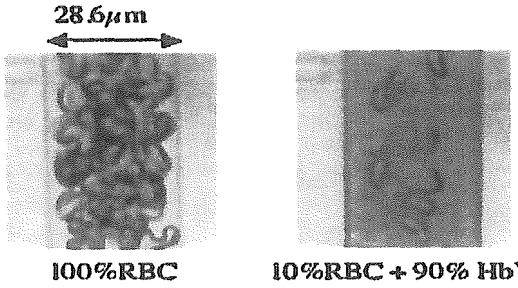

Fig. 2. Flow patterns of RBCs mixed with HbVs suspended in human serum albumin in a narrow tube (diameter, 28.6 μm) [30]. RBCs tended to flow in the centerline, while the HbV particles were homogeneously dispersed in a suspension medium. The individual particles could not be seen at this magnification. However, semitransparent elements were seen in the suspension medium, indicating the presence of HbV. This experimental model, developed by Maeda et al., was used to analyze the O_2 releasing behavior of HbV and RBC. [Hb] = 10 g/dl; centerline flow velocity, 1 mm/s.

diffusion for the facilitated O_2 transport. It has been suggested that the faster O_2 unloading from the HBOCs is advantageous for tissue oxygenation [31]. However, this concept is controversial regarding the recent findings since an excess O_2 supply would cause autoregulatory vasoconstriction and microcirculatory disorders [24,32]. We confirmed that HbV does not induce vasoconstriction and hypertension, due to not only the reduced inactivation of NO as an endothelium-derived vasorelaxation factor, but also possibly the moderate O_2 releasing rate similar to RBC as confirmed in this study.

One characteristic of HbV is that the O_2 affinity (P_{50}) of Hb can be easily regulated by the amount of coencapsulated allosteric effector, pyridoxal 5′-phosphate [21]. It has been clarified by Erni et al. that oxygenation of an ischemic skin flap, where one branch of feeding arteriole was ligated, was improved by infusion of HbV with a high O_2 affinity (low P_{50}) [33,34]. To clarify the underlying mechanism of ischemic tissue oxygenation, we prepared two HbVs with different P_{50}s (8 and 29 mmHg, termed HbV_8 and HbV_{29}, respectively), and observed their O_2 releasing behavior from an occluded arteriole in a hamster skinfold window model [35]. Conscious hamsters received HbV_8 or HbV_{29} at the dose rate of 7 ml/kg bw. In the microscopic view, an arteriole (diameter: 53.0 ± 6.6 μm) was occluded transcutaneously by a glass pipette on a manipulator and the reduction of the intra arteriolar O_2 tension (pO_2) 100 μm down from the occlusion was measured by the phosphorescence quenching of pre-infused Pd-porphyrin. The baseline arteriolar pO_2 (50–52 mmHg) decreased to about 5 mmHg for all the groups. Occlusion after HbV_8 infusion showed slightly slower rate of pO_2 reduction in comparison with that after HbV_{29} infusion. The arteriolar O_2 content was calculated at each reducing pO_2 in combination with the O_2 equilibrium curves of HbVs, and it was clarified that HbV_8 showed significantly slower rate of O_2 release in comparison with HbV_{29} and was a primary source of O_2 (maximum fraction, 0.55) overwhelming RBCs when the pO_2 was reduced (e.g., <10 mmHg) in spite of a small dosage of HbV.

Accordingly, the result of improved oxygenation of the ischemic skin flap, observed by Erni et al., could be explained by low P_{50} HbVs retaining O_2 in the upstream vessels and delivering it to the ischemic tissue via collateral arterioles, even when these may have significantly slower blood flow. Moreover, an advantage of small HBOCs including HbV is that they are homogeneously dispersed in the plasma phase and therefore can deliver O_2 more homogeneously to the periphery than RBCs because microvascular Hct is heterogeneous particularly in pathological states. In such conditions HbV with a higher O_2 affinity (lower P_{50}) should show a slower O_2 unloading which would be effective for oxygenating ischemic tissues. This result supports the possible utilization of HBOCs with lower P_{50} for oxygenation of ischemic tissues.

In summary, observation of microcirculation is important for the development of HBOCs because it is the site where oxygen is unloaded to the target tissues. From the international collaborative evaluation studies of HbV, we have clarified the rheological property, advantages of the cellular structure, and the performances of HbV not only as a transfusion alternative but also for oxygen therapeutics.

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Acute 40 percent exchange-transfusion with hemoglobin-vesicles (HbV) suspended in recombinant human serum albumin solution: degradation of HbV and erythropoiesis in a rat spleen for 2 weeks

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BACKGROUND: Hemoglobin-vesicles (HbVs; diameter, 251 ± 81 nm) are artificial O_2 carriers. Their efficacy for acute exchange transfusion has been characterized in animal models. However subsequent profiles of recovery involving the degradation of HbV in the reticuloendothelial system (RES) and hematopoiesis remain unknown.

STUDY DESIGN AND METHODS: Isovolemic 40 percent exchange transfusion was performed in 60 male Wistar rats with HbV suspended in 5 g per dL recombinant human serum albumin (rHSA; HbV/rHSA, [Hb] = 8.6 g/dL), stored rat RBCs suspended in rHSA (sRBC/rHSA), or rHSA alone. Hematological and plasma biochemical analyses and histopathological examination focusing on the spleen were conducted for the subsequent 14 days.

RESULTS: The reduced hematocrit (Hct) level (26%) for the HbV/rHSA and rHSA groups returned to its original level (43%) in 7 days. Plasma erythropoietin was elevated in all groups: the rHSA group showed the highest value on Day 1 (321 ± 123 mIU/mL) relating to the anemic conditions (HbV/rHSA, 153 ± 22; sRBC/rHSA, 63 ± 7; baseline, 21 ± 3). Simultaneously, splenomegaly occurred in all the groups as HbV/rHSA > rHSA > sRBC/rHSA. Histopathologically, the accumulated HbV in the spleen was undetectable by Day 14, but hemosiderin was deposited in slight quantities for both the HbV/rHSA and sRBC/rHSA groups. Considerable amounts of erythroblasts were apparent in the spleens of both the rHSA and the HbV/rHSA groups.

CONCLUSION: HbVs were phagocytized and degraded in RES, a physiological compartment for the degradation of RBCs, and the elevated erythropoietic activity resulted in the complete recovery of Hct within 7 days in the rat model.

Hemoglobin (Hb)-based O_2 carriers (HBOCs) have been developed progressively for use as a transfusion alternative. Some are now undergoing clinical trials.[1,2] Advantages of HBOCs include the absence of blood-type antigenicity and infectious pathogens and stability for long-term storage when compared with RBC transfusion.[3] Considerably shorter half-life ($t_{1/2}$) of the HBOCs in the blood stream (2-3 days) limit their use,[4] but they are applicable for shorter periods of use as: 1) a resuscitative fluid for hemorrhagic shock during an emergency situation temporarily or for bridging until RBCs are available;[5] 2) a fluid for preoperative hemodilution or perioperative O_2 supply fluid for a hemorrhage during elective surgery to avoid or delay alloge-

ABBREVIATIONS: HBOC(s) = hemoglobin-based O_2 carrier(s); HbV(s) = hemoglobin-vesicle(s); MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; PLP = pyridoxal 5′-phosphate; RES = reticuloendothelial system; rHSA = recombinant human serum albumin; sRBC(s) = stored red blood cell(s).

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neic transfusion;[6] 3) a priming solution for the circuit of an extracorporeal membrane oxygenator during cardiac surgery;[7] and 4) an alternative for use for other potential indications, for example, so-called O_2 therapeutics to oxygenate ischemic tissues.[8,9]

A phospholipid vesicle or liposome-encapsulating concentrated human Hb (Hb-vesicle, HbV) is an HBOC.[10,11] The cellular structure of the HbV (particle diameter, approx. 250 nm) has characteristics that resemble those of natural RBCs because both have lipid bilayer membranes that prevent the direct contact of Hb with blood components and the endothelial lining, thus shielding all side effects of molecular Hb.[12,13] Once in circulation, HbV particles are captured by the phagocytes in the reticuloendothelial system (RES or mononuclear phagocytic system) and are metabolized in the physiologically normal pathway after toploud infusions.[14-17]

We tested the efficacy of HbV suspended in plasma-derived and recombinant human serum albumin (rHSA) in extreme normovolemic hemodilution (80-90% blood exchange) and resuscitation from hemorrhagic shock. They have a comparable O_2-transporting capacity with RBCs.[18-21] However, only a few hours of observation after extensive blood exchange has been reported.

This study undertakes, for the first time, a longer period of observation (2 weeks) after moderate and clinically relevant isovolemic exchange transfusion of a 40 percent estimated blood volume with HbV suspended in a 5 g per dL rHSA solution.[21] We analyzed plasma biochemical, hematological, and histopathological examinations, particularly addressing the degradation of HbV in RES and erythropoietic activity after the reduced Hct. Splenomegaly was more dominant than hepatomegaly after single and repeated infusions of HbV in our previous studies.[14,15,17] Senescent RBCs are known to be captured and degraded in the spleen.[22] For that reason, we conducted infusion of stored homologous RBCs to compare the relative impacts on the spleen.

MATERIALS AND METHODS

Preparation of HbVs suspended in rHSA

HbVs were prepared under sterile conditions, as reported in previous studies.[23,24] The Hb was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dL) contained 14.7 mmol per L pyridoxal 5'-phosphate (PLP) (Sigma-Aldrich Corp., St. Louis, MO) as an allosteric effector at a molar ratio of PLP/Hb of 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. Ltd, Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (NOF Corp., Tokyo, Japan), at a molar composition of 5/5/1/0.033. The lipopolysaccharide content, measured with a modified *Limulus* amebocyte lysate test, was less than 0.1 EU per mL.[25] The physicochemical parameters are P_{50}, 27 Torr; 251 ± 81-nm particle diameter; and less than 3 percent MetHb content. Before use, the HbV suspension ([Hb] = 10 g/dL, 8.6 mL) was mixed with a solution of rHSA (25 g/dL, 1.4 mL; Nipro Corp. Osaka, Japan) to regulate the rHSA concentration in the suspending medium to 5 g per dL. Consequently, the Hb concentration became 8.6 g/dL.[21] Under these conditions, the colloid osmotic pressure and the viscosity (300/sec, 37°C) of the HbV/rHSA were 20 mmHg and 2.9 cP, respectively.

Preparation of stored homologous RBC suspended in rHSA

Blood was withdrawn from donor Wistar rats via the caudal vena cava during ether anesthesia. This was mixed with an RBC preservation fluid, CPDA-1 (C.A. Karmi, Kawasumi Laboratories Inc., Tokyo, Japan) at the volume ratio of 10 percent. The mixture was stored under sterile conditions at 4°C for 1 week because rat RBCs stored for 1 week are reportedly as fragile as the human RBC stored for 4 weeks.[26] After preservation, the stored blood was centrifuged for 10 min at 4000 × *g*, and then the supernatant and the buffy coat were removed. The sedimented RBCs were resuspended in saline and centrifuged. This procedure was repeated twice. Finally, the RBCs were suspended in a 5 g per dL rHSA solution to prepare stored homologous RBCs suspended in rHSA (sRBC/rHSA). The Hb concentration was regulated at 8.6 g per dL, the same Hb concentration of HbV/rHSA.

Exchange transfusion and 2-week observations

Experiments were conducted with 65 male Wistar rats (223 ± 20 g body weight; Saitama Experimental Animals Supply Co., Kawagoe, Japan). During cannulation and exchange transfusion, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (1 mL/kg; Abbott Laboratories, North Chicago, IL). Polyethylene catheters were introduced into the right common carotid artery. Blood withdrawal and sample injection were repeated through one line at 1 mL per 30 seconds. Samples were HbV/rHSA (n = 20), sRBC/rHSA (n = 20), and rHSA only (n = 20). Five rats were used for baseline measurements.

The systemic blood volume was estimated to be 56 mL per kg of the total body weight.[27] Blood was exchanged under the assumption of normovolemia. Therefore, to estimate the necessary amount of HbV, the exchange was assumed to consist of repeating the number of cycles of 1.0-mL withdrawal and sample infusion. The level of exchange, 40 percent, is therefore given as

$$40\% = 100 \times \{1 - [(0.056 \times \text{body weight} - 1.0)/(0.056 \times \text{body weight})]^n\}. \quad (1)$$

The volume exchanged was calculated as $n \times 1.0$ (mL).[28] The sample volume is calculated as 6.0 mL for a rat body weight of 220 g.

After the blood exchange, the catheter was removed, the artery was ligated, and the neck skin was sutured with a stitch. The rats were housed in cages in a barrier room at the animal experimental facility of Keio University. Rats were provided ad libitum access to food and water in a temperature-controlled environment with a 12-hour dark-light cycle.

Five rats were selected randomly from each group at 1, 3, 7, and 14 days for sequential measurements. At each time point, the rats were anesthetized with a 1.5 percent sevoflurane-mixed air inhalation. After measuring the body weight, approximately 150 µL of blood was withdrawn from the tail vein via an indwelling needle (24-gauge; Nipro Corp.) for Hct measurement with glass capillaries, and blood cell counts with an automatic blood cell counter (Model KX-21, Sysmex Corp., Kobe, Japan). The animals were laparotomized and approximately 6 mL of blood was withdrawn from the caudal vena cava for the plasma biochemical tests. The organs were resected en bloc and fixed in a 10 percent formalin neutral buffer solution (Wako Pure Chemical Industries Ltd., Tokyo, Japan) and then embedded in paraffin. Four-micrometer sections were stained with the hematoxylin-eosin, Berlin blue, and Giemsa methods.

The collected blood (approx. 6 mL) was centrifuged ($5,000 \times g$, 10 min) to separate the plasma, which was then ultracentrifuged ($50,000 \times g$, 20 min) to sediment the HbV particles from the plasma at 1 and 3 days after the exchange transfusion with HbV/rHSA to avoid their interference by HbV particles in the plasma biochemical assays.[29] The obtained transparent serum specimens contained no Hb, indicating that no hemolysis of HbV occurred. They were stored at −80°C until biochemical tests at BML, Inc. (Kawagoe, Japan). Erythropoietin (EPO) was measured with radioimmunoassay. Because the rat EPO shows a high degree of homology with human EPO, the rat EPO cross-reacts in the assay of the antihuman EPO.[30]

The experimental protocol was fully approved by the Laboratory Animal Care and Use Committee of School of Medicine, Keio University. It also complied with the *Guide for the Care and Use of Laboratory Animals.*[31]

Statistical analyses

Data are reported as mean ± standard deviation (SD) for all measurements. Differences between the control (baseline) group and a treatment group were analyzed with a one-way analysis of variance followed by Fisher's protected least significant difference test. The changes were considered significant if the p value was less than 0.01.

RESULTS

Body and spleen weights and hematological tests

Rats of all groups tolerated well the 40 percent blood exchange; they survived until their intentional euthanization. The rats survived this intervention because of the normovolemic exchange transfusion while maintaining the blood colloid osmotic pressure with 5 g per dL rHSA as the suspending medium. All rats gained weight until their euthanization (Fig. 1). No noticeable change occurred in their behavior or appearance such as the pilomotor response.

The spleen:body weight ratio increased significantly for the HbV/rHSA group at 1 and 3 days after the exchange. It returned to a level that was comparable to the baseline at 14 days. The rHSA group also showed significant splenomegaly at 3 days, but no splenomegaly at 1 day. At 14 days, the spleen weight reverted to the baseline level. The sRBC/rHSA group also showed moderate, but significant, splenomegaly on Days 1, 3, and 7.

The Hct before the exchange transfusion was approximately 43 percent. It decreased to about 26 percent for the HbV/rHSA and rHSA groups. Both groups showed a monotonic Hct increase; at 7 days, the Hct showed a complete recovery to the baseline level (about 43%) and an overshooting at 14 days (approx. 46%). In the sRBC/rHSA group, the Hct level at 1 day was much higher than that of the other groups because of the sRBC infusion. The Hct level, however, was slightly lower than for the other groups at 7 and 14 days. The mean corpuscular Hb (MCH), mean corpuscular volume (MCV), and mean corpuscular Hb concentration (MCHC) values remained within normal ranges (data not shown); however, MCH and MCHC of the HbV/rHSA group at 1 and 3 days were not measured because of the presence of HbV. The sRBC/rHSA group showed slightly lowered MCV and MCH levels at 1 day. In contrast to Hct, platelet and white blood cell counts showed nonsignificant decreases at 1 day and then maintained rather steady values. The plasma Hb concentration derived from HbV after the exchange transfusion was estimated as 4.4 g per dL, which decreased, respectively, to 1.8 ± 0.1, 1.1 ± 0.1, and 0 g per dL on Days 1, 3, and 7.

Plasma biochemical tests

The plasma EPO level, an indicator of an anemic, hypoxic, or stressed condition, increased significantly from 21 ± 3 IU per L in the normal condition to 312 ± 123 IU per L for the rHSA group at 1 day, which was significantly higher than for the HbV/rHSA group (153 ± 22 IU/L) or the sRBC/rHSA group (63 ± 7 IU/L; Fig. 2). After 3 days, they decreased to less than 100 IU/L; at 7 days, they reverted to the baseline level.

Regarding the other routine analytes, apartase aminotransferase showed slight increases on Day 1 for all

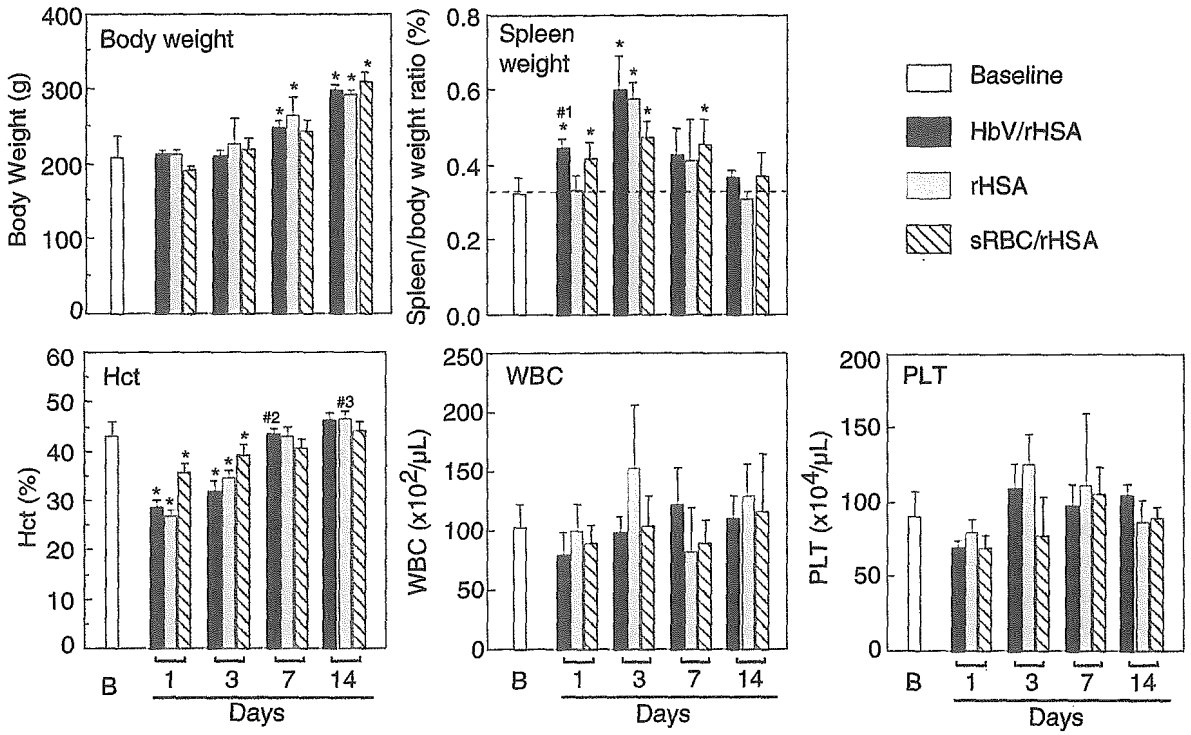

Fig. 1. Changes in body weight, spleen:body weight ratio, and hematological parameters after 40 percent exchange transfusion with HbV/rHSA, rHSA, or sRBC/rHSA. The spleen:body weight ratio (baseline, 0.32 ± 0.04%) increased significantly for the HbV/rHSA group at 1 day (0.45 ± 0.03%) and 3 days (0.60 ± 0.09%). It returned to the baseline at 14 days (0.37 ± 0.02%). The rHSA group also showed significant splenomegaly at 3 days (0.58 ± 0.05%) and returned to 0.31 ± 0.02 percent at 14 days. The sRBC/rHSA group also showed splenomegaly at 1, 3, and 7 days (0.42 ± 0.04, 0.48 ± 0.04, and 0.46 ± 0.06%, respectively). The baseline Hct level was 43 percent; it decreased to about 26 percent for the HbV/rHSA and rHSA groups. At 7 days, they showed complete recovery to approximately 43 percent and then further increased to approximately 46 percent at 14 days. The values are means ± SD. The broken line indicates the baseline value. *Significantly different from the baseline ($p < 0.01$); #1significantly different from the rHSA group ($p < 0.01$); #2$p = 0.0288$ versus sRBC/rHSA; #3$p = 0.0353$ versus sRBC/rHSA. B = baseline.

groups (HbV/rHSA, 70 ± 5 U/L; rHSA, 69 ± 12; sRBC/rHSA, 72 ± 9; baseline, 60 ± 7), but it reverted to the original level, whereas alanine aminotransferase was stable. Alkaline phosphatase and γ-glutamyltransferase showed significant or nonsignificant reductions for all groups throughout the experiment. Creatine phosphokinase was stable for 14 days. For all groups, creatinine and uric acid were maintained at low levels for 14 days (data not shown). Amylase showed some significant reduction, but did not change markedly for 14 days (Fig. 3). In contrast, lipase showed significant and marked increases for the HbV/rHSA group for 3 days, but it tended to decrease after 7 days.

Regarding plasma lipid components in the HbV/rHSA group, the total cholesterol and free cholesterol showed significant increases with maximum values at 3 days (Fig. 3). Nevertheless, they returned to their original levels at 7 days. The β-lipoprotein tended to decrease after the exchange transfusion, showing significant reductions at 3 and 7 days for the rHSA group. The high-density lipoprotein cholesterol also tended to decrease with a significant reduction at 3 days for the rHSA group. Triglyceride tended to decrease for all groups with a significant difference in the HbV/rHSA group at 1 and 3 days, partly because of ultracentrifugation of the plasma fractions, and in the rHSA group at 7 days. At 14 days, they generally recovered to the baseline level. The phospholipid tended to decrease with significant differences for all groups. Free fatty acid tended to increase at 14 days. The serum bilirubin (<0.1 mg/dL) remained at a low level throughout the experiment. Fe^{3+} showed significant reductions at 1 and 3 days for the HbV/rHSA group, at 3 and 7 days for the rHSA group, and at 3 days for the sRBC/rHSA group, but they returned to the original level at 14 days (Fig. 3).

Histopathological study

Histopathological examination revealed no significant changes in the lung, heart, and kidney in all groups. At 1 and 3 days after infusion, significant amounts of HbV phagocytized by macrophages in the marrow and Kupffer

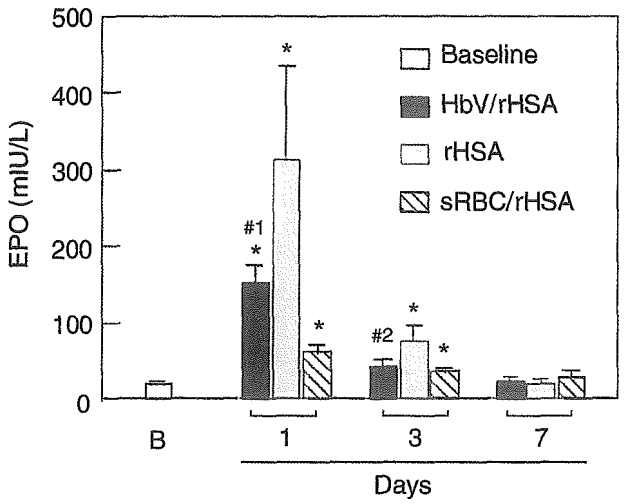

Fig. 2. Plasma EPO activity after 40 percent exchange transfusion with HbV/rHSA, rHSA, or sRBC/rHSA. All groups showed significant increases at 1 day. However, the HbV/rHSA groups showed a lower level than the rHSA group. The values are means ± SD. *Significantly different from the saline group ($p < 0.01$); #1 $p = 0.0222$ versus rHSA; #2 $p = 0.0195$ versus rHSA. B = baseline.

cells in the liver were observed. However, HbV decreased significantly at 7 days and was undetectable at 14 days. At 3 days after infusion, the pancreas in the HbV/rHSA group showed no significant morphological changes in spite of the increased lipase activity.

Sections of the spleen of the HbV/rHSA group, which is stained with Giemsa method, revealed the accumulation of HbV particles in the red pulp zone at 1 and 3 days after the exchange transfusion. The amount of the accumulated HbV decreased at 7 days and then became undetectable at 14 days (Fig. 4). Throughout the period examined in this study, nests composed of erythroblasts and proerythroblasts were formed in the splenic cord, especially at 3 and 7 days, indicating extramedullar erythropoiesis. Nest formation was remarkable for the rHSA group at 3 days. Hematopoietic activity was also observed at 3 days in the marrow of the HbV/rHSA group that contained erythroblastic islets.

The Berlin blue method indicated the presence of hemosiderin in macrophages of the spleen in the HbV/rHSA group at 7 days. This hemosiderin deposition increased until 14 days (Fig. 5). A small amount of hemosiderin was confirmed in the Kupffer cells of the liver at 14 days. Hemosiderin deposition, however, was undetected in the marrow. In addition, in the sRBC/rHSA group, hemosiderin deposition was present in the spleen macrophages at 14 days.

DISCUSSION

A main finding of this study is that the reduced Hct level after the 40 percent exchange transfusion with HbV/rHSA returned to the original level after 7 days; furthermore, the accumulated HbVs in RES became undetectable within 14 days. Significant splenomegaly is attributable to the combination of the accumulation of HbV in the red pulp zone and the considerable presence of nests of erythroblasts in the splenic cord in response to the EPO secretion, but these observations subsided within 14 days.

Extensive studies of circulation kinetics and organ distribution of isotope-labeled HbV clarified that HbV accumulates preferentially in the RES.[11,16] One cause of the splenomegaly is the accumulation of HbV particles in the red pulp zone, as shown in Fig. 4 but this subsided completely within 14 days. Gradual increases in the plasma cholesterol levels by 3 days after infusion and lack of disruption of the HbV in the plasma suggest that the cholesterols are liberated from the RES after the HbVs are captured by the RES and destroyed in the phagosomes of the macrophages.[14,15] In our previous studies of topload HbV infusions, significant increases in the high-density lipoprotein cholesterol, β-lipoprotein, and phospholipids were observed as surplus amounts.[15,17] In contrast, we observed no such significant increases after the 40 percent blood exchange, only decreases. A large demand of nutrients should pertain for hematopoiesis and so on; also, the lipid components from HbV might be utilized efficiently for proliferation.

During the metabolism of Hb, we would expect a release of bilirubin and iron. But they did not increase in the plasma within 14 days. The released heme from Hb in HbV might be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells of the liver and the spleen macrophages.[15,32] Bilirubin would normally be excreted in the bile as a normal pathway, and no obstruction or stasis of the bile should occur in the biliary tree. Berlin blue staining revealed considerable deposition of hemosiderin in the liver and spleen, even after 14 days. Normally, iron from a heme is stored in the ferritin molecule.[33] Both ferritin and hemosiderin release iron. They are anticipated to induce hydroxyl radical production followed by lipid peroxidation.[34] The iron release rate from hemosiderin, however, is substantially less than that from ferritin.[35] Consequently, the excess amount of iron would then normally be stored in an insoluble and less toxic form as hemosiderin. Hemosiderosis is often observed in patients who have received repeated blood transfusions because of the shorter $t_{1/2}$ of the stored RBCs. Moderate splenomegaly and hemosiderin deposition were also confirmed in the spleen in the sRBS/rHSA groups of this study, partly because of the accumulation and degradation of stored RBCs with the lowered membrane deformability and shortened circulation $t_{1/2}$.[26] These results indicate that the metabolism of heme from HbV and the iron storage is within the physiological capacity that has been well characterized for the metabolism of senescent RBCs.[36]

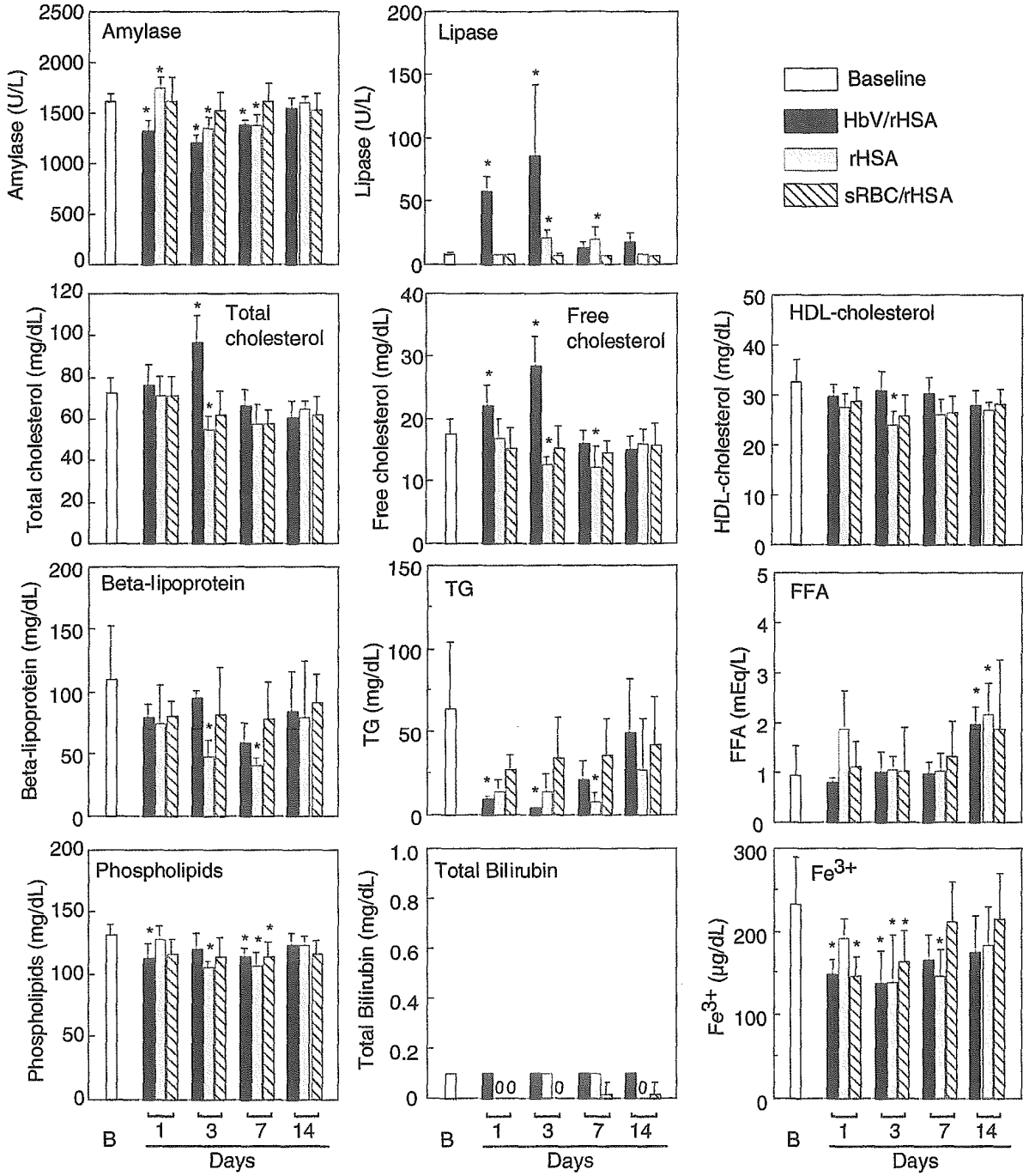

Fig. 3. Plasma biochemical tests representing the metabolism of the components of HbV (lipids and Hb) and pancreatic function after 40 percent exchange transfusion with HbV/rHSA, rHSA, or sRBC/rHSA. The values are means ± SD. *Significantly different from the saline group ($p < 0.01$). TG = triglyceride; FFA = free fatty acid; B = baseline.

Interestingly, not only the HbV/rHSA and sRBC/rHSA groups, but also the rHSA group showed a significant splenomegaly at 3 days, even though the rHSA group showed no symptoms on Day 1. In rats, extramedullary hematopoiesis induced by hypoxia is localized predominantly in the spleen.[37,38] We observed extensive nests of erythroblasts in the splenic cords, especially at 3 days. It is not plausible that the rHSA as an xenogeneic protein accumulates in the spleen macrophages, according to the fact the ^{125}I-labeled rHSA in a rat showed no specific distribution to the spleen.[39,40] Therefore, the splenomegaly for the rHSA group is attributed to the erythropoiesis stimulated by the significant increase in the plasma EPO level.

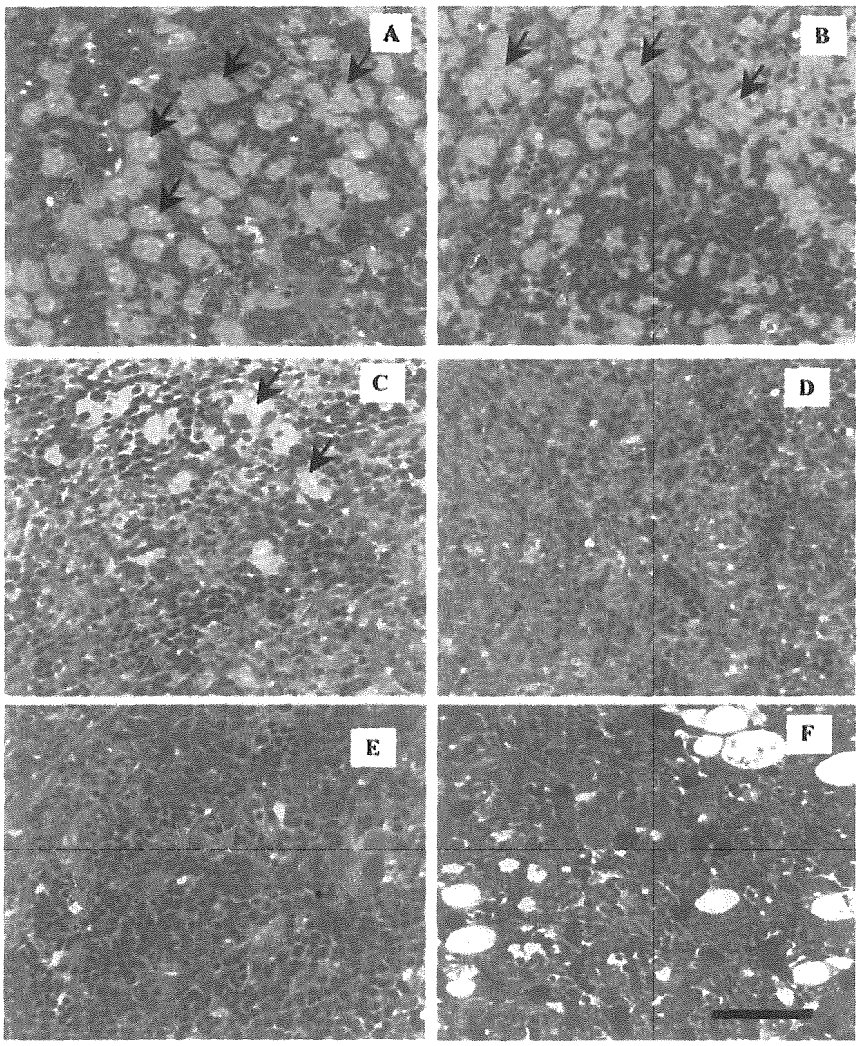

Fig. 4. Histology of rat spleen and marrow after exchange transfusion with HbV/rHSA or rHSA alone. (A-C) Respective images of the spleen of the HbV/rHSA group at 1, 3, and 7 days. Accumulated HbV particles are visible as light-blue areas (black arrows). Nests of erythroblasts are visible as dark blue cells (red arrows). The domain of the HbV particles decreased significantly at 7 days. (D) Spleen of the HbV/rHSA group at 14 days. HbV particles had disappeared, whereas the erythroblast nests remained, as indicated by the red arrows. (E) Spleen of the rHSA group at 3 days. The erythroblast nest formation is remarkable. (F) Marrow of the HbV/rHSA group at 3 days. Hematopoietic activity is visible. Bar = 50 μm (Giemsa method).

Plasma EPO release from the kidney strongly reflects an anemic condition, depending on the O_2-carrying capacity of the circulating blood.[41,42] The highest EPO level was seen in the rHSA group, indicating that its anemic condition was the most severe. Because of the short $t_{1/2}$ and MetHb formation,[43] the HbV/rHSA also showed a significant increase in the EPO level. However, it was considerably lower than that of the rHSA group. The sRBC/rHSA group also showed a moderate increase in the EPO level probably caused by the reduced Hct by the exchange transfusion. Accordingly, the splenomegaly for the HbV/rHSA and sRBC/rHSA groups is also partly attributable to the nests of erythroblasts for erythropoiesis that was sufficient for recovery from the reduced Hct. Interestingly, both HbV/rHSA and rHSA groups tended to show higher Hct values than the sRBC/rHSA group at 7 and 14 days, probably because of the enhanced erythropoiesis caused by the higher levels of EPO excretions than for the sRBC/rHSA group. The MCH, MCV, and MCHC levels were normal overall, supporting our inference of normal erythropoiesis.

Routine plasma biochemical tests showed that the hepatic function was maintained despite the large amount of HbV that were captured and degraded by Kupffer cells. Significant reductions were seen in the amylase activity, whereas a transient increase in lipase activity was observed consistently in our previous topload infusion experiments; this should be due to the up regulation of lipase in response to the infusion of phospholipid vesicles.[15,17,44]

In conclusion, all rats tolerated the 40 percent exchange transfusion with HbV/rHSA and showed complete Hct recovery within 7 days. Although transient splenomegaly and the hemosiderin deposition were confirmed, no excess iron was found in the blood. The recycling or excretion of iron as well as lipid components should be on the physiological pathway that is known for the degradation of senescent RBCs. Although some aspects remain unresolved, the present results offer important information on the safety and handling of HbV during preoperational or perioperational infusion in a clinical setting.

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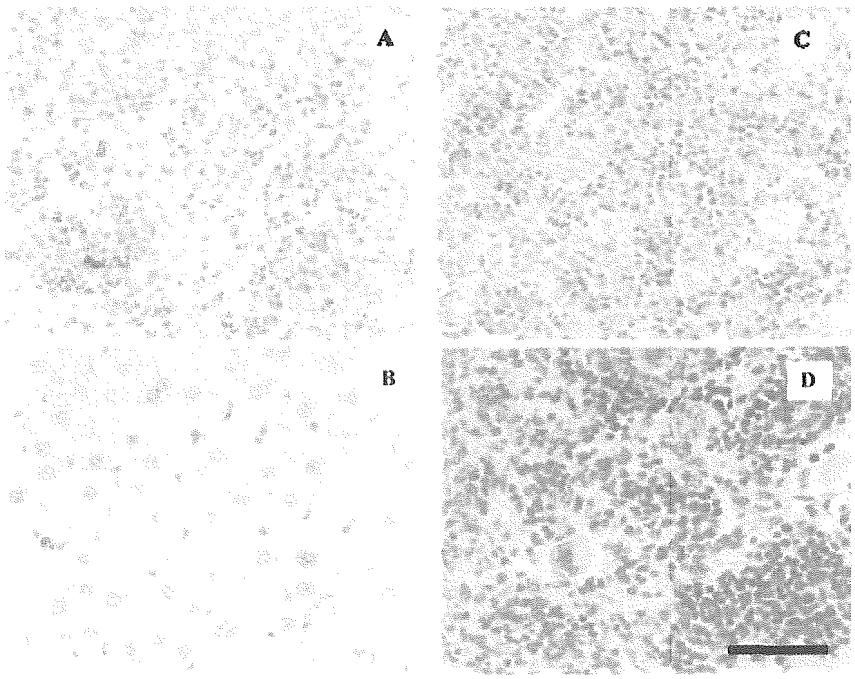

Fig. 5. Histology of rat spleen, liver, and marrow 14 days after exchange transfusion with HbV/rHSA or sRBC/rHSA. Spleen (A), liver (B), and marrow (C) of the HbV/rHSA group. The spleen and liver contained slight hemosiderin deposition, but not the marrow. The spleen of the sRBC/rHSA group (D) also contained slight hemosiderin deposition. Bar = 50 μm (Berlin blue method).

ful discussion of phagocytic and hematopoietic activities. The rHSA was obtained from Nipro Corp.

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