an increase in vascular resistance. On the other hand, HbV (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 that the size of HbV is appropriate for the maintenance of microvascular blood flow.

Biodistribution and Metabolism of HbV, and Influence on Organ Function

In the physiological condition, free Hb released from RBC is rapidly bound to haptoglobin, and removed from the circulation by hepatocytes. However, when the Hb concentration exceeds the haptoglobin binding capacity, unbound Hb is filtered through the kidney where it is actively absorbed. When the reabsorption capacity of the kidney is exceeded, hemoglobinuria and eventually renal failure occur. The encapsulation of Hb completely suppresses renal excretion, although HbV particles as well as phospholipid vesicles (liposomes) in the blood stream are finally captured by phagocytes in the reticuloendothelial system (RES, or mononuclear phagocytic system, MPS) [131].

To examine the precise circulation persistence and biodistribution of HbV, we used radiolabelling technique, ^{99m}Tc-labelled HbV, in collaboration with Prof. Phillips at the University of Texas. The HbV co-encapsulated homocysteine (5 mM) was successfully labeled with ^{99m}Tc by using the hexamethylpropylene amine oxime. The circulation half-life of ^{99m}Tc-HbV was determined to be 35 h. In the gamma camera image, the radioactivity in the blood pool of the heart was gradually decreased and those of the liver and spleen were increased with time. The biodistribution data showed the major organs to eliminate the ^{99m}Tc-HbV from the blood circulation were the liver, bone marrow, and spleen, independent of the injection dose. [132].

The influence of HbV on RES, mainly liver and spleen, was studied with carbon clearance measurements and histopathological examination [133]. The HbV suspension was intravenously infused in male Wistar rats (200 g) at dose rates of 10 and 20 ml/kg, and the phagocytic activity was measured by monitoring the rate of carbon clearance at 8 hs, and at 1, 3, 7 and 14 days after infusion. The phagocytic activity transiently decreased one day after infusion by about 40%, but it recovered and was enhanced at 3 days, showing a maximum of about twice the original level at 7 days, and then returned to the original level at 14 days. The initial transient decreased activity indicates a partly, but not completely, suppressed defensive function of the body. The succeeding increased phagocytic activity corresponds to the increased metabolism of HbV. The histopathological examination with hematoxylin/eosin, and anti-human Hb antibody staining showed that HbV was metabolized within 7 days. Hemosiderin was slightly confirmed with Berlin blue staining at 3 and 7 days in the liver and spleen, although they disappeared at 14 days, indicating that the heme metabolism, excretion, or recycling of the iron ion proceeded smoothly and siderosis was minimal. Electron microscopic examination of the spleen and liver tissues clearly demonstrated the vesicular structure of HbV with a diameter of about 1/40 of RBCs in capillaries, and in phagosomes as entrapped in the spleen macrophages and Kupffer cells one day after infusion. The vesicular structure could not be observed at 7 days. Even though infusion of HbV modified the phagocytic activity for two weeks, it does not seem to cause any irreversible damage to the phagocytic organs from the histological point of view.

We analyzed the influence of HbV on the organ functions by laboratory tests of plasma on a total of 29 analytes [134]. The HbV suspension was intravenously infused into male Wistar rats (20 ml/kg). The blood was withdrawn at 8 hs, and 1, 2, 3, and 7 days after infusion, and the plasma was ultracentrifuged to remove HbV in order to avoid its interference effect on the analytes. Enzyme concentrations, AST, ALT, ALP, and LAP showed significant, but minor changes, and did not show a sign of a deteriorative damage to the liver as one of the main organs for the HbV entrapment and the succeeding metabolism. The amylase and lipase activities showed reversible changes; however, there were no morphological changes in the pancreas. Plasma bilirubin and iron did not increase in spite of the fact that a large amount of Hb was metabolized in the macrophages. Cholesterols, phospholipids, and β-lipoprotein transiently increased showing the maximum at 1 or 2 days, and returned to the control level at 7 days. They should be derived from the membrane components of HbV that are liberated from macrophages entrapping HbV. In conjunction with the previous report of the prompt metabolism of HbV in the reticuloendothelial system by histopathological examination, it can be concluded that HbV infusion transiently modified the values of the analytes without any irreversible damage to the corresponding organs at the bolus infusion rate of 20 ml/kg.

In the series of safety evaluations, the repeated infusion of HbV in Wistar rats was performed at the dose rate of 10 ml/kg/day for 14 days [135]. All the rats tolerated the infusion and body weight increased continuously. The hematological test, serum blood biochemistry, and histopathological examination did not raise any serious concern about the safety of HbV. One day after the final infusion spleen and liver weights increased significantly. Histopathological observation indicated significant HbV accumulation in liver and spleen; however, there was no sign of organ damage. Serum clinical laboratory tests indicated significant increases in lipid components derived provably from HbV particles. After a 2 week interval, spleen and liver weight returned to the original levels; however, a significant amount of hemosiderin was confirmed without serum iron increase. All the concentrations of the lipid

components returned to the original levels. Judging from these results, there was no sign of significant toxicity of HbV at the level of dosage employed.

Summary

The efficacy of HbV as oxygen carriers and their safety have been demonstrated. The advantages of cellular HbV can be summarized as follows:

1. The encapsulated Hb is extremely purified and free from virus, endotoxin, and blood type antigen.
2. There is no chemical modification of Hb. Dissociation of Hb tetramers to dimers is restrained and there is no release of Hb from HbV, preventing renal dysfunction.
3. The oxygen affinity is adequately adjusted and the metHb formation is restrained because both the allosteric effectors and metHb reduction systems can be coencapsulated in the vesicles.
4. HbV can be stored for over 2 years at room temperature, owing to both surface modification with PEG chains and deoxygenation.
5. The surface modification of HbV with PEG chains increases high dispersion stability and is effective to prevent aggregation in blood circulation.
6. The colloid osmotic pressure of the HbV suspension is close to zero. But it is adjustable with the addition of adequate colloids such as HSA, which is important to maintain blood volume. The solution viscosity can be adjusted equivalent to that of blood. This would be important for the shear stress on the vascular wall to regulate vascular tone.
7. HbV suspended in a plasma expander such as HSA and rHSA showed sufficient oxygen transporting capacity comparable with RBC for resuscitation from hemorrhagic shock and extreme hemodilution. It is also applicable for oxygenation of ischemic tissues.
8. The physiological activity of Hb such as binding with NO and CO, production of active oxygen species, heme release, and hemeoxygenase activation, can be minimized by encapsulation. Thus there is less vasoconstriction, hypertension, and oxygen injury.

According to the above achievements, significant efforts have been made to produce HbV with a facility of GMP standard, and to start preclinical and finally clinical trials. The combination of recombinant Hb-vesicles suspended in recombinant albumin would be the most ideal "artificial red blood cells" in the future.

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Oxygen-Carrying Plasma Hemoprotein Including Synthetic Heme

Teruyuki Komatsu and Eishun Tsuchida

Summary. Recombinant human serum albumin (rHSA) incorporating tetraphenylporphyrinatoiron(II) derivative with four pivaloylamino substituents (FepivP), albumin-heme, is an entirely synthetic hemoprotein that can reversibly bind and release O_2 under physiological conditions. We have recently found that replacing the substituent groups of FepivP with more hydrophobic 1-methylcyclohexanoylamino groups, affording FecycP, substantially stabilizes the formed O_2–adduct complex. The O_2- and CO-binding abilities and blood compatibility of this new rHSA-heme hybrid (rHSA-FecycP) have been investigated by spectroscopy. The maximum number of FecycP binding to one albumin was determined to be eight. Because the isoelectric point and circular dichroism (CD) spectral pattern were identical to those of rHSA itself, the two-dimensional structure of the host albumin could be unchanged after the incorporation of FecycP. Laser-flash photolysis experiments gave the association and dissociation rate constants for O_2 and CO (k_{on}, k_{off}). The rebinding kinetics of these gaseous ligands consists of multiple exponentials. We conjectured that the O_2- and CO-binding reactions are affected by the molecular environment around each of the active heme sites. rHSA-FecycP showed almost the same O_2-binding affinity($P_{1/2}{}^{O_2}$ 34 torr at 37°C) and thermodynamic parameters (ΔH, ΔS) for the oxygenation as rHSA-FepivP. In contrast, the half-life of the O_2-adduct complex (9 h, 37°C) became significantly longer than that of rHSA-FepivP (by a factor of 4.5), which is close to that of myoglobin. The obtained red solution was stable and demonstrated a long shelf life (>2 years) at room temperature. The equivalent mixture of rHSA-FecycP and whole blood exhibited no coagulation or precipitation, indicating its high blood compatibility.

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Key words. Human serum albumin, Albumin-heme, Synthetic hemoprotein, Oxygen-binding ability, Red blood cell substitute

Introduction

Human serum albumin (HSA) used for clinical treatment in Japan amounted to 1.9 million l (in terms of a blood source) in 2002 [1]. Most was administered to hemorrhagic shocked patients as a resuscitation fluid. If HSA can transport oxygen (O_2) like red blood cells, it could be of extreme medical importance not only as a blood replacement but also as an O_2 therapeutic agent.

In our circulatory system, free hemin, an iron(III) complex of protoporphyrin IX dissociated from methemoglobin, is potentially toxic because it may (1) intercalate phospholipid membranes, (2) be a major source of iron for bacterial pathogens, and (3) catalyze the formation of free radicals. Hemopexin has high affinity for binding protein with hemin, having the highest binding constant of any known protein ($K > 10^{12}\,M^{-1}$), but it releases it into liver cells via specific surface receptors [2]. Crystal structure analysis of the hemopexin-hemin complex revealed that the hemin is tightly bound by double histidine coordinations to the central ferric ion and multiple hydrogen bondings with the amino acid residues [3]. Nevertheless, the concentration of hemopexin in the plasma is rather low (<17 μM). HSA may also provide reserve binding capacity of hemin in various conditions (e.g., trauma, inflammation, hemolysis). In fact, HSA binds hemin with a relatively high affinity ($K = 10^8\,M^{-1}$) [4]. We have determined the single crystal structure of the HSA-hemin-myristate complex with a resolution of 3.2 Å [5]. Hemin is accommodated into the narrow D-shaped pocket in subdomain IB; and proximal coordination with Tyr-161 and three hydrogen bondings with basic amino acids contribute to maintaining the assembly. Addition of a sodium dithionate into this solution under an N_2 atmosphere reduced the central ferric ion to the ferrous state, although exposure to O_2 gas immediately oxidized the iron(II) center (T. Komatsu, N. Ohmichi, E. Tsuchida, unpublished data, 2004).

We have found that tetraphenylporphyrinatoiron(II) derivative with four pivaloylamino substituents (FepivP) (Fig. 1) was also incorporated into HSA, and the obtained albumin-heme (HSA-FepivP) can reversibly bind and release O_2 under physiological conditions in the same manner as hemoglobin (Hb) and myoglobin (Mb) [6–12]. Because recombinant HSA (rHSA) was manufactured on a large scale by expression in *Pichia pastoris* [13], rHSA-heme hybrid has become entirely synthetic and absolutely free of infectious pathogens. Our animal experiments have also demonstrated that rHSA-heme works as an "oxygen-carrying plasma hemoprotein" in the bloodstream [14; T. Komatsu et al., unpublished data, 2004].

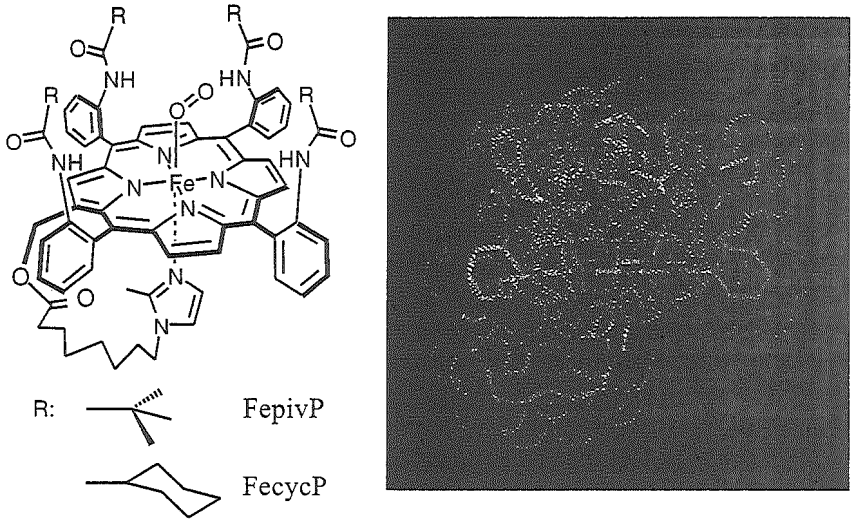

FIG. 1. Structures of the new tetraphenylporphyrinatoiron(II) derivative with more hydrophobic 1-methylcyclohexanoylamino groups on the porphyrin ring plane (FecycP) and pivaloylamino substituents (FepivP), and the simulated structure of oxygenated FecycP. The extensible systematic forcefield (ESFF) simulation was performed using an Insight II system (Molecular Simulations, San Diego, CA, USA). The structure was generated by alternative minimization and annealing dynamic calculations from 1000 K to 100 K. The dielectric constant was fixed at 2.38 D, corresponding to the toluene solution. The *dotted surface* represents the van der Waals radius

Half of the Hb-based O_2 carrier in advanced clinical trials still exhibited vasoconstriction, which increased blood pressure and decreased cardiac output [15–19]. The precise mechanism of this hypertension is controversial, but many investigators suspect that the Hb molecules penetrate the vascular endothelium and bind the endothelial-derived relaxing factor (EDRF), namely nitric oxide [20–27]. Others believe that excessive delivery of O_2 to arteriolar vascular walls induces autoregulatory vasoconstriction [28–33]. Interestingly, rHSA-heme does not induce such a vasopressor effect [34]. The electrostatic repulsion between the albumin surface and glomerular basement membrane around the endothelial cell retards rapid leakage of the rHSA-heme molecule and quick scavenging of NO. Albumin-heme is now recognized to be one of the most promising materials as a new class of red blood cell substitute.

To improve the O_2-binding ability of rHSA-FepivP, we have synthesized new tetraphenylporphyrinatoiron(II) derivative with more hydrophobic 1-methylcyclohexanoylamino groups on the porphyrin ring plane (FecycP) (Fig. 1) [35]. rHSA-FecycP forms a significantly stable O_2-adduct complex with

a long half-life compared to that of FepivP (by a factor of 4.5). We herein report the O_2- and CO-binding abilities of this entirely synthetic albumin-based O_2 carrier.

Incorporation of Heme into rHSA

Based on quantitative analysis of the absorption intensity for the Soret band of aqueous rHSA-FecycP, the maximum number of FecycP binding to an rHSA was determined to be eight using a molar extinction coefficient [35]. FecycP is accommodated into certain domains of rHSA with binding constants of 10^6–$10^4\,M^{-1}$.

The isoelectric points (*p*I) of the obtained rHSA-FecycP hybrid (FecycP/rHSA = 1–8 mol/mol) were 4.8, exactly the same as those of rHSA. Fatty acid binding, for example, induced a reduction in the *p*I value due to partial neutralization of the surface charge. The FecycP molecule without any ionic side chain interacts nonspecifically with a hydrophobic subdomain of rHSA, so its surface charge distributions are unaltered. Consequently, the essential biological roles as serum albumin [i.e., control of colloid osmotic pressure (COP) and plasma expansion] are essentially sustained after the incorporation of FecycP.

The secondary and tertiary structures of rHSA and the deformation upon FecycP binding were measured by circular dichroism (CD) spectroscopy. The spectral pattern showed typical double-minimum negative peaks in the ultraviolet (UV) region independent of the number of FecycP molecular bound (Fig. 2). The estimated α-helix content was approximately 67%, suggesting that the FecycP association did not cause any high-ordered structural change in the host albumin. Moreover, rHSA-FecycP showed no induced CD in the Soret region (400–500 nm). The hemin binding to the serum albumin is accompanied by a rise in the extrinsic negative Cotton effect in the Soret region because it binds to albumin through axial coordination, allowing a large degree of immobilization [36,37]. We concluded that hydrophobic interaction is the major molecular force of FecycP binding, and its incorporation does not induce any changes in the highly ordered structure or in the surface net charges of rHSA.

O_2-Binding Property of rHSA-Heme

The UV-visible absorption spectrum of the aqueous rHSA hybrid that included carbonyl FecycP showed the formation of the typical CO-coordinated low-spin tetraphenylporphyrinatoiron(II) derivative (λ_{max}: 429, 545 nm). Light irradiation of this solution under an O_2 atmosphere led to

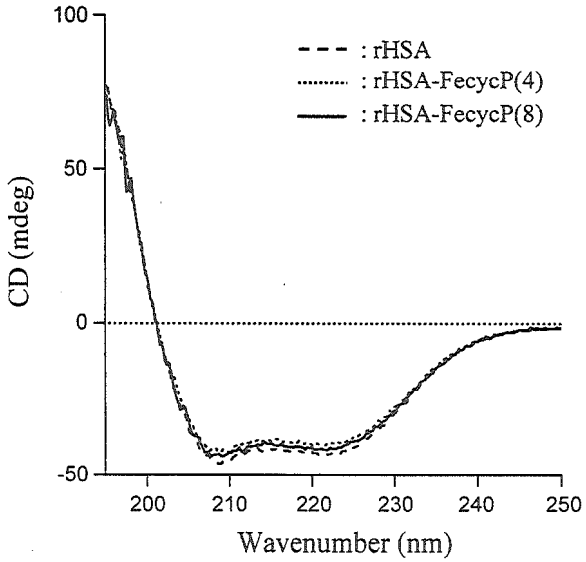

FIG. 2. Circular dichroism (*CD*) spectra of recombinant human serum albumin (*rHSA*) and rHSA-FecycP in water at 25°C

CO dissociation, giving the O_2-adduct complex (λ_{max}: 428, 555 nm). Upon exposure of the oxygenated rHSA-FecycP to N_2, the UV-visible absorption pattern changed to that of the five-*N*-coordinated high-spin iron(II) complex with an intramolecularly coordinated proximal imidazole (λ_{max}: 445, 543, 567 nm). This oxygenation was reversibly dependent on the O_2 partial pressure and sufficiently stable under physiological conditions (37°C, pH 7.4) (Fig. 3). The rate of irreversible oxidation is satisfactorily slow (vide infra).

The O_2 coordination to FecycP in human serum albumin is expressed by Eq. 1.

$$\mathrm{FeP} + \mathrm{O_2} \underset{k_{off}^{O_2}}{\overset{k_{on}^{O_2}}{\rightleftarrows}} \mathrm{FeP\text{-}O_2} \qquad (1)$$

$$\left[P_{1/2}^{O_2} = \left(K^{O_2}\right)^{-1} = k_{on}^{O_2} / k_{off}^{O_2}\right]$$

The O_2 association and O_2-dissociation rate constants ($k_{on}^{O_2}$, $k_{off}^{O_2}$) were explored by laser flash photolysis (Table 1) [9,35,38–40]. The detailed kinetic evaluation of rHSA-FecycP gave the following results.

1. The absorption decays accompanying O_2 recombination were composed of three phases of first-order kinetics; the curves were fit by a triple-exponential equation [9]. The minor (<10%) and fastest component was

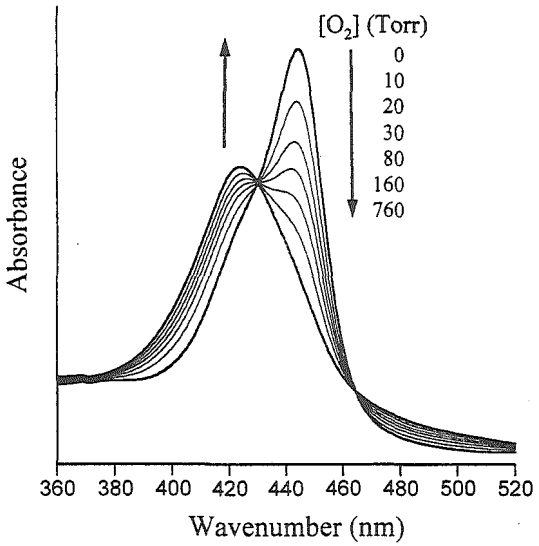

FIG. 3. Ultraviolet-visible. Absorption spectral changes of rHSA-FecycP(4) dependent on the O_2 partial pressure in phosphate-buffered solution (pH 7.3) at 37°C. The number in parenthesis is molar ratio of FecycP and rHSA

TABLE 1. O_2 association and dissociation rate constants for rHSA-FecycP in phosphate-buffered solution (pH 7.3) at 25°C

	$k_{on}[M^{-1}s^{-1}]$		$k_{off}[s^{-1}]$	
Substance	Fast	Slow	Fast	Slow
rHSA-FecycP(8)	4.6×10^7	7.3×10^6	9.8×10^2	1.6×10^2
rHSA-FepivP(8)[a]	3.4×10^7	9.5×10^6	7.5×10^2	2.0×10^2
Hb (T-state)α[b]	2.9×10^6		1.8×10^2	

rHSA, recombinant human serum albumin; FecycP, tetraphenylporphyrinatoiron(II) derivative with 1-methyl cyclohexanoylamino groups; FepivP, tetraphenyl porphyrinatoiron (II) with pivaloyamino substitute; Hb, hemoglobin.
[a] Ref. [9].
[b] pH 7, 20°C; Ref. [40].
The numbers in parenthesis is molar ratio of porphyrin and rHSA.

independent of the O_2 concentrations. It should be correlated with a base elimination [41].

2. Based on careful inspection of the two slower phases, the association rate constants for the fast and slow rebinding [k_{on}(fast) and k_{on}(slow)] of O_2 were calculated. The k_{on}(fast) values are four- to fivefold higher than the k_{on}(slow) volues.
3. The concentration ratios of the fast and slow reactions were 2:1 to 3:1.

Based on these findings, we can conclude that the O_2 association with FecycP in the hydrophobic domains of rHSA is influenced by the molecular

TABLE 2. O_2-binding equilibrium parameters and half-lifetime of rHSA-FecycP in phosphate-buffered solution (pH 7.3)

Substance	$P_{1/2}$(torr)[a]	ΔH[kJ mol^{-1}]	ΔS[J K^{-1}mol^{-1}]	$\tau_{1/2}$[h][a]
rHSA-FecycP(4)	34	−59	−108	9
rHSA-FecycP(8)	35	−59	−107	9
rHSA-FepivP(4)[b]	36	−60	−114	2
rHSA-FepivP(8)[b]	33	−60	−112	2
Red cells[c]	27			
Hbα	40[d]	−57 to −65[e]	−116 to −133[e]	35[f]
Mb[d]	40[d]	−57 to −65[e]	−116 to −133[e]	12[g]

[a] At 37°C.
[b] Ref. [8].
[c] pH 7.4; ref. [42].
[d] T-state, pH 7, 20°C; ref. [40].
[e] pH 7.4; ref. [43].
[f] At 37°C, pH 7.2; ref. [44].
[g] At 35°C, pH 7.0; ref. [45].
The number in parenthesis is molar ratio of porphyrin and rHSA.

microenvironment around each O_2 coordination site (e.g., steric hindrance of the amino acid residue and difference in polarity).

The O_2-binding affinity for such oxygenation could be directly determined. Adequate isosbestic behavior was maintained during the course of a spectrophotometric titration of O_2 (Fig. 3). According to the kinetic experiments, the $P_{1/2}$ values were divided into two components using our previously reported equation [9]. The calculated $P_{1/2}$ for the fast and slow phases were identical in each case (Table 2). The thermodynamic parameters (ΔH, ΔS) of oxygenation were also measured by the van't Hoff plots of the K^{O_2} values (Fig. 4) [8]. The $P_{1/2}{}^{O_2}$, ΔH, and ΔS values for oxygenation of rHSA-FecycP resembled those of Hb and Mb [8,40,42–45]. Moreover, we could not find significant differences in these parameters for rHSA-FepivP and rHSA-FecycP. This result indicates that the substituent structure on the porphyrin plane does not cause any substantial change in the O_2 equilibria and kinetics of rHSA-heme.

Stability of O_2-Adduct Complex of Albumin-Heme

Accompanying the autooxidation of the central iron(II), the absorption band (λ_{max} 555 nm) slowly disappeared at 37°C, leading to formation of the inactive ferric porphyrin. The effect of the heme structure on the half-life of the O_2-adduct complex against the ferric state ($\tau_{1/2}$) was marked. The rHSA-FecycP had a $\tau_{1/2}$ of 9 h, which is 4.5-fold longer than that of rHSA-FepivP and close to that of the Mb (12 h at 37°C) [46].

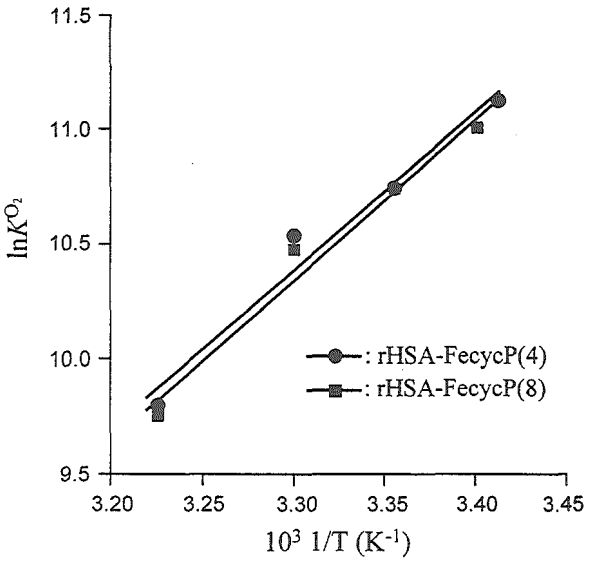

FIG. 4. Van't Hoff plots of O_2-binding affinity of rHSA-FecycP in phosphate-buffered solution (pH 7.3)

CO-Binding Property of rHSA-Heme

Upon addition of CO gas through the deoxy or oxy state of the rHSA-FecycP solution, the spectrum immediately exhibited formation of the carbonyl complex. The CO-binding affinity ($P_{1/2}^{CO}$) of rHSA-FecycP became 2.5-fold higher than that of rHSA-FepivP (Table 3) [9,47,48]. Kinetically, this is due to the low CO dissociation rate constant, k_{off}^{CO}. More recently, CO/O_2 discrimination of Hb and Mb has not been based mainly on distal steric constraints in the heme pocket; the emphasis has shifted to polar interactions in the binding pocket [49,50]. That is, a polar environment could favor the highly polarized coordinated Fe-O_2 unit over the apolar Fe-CO moiety. In FecycP, the hydrophobic cavity around the central ferrous ion probably contributes to the rise in CO-binding affinity. This interpretation is in good agreement with assumptions by other investigators.

Blood Compatibility

The red rHSA-FecycP solution showed a long shelf life (>2 years) at temperatures of 4°–37°C without any aggregation or precipitation. The solution properties also satisfied physiological requirements. The specific gravity was 1.013 (FecycP/rHSA = 1–8 mol/mol). The viscosity of 1.2 cP (at a high shear

TABLE 3. CO-binding parameters of rHSA-FecycP in phosphate-buffered solution (pH 7.3) at 25°C

Substance	$P_{1/2}^{CO}$(torr)	$k_{on}(M^{-1}s^{-1})$ Fast	$k_{on}(M^{-1}s^{-1})$ Slow
rHSA-FecycP(8)	0.04	5.9×10^6	8.9×10^5
rHSA-FepivP(8)	0.10	4.9×10^6	6.7×10^5
Hb (T-state)α[a]	0.30	2.2×10^5	

[a] Aqueous, pH 7.0–7.4, 20°C; refs. [47, 48].
The number in parenthesis is molar ratio of porphyrin and rHSA.

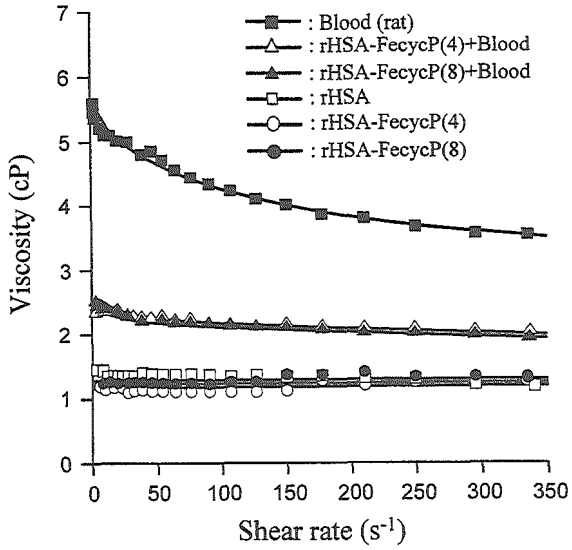

FIG. 5. Viscosity of rHSA-FecycP solution with whole blood at 37°C

rate of 230 s^{-1}) was much lower than that of whole blood (4.0 cP) and exhibited Newtonian-type shear rate dependence similar to that of rHSA itself (Fig. 5). Furthermore, the viscosity of the mixed dispersion with freshly drawn blood (1:1, v/v) showed 2.0 cP (at 230 s^{-1}), indicating that rHSA-FecycP had good compatibility with blood. Optical microscopic observations also revealed that the homogeneous morphology of the red blood cells was not affected by mixing with whole blood (not shown).

Conclusions

Human serum albumin incorporating synthetic heme formed an O_2-adduct complex under physiological conditions. In particular, oxygenated rHSA-FecycP showed high stability compared to the previous rHSA-FepivP, and its half-life reached a value similar to that of the native Mb. It has been also found

that another rHSA-heme complex incorporating an FecycP analogue with a histidyl base at the porphyrin periphery had an extremely long half-life of the oxygenated complex (25 h) under the same conditions (in this case the O_2-binding affinity is quite high) [35]. rHSA–FecycP with a $P_{1/2}$ value (34 torr at 37°C) similar to that of red blood cells is now the most promising material to be used as an artificial O_2 carrier. Exchange transfusion with rHSA-FecycP into anesthetized beagles to evaluate its clinical safety and efficacy is now under investigation.

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Studies on Red Cell Substitutes in Japan and Future Perspectives

Masuhiko Takaori

Key words. Artificial blood, Chemical property, Preclinical assessment, Clinical trial, Clinical efficacy

In 1966 Toyoda [1] at the Department of Surgery, Tokyo University School of Medicine, synthesized polystyrene encapsulated hemoglobin vesicles as artificial red cells and infused them in rabbits intravenously. However, they could not confirm whether or not those vesicles actually transported oxygen to the tissues. In 1979, Naito and Yokoyama [2] of Green Cross, Japan, produced Fluosol DA (Green Cross, Osaka, Japan), mixture of perfluorodecalin and perfluorotripropylamine, which looked like milk and was called "white blood". They performed some clinical trails in Japan. We used this product for a patient who suffered from unexpected massive bleeding and could not get proper blood for transfusion. The patient recovered uneventfully. However, it could be certain whether the Fluosol DA (Green Cross) was absolutely effective for oxygen transport, as it failed to transport sufficient amounts of oxygen to the tissues under normal atmospheric pressure without a high concentration of Fluosol DA [3].

Pharmaceutical companies such as Baxter, Biopure, Hemosol, and Alliance developed various artificial blood products in the past [4]. Data from some of the clinical trials which were performed in Europe [5] and the USA [6] have been published; however, none of the products progressed to clinical use. Nevertheless, the development of artificial blood for clinical use remains an urgent problem, particularly in the face of an expected shortage of blood for transfusion in the near future. In Japan we already occasionally experience

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shortfalls in blood supply for transfusion, and anticipate a very severe shortfall in the future. Watanabe et al. [7] demonstrated that demand for blood will exceed supply by 2005. We are collecting outdated blood and extracting hemoglobin (Hb) for storage now as a possible solution. We hope to convert the collected blood products into artificial blood for use as a supplement for blood for transfusion in the future.

Recent Progress in Artificial Blood

Since 1990 the research group at Waseda University has developed a liposome encapsulated hemoglobin vesicle (HbV) as a substitute for red blood cells. In 1995 the Terumo company, which was associated with Waseda's scientific research, produced a similar HbV called the Neo Red Cells (NRC) (Terumo, Tokyo, Japan). We tested this product in animal experiments and found that it could transport oxygen to the tissues [8]. Under hemodilution, in which the animal's hematocrit value decreased to about 12%, the mixed venous oxygen saturation could be maintained at mostly normal with NRC's (Terumo) infusion but not with a plasma substitute such as hydroxyethyl starch (HES) solution. Oxygen consumption was maintained sufficiently with NRC (Terumo) but not with HES solution (Table 1).

Similarly Motoki et al. [9] noted that NRC (Terumo) released a greater amount of oxygen in the peripheral tissues compared with autologous blood transfusion (Fig. 1). Cardiac output in our study [6] was maintained satisfactorily with NRC (Terumo). Furthermore, it was noted that the circulating blood volume in the same situation could be maintained at a normal level without adverse effects, particulary life-threating complications. Therefore we believe that NRC can be used in practice.

Yoshizu et al. [10] at Keio University hemodiluted rats in which the hematocrit value was decreased to around 20% with either albumin solution or NRC. They measured tissue oxygen tension in the kidney polarographically and noted that NRC could maintain oxygen tension higher than the albumin solution or autologous blood transfusion did. A similar trend was also noted in skeletal muscle.

Subsequently the Waseda and Keio groups improved the efficiency of HbV for oxygen transport, and recently presented the physicochemical properties [11] (Table 2).

In the meantime, Nishi and Kida [12] at the University of Kumamoto, School of Medicine, Department of Pharmacology, formed pyridoxal phosphate polyethylene conjugated hemoglobin dimer (Fig. 2) as artificial red cells and used them for organ perfusion. This product obtained 24 h half life in the circulation of rats. It was noted, however, that this product scavenged nitric

Table 1. Changes in oxygen partial pressure (PvO_2), oxygen saturation (SvO_2), oxygen content (CvO_2) in mixed venous blood and oxygen consumption (VO_2) following hemodilution with Neo Red Cell and HES (hydroxyethyl starch) solution

		Initial	4× hemodilutions	8× hemodilutions	1 h after hemodilution	2 h after hemodilution
PvO_2	H	62 ± 5	52 ± 4	46 ± 6	41 ± 7	41 ± 7
mmHg	N	64 ± 3	50 ± 4	45 ± 4	42 ± 6	42 ± 5
SvO_2	H	73 ± 4	66 ± 5	41 ± 4	38 ± 6	37 ± 9
%	N	73 ± 5	67 ± 6	51 ± 6**	55 ± 6**	52 ± 5**
CvO_2	H	14.7 ± 0.7	8.2 ± 0.4	2.2 ± 0.4	2.4 ± 0.5	2.4 ± 0.4
ml/dl	N	15.1 ± 0.5	8.0 ± 0.5	3.2 ± 0.4**	3.2 ± 0.6**	2.9 ± 0.5**
VO_2	H	91.4 ± 7.4	68.4 ± 5.3	56.2 ± 5.9	46.3 ± 7.5	42.4 ± 6.2
ml/min	N	94.9 ± 7.3	74.3 ± 8.6	74.1 ± 8.4**	77.2 ± 6.1**	80.5 ± 6.9**

mean ± SD.
H, hydroxyethyl starch group; N, Neo Red Cells group.
Source: Takaori M, Fukui A (1996) Treatment of massive hemorrhage with liposome encapsulated hemoglobin (NRC) and hydroxyethyl starch (HES) in beagles.
Artif Cells Blood Substs Immunob Biotechnol 24; 643–653.
Comparison between groups **: $p < 0.01$.

TABLE 2. Physicochemical Properties of HbV

Hb	10.0 g/dl
Hb/Lipid	1.7
PEG-DSPE	0.3 mol %
diameter	247 μm
allosteric effector (Pyridoxal Phosphate/Hb)	2.5 mol/mol
P50	33 mmHg
MetHb	<1.0%
HbCO	<2.0%
Suspension	Saline

Hb, hemoglobin; PEG-DSPE, polyethylene glycole; MetHb, methemoglobin; HbCO, carboxylhemoglobin.

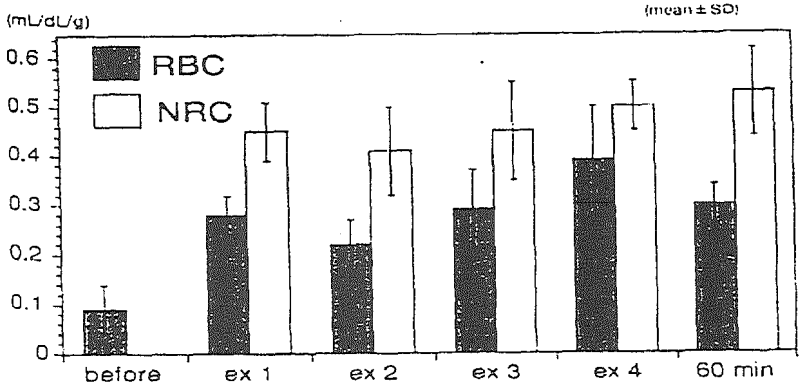

FIG. 1. AV (arteriovenous) oxygen content difference/Hb. Source: Usuba et al. [7]

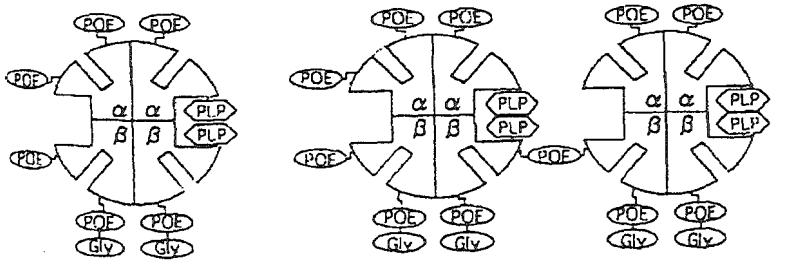

FIG. 2. Molecular structure of PHP. Source: Nishi and Kida [9]. *POE*, polyoxyethylene; *Gly*, glycine; *PLP*, phosphoenolpyruvate

oxide from the endothelium. Therefore, this product was excluded from clinical application.

Design of Artificial Red Cells

Although a search has been attempted for a substance which transports oxygen and releases it at the peripheral tissues more efficiently than hemoglobin, no such substance has yet been found. Therefore we concluded that natural Hb is best for this purpose at the present time.

We were concerned which type of artificial red cell is better, the cellular or acellular type. We concluded that the cellular type is superior to the acellular type for the following reasons:

1. Some substances, for example allostetic substances, such as pyridoxal phosphate as a substitute for 2.3 DPG (diphosphoglycerol), and a reductive substance such as homocysteine as substitute a for methemoglobin reductase, can be encapsulated in a vesicle accompanied with hemoglobin.
2. Greater blood flow may be maintained in the coronary and peripheral minute vessels, since the fluid contained in cellular particles can maintain a more similar blood viscosity, even under high-graded hemodilution.
3. Longer persistence of the artificial red cell in the circulating blood can be obtained with a cellular type. Rapid excretion in urine and in exhaled air, as seen with conjugated hemoglobin and with perfluorocarbon emulsion, respectively, will not occur with the cellular type.
4. Encapsulated Hb can be kept from direct contact with surrounding tissues, such as the endothelium and circulating blood cells, meaning the oxygen-carrying substance is protected and does not affect the surrounding tissues.

The Waseda research group enclosed pyridoxal phosphate in HbV and controlled P50 of hemoglobin at 33 mmHg (Table 2).

It has been found that extremely severe hemodilution with less than 10% of hematocrit value causes an increase in pulse pressure even when systolic pressure decreases slightly. Diastolic pressure is decreased markedly at 40 mmHg, which approaches the critical pressure for corony perfusion. However, when hemodilution can be done, with artificial blood which contains cellular type red cells and viscosity that can be maintained similarly to physiological blood, the perfusion pressure that will be created from peripheral vessel back pressure will be normal.

Retention time of artificial red cells in circulation after infusion needs to be longer than 24 h. If not, oxygen transport to the tissues will be reduced sharply. Since some physiological adaptation is needed, such as hematopoiesis

(2 ml/kg per day for red cell mass), an increase in 2,3 DPG in the red cells would need to be induced in accordance with gradual extravasation of the artificial red cells. Thus we can not safely use artificial blood with a short half life in clinical practice. The half-life of HbV is estimated as 16–18 h by the Waseda study in the rat [11] (Fig. 3). In addition, Tsutsui et al. [13] (Fig. 4), reported that the half-life is about 16–18 h in rats. However, we were recently informed that the half-life of HbV was 36–40 h in a primate (personal/communication, Ogata, Terumo).

Both the above studies present the perplexing problem of the rapid conversion from hemoglobin to metHb in vivo. To address this problem Waseda's investigators tried to encapsulate catalase, as reductase, in the vesicle. They encapsulated various doses of catalase in the vesicle, and found that encapsulation of catalase of 6000 unit/ml reduced the conversion of Hb to MetHb to $^1/_3$ of that of homocysteine alone. They observed that the suppresion of conversion seemed to be dose dependent and its optimum dose has not yet been decided [11].

With regard to direct contact of Hb with the surrounding tissues, Waseda and Keio's investigators bled 50% of circulating blood from rats and left the

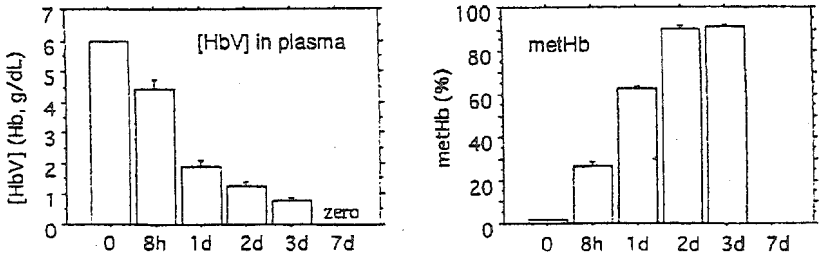

FIG. 3. Retention volume of HbV in plasma and metHb content. Source: Sakai et al. [12]

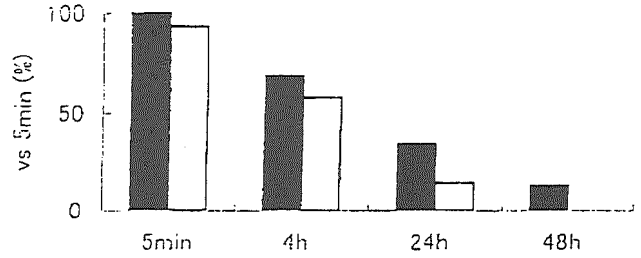

FIG. 4. Blood concentration of NRC on topload model. *Shaded*, total NRC-crit (%. vs 5 min); *unshaded*, NRC-crit with function estimated by NRC-crit and methemoglobin ratio (%. vs 5 min of total NRC-crit). Source: Tsutsui et al. [13]

blood in place for 15 min. They then replaced the blood loss with the withdrawn blood, human albumin solution, and the HbV suspended in 5% human albumin. They noted that no increase in blood pressure occurred immediately after the infusion of HbV (Fig. 5). It is suggested that encapsulation of Hb with liposome membrane blocked nitric oxide scavenging and prevented vasoconstriction [12]. Incidentally, rats treated with the HbV or autologous blood all survived but rats treated with albumin solution did not. In addition, blood lactate levels elevated transiently in a shock state and recovered rapidly after the infusion of HbV and autologous blood. However, lactate levels with the infusion of human albumin recovered in surviving animals, but may remain or elevate in nonsurvival.

On the other hand, some objections could be made against our recommendation for cellular types such as:

1. The complexity of production process. Many processes for the encapsulation of hemoglobin, allostetic substances and metHb reductase, PEGylation, such as coating the surface of HbV with polyethylene glycol, and the extraction of carbon monoxide are required.
2. The cost for cellular artificial red cells would be higher than that of acellular types.
3. Apprehension about phagocytotic tissue retention of the HbV and subsequent immunosuppression is not excluded.

Waseda's investigators observed microscopically that HbVs were captured in microphages and bone marrow cells. Therefore the weight of the liver and spleen was increased respectively. They noted a paradoxical phenomenon that phagocytotic activity for carbon particles infused intravenously increased temporarily after infusion of HbV but recovered completely 7 days later.

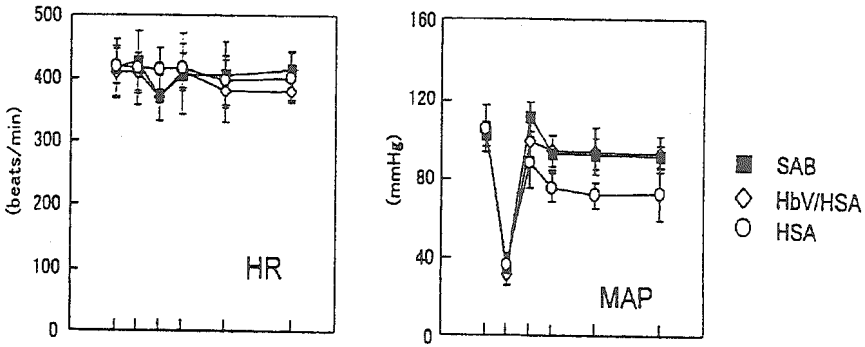

FIG. 5. *HR*, heart rate; *MAP*, mean arterial pressure; *SAB*, autologous blood; *HbV/HSA*, HbV/human albumin; *HSA*, human albumin. Source: Sakai et al. [12]

Abe et al. [15] at the Hokkaido Blood Supply Center tested the effect of HbV on complement hemolytic activity and the complement killing activity of bacteria. They noted that infusion of HbV reduced the hemolytic activity in a dose-dependent manner, and that HbV infusion suppressed the bacteria-killing activity with complements. However, they concluded that the human defense system against bacterial infection would be little influenced [15].

Yanagida et al. [16] noted a severe decrease in immunoglobulins after hemodilution with NRC, in which animal hematocrit was decreased to 7.1%. However, circulating neutrophils and lymphocytes increased after the hemodilution. These changes are similar to those we observed in severe hemodilution with dextran and HES [17]. Therefore, changes observed seem unlikely specific responses to NRC. Incidentally, changes in immunoglobulins and leukocytes recovered within a few days.

Hoka et al. [18] of the Kitazato University School of Medicine, Department of Anesthesiology, studied the effect of HbV on interaction between the neutrophil and the endothelium. Under vital microscopic observation they found in the golden hamster that the migration of the neutrophil through the endothelium layer of the buccal mucous pouch was markedly attenuated with infusion of HbV.

Safety and Efficacy of Artificial Blood

In accordance with the accumulation of data about the safety and efficacy of HbV in pre-clinical studies, a project team supported by the Japanese Ministry of Health and Welfare, sought to make safety and efficacy criteria for the clinical use of HbV.

Several studies referring to chemical and biological safety have been reported [19–22]. However, particularly with HbV, we proposed several measures recently. In the process of encapsulation of Hb in liposome vesicles, it is necessary convert Hb to carbon monoxide Hb. Therefore, it must be confirmed whether or not there will be residual carbon monoxide Hb and carbon monoxide in the solution. Likewise there must be checks to see if excess phospholipid of lipsosome or polyethylene glycol remain in the solution. Obviously endotoxin contamination cannot be allowed. It must be also kept absolutely sterile during storage. The physicochemical properties of HbV as shown in Table 2 have been tested and guaranteed to be absolutely safe.

Concerning the clinical safety of artificial blood, several studies have reported as above. However, a check list for finding adverse effects of HbV on vital functions is shown in Table 3.

Most of those have been tested in pre-clinical studies. However, due to present inability to mass produce HbV, a few items remain to be tested in the near future. Incidentally, the criteria of the Japanese Ministry of Health and Welfare regarding the severity of adverse effects should be applied in pre-clinical studies and clinical trials.

TABLE 3. Safety of HbV solution

Clinical Assessment	
Psychological function and behavior	Mental and nervous function
Tendon and muscle function	Heart and circulartory function
Respiratory and gas exchange function	Hepatic function
Body fluid buffering capacity	Serum electrolyte composition
Renal function	Digestive function
Hemostatic and fibrinolytic function	Hematopoietic function
Endocrine functions	Defensive and immune function
Reproductive function	Teratogenicity
Tumorigenicity	Interaction with commonly used drugs

Regarding the efficacy of artificial blood, however, few criteria have been established. As with the safety criteria, efficacy of HbV as artificial blood should be evaluated from physicochemical activity and clinical efficacy points of view. The following should be considered: (1) oxygen-deliverying capacity, which is mainly controlled by the amount of Hb and the oxygen dissociation curve of Hb inside of the vesicles; (2) conversion rate of Hb to metHb during storage and in vivo after infusion; (3) dispersibility of the vesicles in the solution and blood; (4) size of the vesicle; (5) viscosity of the solution; (6) homogeneity and stability of the vesicles in the solution; and (7) pH of the solution.

The most important property in clinical efficacy is an ability for the oxygen supply to reach tissues. It depends on good pulmonary oxygenation, oxygen extraction in the tissues, a lower conversion rate from Hb to metHb, and having a cardiac output associated with normal circulating blood volume after infusion for blood loss. Finally it depends on adequate retention of the vesicles in the circulation.

After confirmation of safety and efficacy, clinical trials must be performed before use in practice. The Society of Blood Substitutes, Japan, provided guidelines for clinical trials of artificial blood 6 years ago. We intend to follow those guidelines to clinical trial.

Design of Clinical Trials

Clinical trials should satisfy the following two points: (1) good design to obtain definite results for evidence, and (2) obtaining proper informed consent from subjects.

Table 4. Ethical considerations for clinical trials

Clinical trials should be done for the treatment or therapy for patients
Clinical trials should be equivalent or superior to conventional treatment or therapy
Clinical trials must be performed with informed consent

Table 5. Criteria for selection for clinical trial

Replacement of surgical blood loss (15–20 ml/kg)
Hemodilutional autologous blood transfusion
Blood transfusion for unexpected intraoperative bleeding
Blood transfusion for emergent surgery without proper blood
Transfusion for patients with uncommon blood types whose surgery is relatively urgent

Limit to anticipated blood loss of 15–20 ml/kg.

For achievement of definite satisfactory results, four items should be prepared, namely: (1) proper setting of controls for the procedure or treatment, (2) establishing control measures before the procedure or treatment begins, (3) application of routine laboratory tests and simple procedures, and (4) exclusion of subjects with severe illness or subjects with complicated or unstable conditions.

Regarding medical ethics, we must recognize and observe three items listed in Table 4, namely: (1) clinical trials should be performed for treatment or therapy of the patient, (2) the treatment or therapy in clinical trials should be equivalent or superior to conventional treatment or therapy, and (3) clinical trials must be done with the proper informed consent of the patient.

Considering the above restrictions, we designed a clinical trial (Table 5). It will be limited to treatment of 15–20 ml/kg blood loss, such as hemodilutional autologous blood transfusion, transfusion for unexpected surgical bleeding, transfusion in emergent surgery without proper blood preparation, and transfusion for patients with an uncommon blood type whose surgery is relatively urgent.

Some other applications might be proposed (Table 6). We would like to avoid other applications in the first clinical trial, since pathological conditions or illness influence procedures for measurement and accuracy, and so data obtained from those patients would skew the results.

Conclusions

Further assessment and confirmation remain to be done for the safety of HbV. Further improvement of the physicochemical properties of HbV and its mass production should be done in the near future. For example, a fine filter with

Table 6. No program projected first clinical trial for new artificial blood

Post-traumatic hypovolemia
Hemorrhagic shock
Myocardial and cerebral ischemia
Supplemental treatment for idiopathic anemia
Priming for extraorporeal circuit
Superoxygenation for malignant neoplasma therapy
Liquid ventilation
Perfusion and preservation for organ transplantation

Table 7. Social needs for artificial blood

Disaster use	long-term shelf storage
Emergent use	before arrival of matched or O-blood
Shortage of blood for transfusion	
Recycle use of hemoglobin from donated blood	
Application as universal blood	
Avoiding transfusion complications and human errors	
Transfusion for patients with uncommon blood type or with refusal of donated blood	

0.2 μ diameter is used for elimination of bacteria from the product at the present time; however more definite methods for asepsis such as carbon dioxide replenishment or gamma radiation, should be introduced.

Social needs for artificial blood are shown in Table 7. They are all urgent. In my view, blood transfusions as routinely performed in practice today do not fit well with sophisticated medicine. It seems that, in the future, transfusion medicine, particularly red cell transfusion, will be replaced by infusion of artificial blood. Therefore I earnestly hope for great development of artificial blood and its use in clinical practice in the near future.

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