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Hemoglobin-Vesicle, a Cellular Artificial Oxygen Carrier that Fulfils the Physiological Roles of the Red Blood Cell Structure

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Abstract Hb-vesicles (HbV) are artificial O_2 carriers encapsulating concentrated Hb solution (35 g/dL) with a phospholipid bilayer membrane (liposome). The concentration of the HbV suspension is extremely high ([Hb] = 10 g/dL) and it has an O_2 carrying capacity that is comparable to that of blood. HbV is much smaller than RBC (250 vs. 8000 nm), but it recreates the functions of RBCs; (i) the slower rate of O_2 unloading than Hb solution; (ii) colloid osmotic pressure is zero; (iii) the viscosity of a HbV suspension is adjustable to that of blood; (iv) HbV is finally captured by and degraded in RES; (v) co-encapsulation of an allosteric effector to regulate O_2 affinity; (vi) the lipid bilayer membrane prevents direct contact of Hb and vasculature; (vii) NO-binding is retarded to some extent by an intracellular diffusion barrier, and HbV does not induce vasoconstriction. (viii) Both RBC and HbV can be a carrier of not only O_2 but also exogenous CO. However, HbV has limitations such as a shorter functional half-life when compared with RBCs. On the other hand, the advantages of HbV are that it is pathogen-free and blood-type-antigen-free; moreover, it can withstand long-term storage of a few years, none of which can be achieved by the RBC transfusion systems.

1 Introduction

Biconcave RBCs deform to a parachute-like configuration to flow through a narrower capillary. This profile is believed to be effective to increase the surface-to-volume ratio and stir the intracellular viscous Hb solution to facilitate the gas exchange. On the other hand, physicochemical analyses have revealed that O_2 unloading of Hb is significantly retarded by compartmentalization in RBC. Why has nature selected such an inefficient cellular structure for gas transport? Interestingly, some of the answers to this question have been revised by the

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research of blood substitutes. They are, (i) retardation and targeting of O_2 unloading at microcirculation to avoid autoregulatory vasoconstriction; (ii) reduction of a high colloidal osmotic pressure, COP, of an Hb solution to zero, to increase blood Hb concentration; (iii) rheology control of blood, a RBCs dispersion, to a non-Newtonian viscous fluid; (iv) prevention of extravasation or excretion through renal glomeruli; (v) preservation of the chemical environment in cells, such as the concentrations of electrolytes and enzymes; (vi) prevention of direct contact of toxic Hbs and endothelial cell lying, and (vii) modulation of reactions with NO as an endothelium derived relaxation factor, EDRF. These observations reassure the importance of the cellular structure of RBCs to design Hb-based oxygen carriers.

Hb-vesicles (HbV) are artificial O_2 carriers encapsulating concentrated Hb solution (35 g/dL) with a phospholipid bilayer membrane [1]. Concentration of the HbV suspension is extremely high ([Hb] = 10 g/dL, [lipids] = 6 g/dL, volume fraction, ca. 40 vol%) and it has an oxygen carrying capacity that is comparable to that of blood. In this review paper, we summarize the characteristics of HbV that can fulfill some of the physiological roles of the cellular RBC structure.

2 Structural Stability and Suspension Properties

Many people think liposomes are unstable capsules. However, it depends on the lipids and the composition. In the case of RBCs, the sophisticated cytoskeleton network structure stabilizes the cellular structure. However, hypotonic hemolysis easily occurs. We confirmed that HbV are more resistant than RBCs to hypotonic shock, freezing by liquid N_2 and thawing, enzymatic attack (phospholipase A_2) [2], and a shear stress (1500 s^{-1}). Moreover, it can be stored at room temperature over 2 years [3]. In spite of such high stability, we confirmed with animal experiments that HbV are eventually captured by reticuloendothelial system (RES) and degraded promptly within 2 weeks without decomposing (hemolysis) in blood circulation [4]. Phospholipid vesicles for the encapsulation of Hb would be beneficial for heme detoxification through their preferential delivery to the RES, a physiological compartment for degradation of senescent RBCs, even at doses greater than putative clinical doses [5].

Colloid osmotic pressure (COP) is an important factor to determine blood volume in the body. Hb solution (5 g/dL) showed the COP of 16 Torr [6]. Polymerization of Hb reduces COP depending on the resulting molecular weight. PEG-conjugated Hb shows the largest COP, which is about 3 times higher than blood (ca. 25 Torr) due to the highly hydrated PEG chains [7]. On the other hand, both HbV and washed RBCs showed 0 Torr because of the suspension of large particles: the number of total particles of HbV is less than 1/100 of the number of Hb molecules at the same Hb concentration [6]. COP acts in opposition to hydrostatic pressure to balance the distribution of fluid between blood and interstitial compartments [7]. COP is a colligative property,

depending proportionally on the concentration of protein exerting the force and specifically on the macromolecular properties of that protein. Solutions with high COP cause significant transcapillary filtration of water in the direction from the interstitial space into the vascular lumen. An increase in blood volume is advantageous to increase cardiac output for resuscitation, though the composition of other components of blood and tissue will also be compromised. HbV, on the other hand, does not have an oncotic effect, and the particle should be suspended in a plasma expander (plasma substitute, water-soluble polymer). The COP of the resulting suspension is identical to that of the suspending medium. When HbV is suspended in 5% rHSA, the suspension shows 20 Torr at any Hb concentration. HbV can create a suspension of [Hb] = 10 g/dL at the physiologic COP, that cannot be attained easily by other chemically modified Hb solutions.

The HbV suspended in rHSA ([Hb] = 10 g/dL) was nearly Newtonian [8]. Other plasma substitute polymers -hydroxylethyl starch (HES), dextran (DEX), and modified fluid gelatin (MFG)- induced HbV flocculation, possibly by depletion interaction, and rendered the suspensions as non-Newtonian with a shear-thinning profile. These HbV suspensions showed a high storage modulus (G') because of the presence of flocculated HbV. However, HbV suspended in rHSA exhibited a very low G'. The viscosities of HbV suspended in DEX, MFG, and high-molecular-weight HES solutions responded quickly to rapid step changes in shear rates of $0.1-100\ s^{-1}$ and a return to $0.1\ s^{-1}$, indicating that flocculation is both rapid and reversible. The HbV suspension viscosity was influenced by the presence of plasma substitutes. The HbV suspension provides a unique opportunity to manipulate rheological properties for various clinical applications.

3 The Rate of O_2-Unloading, and NO- and CO-Bindings

The O_2-release from flowing HbVs was examined using an O_2-permeable, fluorinated ethylenepropylene copolymer tube (inner diameter, 28 μm) exposed to a deoxygenated environment [9]. Measurement of O_2 release was performed using an apparatus that consisted of an inverted microscope and a spectrophotometer, and the rate of O_2 release was determined based on the visible absorption spectrum in the Q band of Hb. HbVs and human RBCs were mixed in various volume ratios at [Hb] = 10 g/dl, and the suspension was perfused at the centerline flow velocity of 1 mm/s through the narrow tube. The mixtures of cell-free Hb solution and RBCs were also tested. Because HbVs were homogeneously dispersed, increasing the volume of the HbV suspension resulted in a thicker marginal RBC-free layer. Irrespective of the mixing ratio, the rate of O_2 release from the HbV/RBC mixtures was similar to that of RBCs alone. On the other hand, the addition of 50 vol% of acellular Hb solution to RBCs significantly enhanced the rate of deoxygenation. This difference 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 diffusion for the facilitated O_2

transport. It has been suggested that faster O_2 unloading from the HBOCs is advantageous for tissue oxygenation. However, this concept is controversial with regard to the recent findings, because an excess O_2 supply would cause autoregulatory vasoconstriction and microcirculatory disorders. We confirmed that HbVs do not induce vasoconstriction and hypertension. This is not only owing to the reduced inactivation of NO (see below) but also possibly due to the moderate O_2 release rate that is similar to RBCs.

One of the important roles of the RBC structure is the retardation of NO-binding. However, the mechanism has been controversial, whether, (i) an unstirred layer surrounding the cell should be the diffusion barrier, (ii) cytoskeletal cell membrane can be the diffusion barrier, or (iii) the highly concentrated Hb solution can be the barrier. To clarify the mechanism, we analyzed HbVs with different intracellular Hb concentrations, $[Hb]_{in}$, and different particle sizes using stopped-flow spectrophotometry [10]. In the case of different $[Hb]_{in}$ (1- 35 g/dl), NO-binding is retarded at a higher $[Hb]_{in}$, on the other hand, CO-binding did not show such retardation. In the case of different particle diameter of HbV at constant $[Hb]_{in}$, 35 g/dl, NO-binding is retarded with a larger particle, while the CO-binding did not show such changes. The computer simulations can recreate these tendencies. The two-dimensional concentration changes of free-NO and unbound free-hemes in one HbV at the $[Hb]_{in}$ was 1 g/dl showed that NO diffuses rapidly into HbV and the reaction proceeds quite homogeneously. On the other hand, HbV at $[Hb]_{in} = 35$ g/dl showed heterogeneous distribution. The concentration gradients of both NO and heme change from the interior surface to the core. The intrinsically fast NO-binding induces an intracellular diffusion barrier in a highly concentrated Hb solution, but not for the slow CO-binding. We can estimate the apparent binding rate constant of a particle encapsulating a 35-g/dl Hb with 8000-nm diameter, and they are similar to the reported values for RBCs. The mechanism of retardation of NO-binding is controversial, but from these data, we estimate that (i) rapid NO-binding reaction induces intracellular diffusion barrier, (ii) cellular membrane cannot be a barrier for gas diffusion, and (iii) a higher $[Hb]_{in}$ and larger size are the factors for retarding NO-binding. However, we have to admit that NO-binding of HbV is much faster than that of RBC. The absence of vasoconstriction for HbV cannot be explained with these data. We believe that small Hb would permeate across the endothelium to reach to the site where NO is produced and transferred to the smooth muscle. It was recently reported that dextran conjugated Hb permeates through the endothelium. However, much larger HbV would remain in the lumen and does not bind NO in the endothelium.

4 Resuscitation from Hemorrhagic Shock with HbV

The first attempt of HbV to restore the systemic condition after hemorrhagic shock was conducted using anesthetized Wistar rats. After 50% blood withdrawal, the rats showed hypotension and considerable metabolic acidosis and

hyperventilation. They received HbV suspended in rHSA, shed autologous blood (SAB), or rHSA alone. The HbV group restored mean arterial pressure, similar to the SAB group, which was significantly higher than the rHSA group. No remarkable difference was visible in the blood gas variables between the resuscitated groups. However, two of eight rats in the rHSA group died before 6 h [11]. After removing the catheters and awakening, the rats were housed in cages for upto 14 days. The HbV group gained body weight; the reduced Hct returned to the original level in 7 days, indicating elevated hematopoiesis. Both groups showed transient elevation of AST and ALT at 1 day. Splenomegaly was significant in the HbV group at 3 days because of the accumulation of HbV. However, it subsided within 14 days. Histopathological observation indicated that a significant amount of HbV accumulated in the spleen macrophages, and complete disappearance within 14 days. These results indicate that HbV is useful as a resuscitative fluid for hemorrhagic shock. Its performance is comparable to that of SAB. Similar experiments using beagles have shown 1-year survival after resuscitation with HbV.

The above elevations of AST and ALT after resuscitation with HbV or RBC indicate the systemic reperfusion injury. Recent reports on cytoprotective effects of exogenous CO urged us to test infusion of CO-bound HbV and RBC in hemorrhagic-shocked rats to improve tissue viability [12]. Using the similar model, hemorrhagic shocked Wistar rats received CO-HbV, CO-RBC, O_2-HbV, or O_2-RBC suspended in 5% rHSA. All groups showed prompt recovery of blood pressure and blood gas parameters, and survived for 6 h of observation period. Plasma AST, ALT and LDH levels were elevated at 6 h in the O_2-HbV and O_2-RBC groups. They were significantly lower in the CO-HbV and CO-RBC groups. Blood HbCO levels (26–39%) decreased to less than 3% at 6 h while CO was exhaled through the lung. Both HbV and RBC gradually gained the O_2 transport function. Collectively, both CO-HbV and CO-RBC showed a resuscitative effect and reduced oxidative damage to organs. Adverse and poisonous effects of CO gas were not evident for 6 h in this experimental model. Further study is necessary to clarify the neurological impact of a longer observation period, though the results suggest a possible new clinical indication.

In conclusion, HbV can mimic the functions of RBCs. However, the half-life of HbV is much shorter than that of RBCs, and limits their use. On the other hand, the advantages of HbV are that it is pathogen-free and blood-type-antigen-free; moreover, it can withstand long-term storage of a few years, none of which can be achieved by the RBC transfusion systems. We continue further development of HbV aiming at the eventual realization and contribution to the clinical medicine.

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27

Hemoglobin Vesicles as a Cellular-type Hemoglobin-based Oxygen Carrier

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27.1 Introduction

Hemoglobin (Hb) is the most abundant protein in blood (12–15 g/dL), and should be the most essential protein. However, Hb becomes toxic once it is released from red blood cells (RBCs), which is evident in some pathological hemolytic diseases. Chemically modified cell-free Hb-based oxygen carriers (HBOCs), such as intramolecularly crosslinked, polymerized, and polymer-conjugated Hbs, have been synthesized to prevent the toxic effect of cell-free Hbs. However, no product is commercially available yet. Some safety issues arose during the final stage of clinical trials. It seems difficult to completely eliminate the side effect of cell-free Hbs by chemical modification. Now is the time to reconsider the physiological importance of the cellular structure of RBCs. Why is Hb compartmentalized in RBCs with such a complicated corpuscular structure? Hb vesicles

* Emeritus Professor Eishun Tsuchida passed away during the submission of this manuscript.

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(HbVs) are artificial oxygen carriers encapsulating concentrated Hb solution (35 g/dL) with a phospholipid bilayer membrane. HbVs are designed to mimic or overcome the function of RBCs. In this chapter, we focus on the concept of Hb encapsulation and recent topics concerning HbVs, especially reactions with gaseous molecules (O_2, NO, CO), which greatly relate to its safety and a new application.

27.2 The Concept of Hb Encapsulation in Liposomes

Hb encapsulation was first performed by Chang in the 1950s [1], using a polymer membrane. Some Japanese groups also tested Hb encapsulation with gelatin, gum Arabic, silicone, and so on. Nevertheless, it was extremely difficult to regulate the particle size to be appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility. After Bangham and Horne reported in 1964 [2] that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior, it seemed reasonable to use such vesicles for Hb encapsulation. Djordjevici and Miller [3] prepared a liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acid, and so on. Since then, many groups have tested encapsulated Hbs using liposomes [4–7]. Some failed initially, and some are progressing with the aim of clinical usage. The Naval Research Laboratory presented remarkable progress on LEH [8], but it suspended development about 10 years ago. What we call HbVs with high-efficiency production processes and improved properties have been established by our group, based on nanotechnologies of molecular assembly and pharmacological and physiological aspects [9]. In spite of such a large number of studies of HBOCs in general, no product has so far been tested clinically because of the difficulty of the production method. Chemically modified cell-free HBOCs are much easier to produce, therefore more researchers have tested the cell-free types, and they have been more advanced than the cellular type in entering clinical trials. However, during the long history of R&D, some unexpected problems arose for cell-free HBOCs, presumably due to the direct exposure of Hb to vasculature.

It has been well understood that the compartmentalization of Hb in RBCs is important for: (i) prevention of extravasation or excretion through renal glomeruli; (ii) preservation of the chemical environment in cells, such as the concentrations of electrolytes and enzymes; and (iii) rheology control of blood, an RBC dispersion, to a non-Newtonian viscous fluid. Moreover, for us it seems that RBCs are evolutionally designed for: (iv) retardation and targeting of O_2 unloading at microcirculation to avoid autoregulatory vasoconstriction; (v) reduction of a high colloidal osmotic pressure of Hb solution to zero, to increase blood Hb concentration; and (vi) modulation of reactions with NO as an endothelium-derived relaxation factor (EDRF). Now we have to consider the physiological importance of RBC structure, and mimic the structure to design the optimal HBOCs.

Our HbVs are artificial oxygen carriers encapsulating concentrated Hb solution (35 g/dL) with a phospholipid bilayer membrane [7]. Concentration of the HbV suspension is extremely high ([Hb] = 10 g/dL, [lipids] = 6 g/dL, volume fraction ~40%). HbV has an oxygen-carrying capacity that is comparable to that of blood. HbV is much smaller than RBCs (250 vs 8000 nm), but it recreates the functions of RBCs, as has been confirmed by many animal experiments testing its effectiveness as a resuscitative

Table 27.1 *Preclinical studies of HbV as a transfusion alternative and for other therapeutics.*

	Indication	Ref.
1.	Resuscitative fluid for hemorrhagic shock	[11–13]
2.	Hemodilution	[14]
3.	Priming fluid for extracorporeal membrane oxygenator (ECMO) for cardiopulmonary bypass	[10]
4.	Perfusate for resected organs	[15, 16]
5.	Oxygenation of ischemic brain (stroke)	[17]
6.	Oxygenation of ischemic skin flap (plastic surgery)	[18, 19]
7.	Tumor oxygenation for irradiation sensitization	[20]
8.	CO carrier for cytoprotection at reperfusion	[21]
9.	Measurement of brain oxygen consumption by positron emission tomography (PET)	[22]

fluid for hemorrhagic shock, hemodilution, and a prime for cardiopulmonary bypass [10–12] (Table 27.1). Other characteristics similar to those of RBCs include: (i) the rate of O_2 unloading is slower than Hb solution [23]; (ii) colloid osmotic pressure is zero at [Hb] = 10 g/dL, and it has to be co-injected with or suspended in a plasma substitute such as albumin or HES [24]; (iii) the resulting viscosity of an HbV suspension is adjustable to that of blood [25]; (iv) HbV is finally captured by RES and the components are degraded and excreted [13, 26, 27]; (v) the HbV particle of itself is not eliminated through glomeruli [28]; (vi) PLP is co-encapsulated as an allosteric effector, instead of 2,3-diphosphoglyceric acid, to regulate oxygen affinity [18, 29]; (vii) no hemolysis occurs during circulation and the lipid-bilayer membrane prevents direct contact of Hb and vasculature; and (viii) reaction of NO is retarded to some extent by an intracellular diffusion barrier, and HbV does not induce vasoconstriction [30–32].

In the next section we focus on the gas reactions of cell-free Hb and cellular HbV.

27.3 Hb Encapsulation Retards Gas Reactions

The major remaining hurdle before clinical approval of the earliest generation of HBOCs is vasoconstriction and resulting hypertension, which is presumably attributable to the high reactivity of Hb with NO [33]. It has been suggested that Hbs permeate across the endothelial cell layer to the space near the smooth muscle and inactivate NO. However, cellular HbVs induce neither vasoconstriction nor hypertension [30]. A physicochemical analysis using stopped-flow rapid-scan spectrophotometry clarified that Hb encapsulation in vesicles retards NO binding in comparison to Hb because an intracellular diffusion barrier of NO is formed. The requisites for this diffusion barrier are (i) a more concentrated intracellular Hb solution and (ii) a larger particle size [31, 32] (Figure 27.1a). Even though various kinds of liposome-encapsulated Hb have been studied by many groups [7], our HbV encapsulates a highly concentrated Hb solution (>35 g/dL) with a regulated large particle diameter (250–280 nm) and attains 10 g/dL Hb concentration in the suspension. The absence of vasoconstriction in the case of intravenous HbV injection might

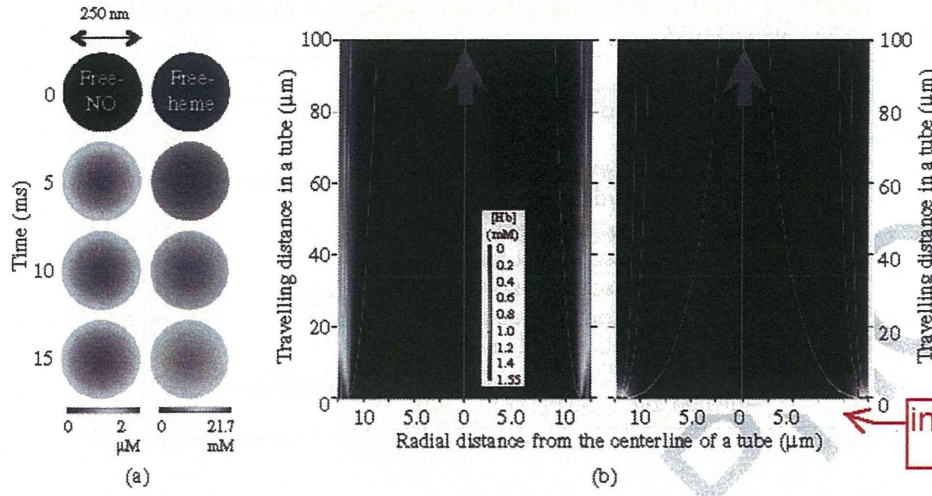

Figure 27.1 *Encapsulation of Hb in vesicles retards NO binding. (a) Schematic two-dimensional representation of the simulated time courses of distributions of unbound free NO and unbound free heme (deoxy form) in one HbV (250 nm) after immediate mixing of NO and HbV by stopped-flow method. Computer simulation shows that both free NO and unbound hemes are distributed heterogeneously. The concentration changes gradually from the surface to the core. The determinant factor of retardation of NO binding should be the intracellular diffusion barrier, which was induced by: (i) intrinsically larger binding rate constant of NO to a heme in an Hb molecule; (ii) numerous hemes as sites of gas entrapment at a higher Hb concentration; (iii) a slowed gas diffusion in the intracellular viscous Hb solution; and (iv) a longer gas diffusion distance in a larger capsule [31]. (b) Schematic representation of the simulated density distribution and track of HbV (left) and Hb (right) in a narrow tube (<100 μm traveling distance). We assumed that two different solutions with the same physicochemical properties enter and flow through the same tube. The radius of the tube was 12.5 μm: component 1 (blue color) enters the core of the tube (radial distance from the centerline, 0–11 μm) and component 2 (red color) enters near the wall (radial distance from the centerline, 11–12.5 μm). Finally, both components are mixed completely. The diffusivity of HbV is much slower than that of Hb, resulting in the retarded gas reactions in microvessels. The concentration of Hb is expressed as heme concentration ([Hb] = 1.55 mM at 10 g/dL) [39]. (Reproduced with permission from* Am J Physiol. Heart Circ. Physiol., ***298****, H956–H965 (2010)).*

be related to the lowered NO binding rate constant, though it is much larger than that of RBCs, and the lowered permeability across the endothelial cell layer in the vascular wall.

The proposed mechanism of vasoconstriction induced by HBOCs in relation to gaseous molecules is not limited to NO scavenging. For example, endogenous carbon monoxide (CO) is produced by constitutive hemeoxygenase-2 in hepatocytes, serving as a vasorelaxation factor in hepatic microcirculation. Hb permeates across the fenestrated endothelium, scavenges CO, and induces constriction of sinusoids and augments peripheral resistance [15]. Oversupply of O_2 induces autoregulatory vasoconstriction to regulate the O_2 supply [34]. Injection of small HBOCs induces vasoconstriction, probably because of the facilitated O_2 transport [35].

These reports imply the importance of studying the reaction profiles of HBOCs with NO, CO, and O_2. Stopped-flow rapid-scan spectrophotometry and flash photolysis are common methods of defining the binding and dissociation rate constants of Hb [31, 32, 36, 37]. However, Hb concentration in a cuvette must be diluted extremely, for example to 2 μM heme concentration ([Hb] = 0.003 g/dL). On the other hand, gas-permeable narrow tubes enable the measurement of the O_2-releasing rates of HBOCs and RBCs during their flow through the tubes at a practical Hb concentration (6–13 g/dL) [23, 35, 38]. We used gas-permeable narrow tubes made of perfluorinated polymer to study not only O_2-release but also NO-binding and CO-binding profiles. We examined these gas reactions when Hb-containing solutions of four kinds were perfused through artificial narrow tubes at a practical Hb concentration (10 g/dL). Purified Hb solution, polymerized bovine Hb ($Poly_BHb$), encapsulated Hb (HbV, 279 nm), and RBCs were perfused through a gas-permeable narrow tube (25 μm inner diameter) at 1 mm/second centerline velocity. The level of reactions was determined microscopically based on the visible-light absorption spectrum of Hb. When the tube was immersed in NO and CO atmospheres, both NO binding and CO binding of deoxygenated Hb and $Poly_BHb$ in the tube were faster than those of HbV and RBCs, and HbV and RBCs showed almost identical binding rates. When the tube was immersed in an N_2 atmosphere, oxygenated Hb and $Poly_BHb$ showed much faster O_2 release than did HbV and RBCs. $Poly_BHb$ showed a faster reaction than Hb because of the lower O_2 affinity of $Poly_BHb$ than of Hb [39].

The diffusion process of the particles was simulated using Navier–Stokes and Maxwell–Stefan equations (Figure 27.1b). Results clarified that small Hb (6 nm) diffuses laterally and mixes rapidly. However, the large-dimension HbV shows no such rapid diffusion. The NO and CO molecules, which diffuse through the tube wall and enter the lumen, would immediately react with Hb-containing solutions at the interface. Therefore, the fast mixing would be effective in creating more binding sites of these gas molecules. In the case of O_2 release, O_2 can be removed more easily at the tube wall, where the O_2 concentration gradient is the greatest. The fast mixing would create a higher concentration gradient and fast O_2 transfer. The purely physicochemical differences in diffusivity of the particles and the resulting reactivity with gas molecules are one factor inducing biological vasoconstriction of HBOCs.

27.4 HBOCs as a Carrier of not Only O_2 but Also CO

CO, biliverdin, and bilirubin are produced during oxidative heme degradation that is catalyzed by a stress protein: heme oxygenase (HO). They mediate antioxidative, antiproliferative, and anti-inflammatory effects [40]. Endogenous CO shows a vasorelaxation effect, as does NO [41]. Motterlini *et al.* [42] synthesized a series of CO-releasing metal complexes; subsequent *in vivo* studies clarified some pharmacological effects. Despite the poisonous effect of CO gas, low-concentration CO inhalation (250 ppm) was tested in animal models of hemorrhagic shock, septic shock, and ischemia-reperfusion [43]. Some cytoprotective effects were obtained and the mechanism has been studied extensively. Cabrales *et al.* [44] recently reported CO-bound RBC injection to hemorrhaged hamsters and clarified its cytoprotective effect in subcutaneous microcirculation. These studies have led us to test intravenous injection of CO as a ligand of heme in HBOCs that have been extensively studied as transfusion alternatives.

A traumatic hemorrhage might cause a shock state, which subsequently causes a systemic inflammatory response, in some cases leading to multiple organ failure (MOF). Resuscitation with transfusion or HBOCs with an O_2-carrying capacity induces reperfusion injury, as evidenced by elevations of plasma enzyme levels and tissue cytokine levels [12, 45, 46]. Actually, we observed elevation of plasma enzyme levels 6 hours after resuscitation from hemorrhagic shock by administration of O_2-bound RBCs and HbVs in a rat model [11]. It is expected that co-injection of cytoprotective CO would improve resuscitative effects. For this study, using the same experimental model, we tested injection of CO-bound HbVs for the first time as an exogenous CO supplier for fluid resuscitation. In comparative experiments, we also tested empty vesicles (EVs) which carry neither O_2 nor CO, and CO-bound RBCs. All fluids showed restoration of blood-pressure and blood-gas parameters, and the rats survived for 6 hours of observation period. No remarkable difference was found among the groups, except that the EV group showed significant hypotension. Plasma enzyme levels (AST and ALT) were elevated, especially in the O_2-HbV, O_2-RBC, and EV groups. They were significantly lower in the CO-HbV and CO-RBC groups than in the O_2-bound fluids. Blood HbCO levels (26–39% immediately after infusion) decreased to less than 3% at 6 hours, while CO was exhaled through the lung, as detected by gas chromatography. Both HbV and RBC gradually gained the O_2 transport function. Accordingly, both CO-HbV and CO-RBC showed a resuscitative effect for hemorrhagic-shocked rats. They reduced oxidative damage to organs in comparison to O_2-HbV and O_2-RBC. Adverse and poisonous effects of CO gas were not evident in this experimental model [21].

Hemorrhagic shock and resuscitation typically entail systemic ischemia-reperfusion injury. Activated neutrophils and macrophages produce reactive oxygen species (ROS) [47], with NADPH-oxidase involved as a major source. This enzyme contains two hemes that catalyze the NADPH-dependent reduction of oxygen to form O_2^- [48]. However, CO can bind to the hemes and modulate the enzymatic activity [49]. During hemorrhagic shock, there should be an initiation of inflammatory cytokine production and NO release from the inducible form of NO synthase (NOS) in organs such as the liver and lung. In fact, CO gas potently inhibits the conversion of L-arginine to NO and citrulline by neuronal and macrophage NOS because two heme moieties are contained in the active enzymes. CO would modulate overproduction of NOS-derived NO [50]. Together, O_2^- and NO react to form peroxynitrate, $ONOO^-$, a potent cytotoxic molecule that promotes nitration of tyrosyl residues in proteins [51]. The possibility exists that the injected CO reduces production of both NO and O_2^-, and the resultant $ONOO^-$. Actually, our immunohistochemical observations of the liver and lung clarified that injection of CO-HbV and CO-RBC reduced the formation of nitrosotyrosine on the proteins.

To our knowledge, the present study is the first to use an HBOC to administer CO in a shock state for a pharmacological effect. Although further research is definitely necessary to clarify the mechanism and clinical relevance of our experimental results using small animals, the data would suggest that both RBCs and HBOCs can be effective CO-carriers. Vandegriff *et al.* also reported that CO-bound PEG-Hb reduces myocardial infarction [52]. The advantages of CO-bound HBOC injection are: (i) carbonylhemoglobin is stable for a longer-term storage; (ii) special equipment to inhale CO gas is not necessary in an emergency situation; (iii) the CO dosage is strictly definable; and (iv) the fluid functions initially as a CO carrier to prevent pro-oxidative damage and then as an O_2 carrier.

27.5 Conclusion

Historically, the starting point of the development of HBOCs simply aimed at transporting oxygen to peripheral tissues as blood does. However, the development became complicated after the discovery of endogenous NO and CO, which have strong affinity to HBOCs and influence on their safety. Actually, RBCs are designed to retard the gas reactions, and we have to reconsider the physiological significance of the RBC structure when designing HBOCs. In this chapter, we also demonstrated the potential of HBOCs as CO carriers. Of course, CO is a toxic gaseous molecule, but it shows cytoprotective effect depending on the dose.

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Superior Plasma Retention of a Cross-Linked Human Serum Albumin Dimer in Nephrotic Rats as a New Type of Plasma Expander

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ABSTRACT:

Human serum albumin (HSA) is used clinically as a plasma expander in patients with hypoalbuminemia and can also function as a drug carrier. However, the administered HSA is readily eliminated from the blood circulation under pathological conditions, especially the nephrotic syndrome. In this study, we present data on the pharmacokinetics of a structurally defined HSA dimer [two HSA molecules that are cross-linked by reaction with 1,6-bis(maleimido)hexane via Cys34] in nephrotic rats and its superior circulation persistence, owing to the molecular size effect. The half-time ($t_{1/2}$) of the HSA dimer persisted in the circulation 1.3 times longer than that of monomeric HSA in normal rats, primarily because of the suppression of the accumulation of the HSA dimer in the skin and muscle. In nephrotic rats, the $t_{1/2}$ of the HSA monomer decreased considerably, whereas the HSA dimer remained unaltered in the blood stream, similar to that for normal rats. As a result, the $t_{1/2}$ of the HSA dimer was 2-fold longer than that of the HSA monomer. This longer $t_{1/2}$ can be attributed to the fact that accumulation in the kidney and urinary excretion of the HSA dimer were significantly suppressed. The cross-linked HSA dimer shows a longer blood circulation than native HSA monomer in nephrotic rats, which can be attributed to the suppression of renal filtration and leakage into the extravascular space. This HSA dimer has the potential for use as a drug carrier, new plasma expander, and an artificial albumin-based oxygen carrier under a high glomerular permeability condition such as nephrosis.

Introduction

In clinical settings, a critically ill patient is typically given a plasma expander to maintain colloid osmotic pressure and to increase the plasma volume. As of this writing, dextran, hydroxylethyl starch, and albumin have all been developed as plasma expanders and all are frequently used in critical situations.

As mentioned above, human serum albumin (HSA) is used as a plasma expander and is particularly useful when it is given as an infusion to patients with hypoalbuminemia, such as the nephrotic syndrome. For many years, it was generally thought that an albumin infusion improved life expectancy (Wilkes and Navickis, 2001; Vincent et al., 2004). However, there has been little hard evidence to support its widespread clinical use. In fact, the *meta*-analysis report concluded that an HSA infusion can be potentially harmful to critically ill patients, and evidence in support of the administration of HSA reducing mortality in critically ill patients with hypoalbuminemia is lacking (Cochrane Injuries Group Albumin Reviewers, 1998). In such clinical conditions, infused HSA does not appear to play a primary role as a plasma expander, because the blood retention of HSA would likely be decreased. It is well known that capillary protein permeability is increased in many pathological and physiological conditions and that this increased response is accompanied by an increased HSA flux to the extravascular compartment. Under such conditions, the administered HSA is transported to organs or extravasated, causing a formation of edema and, hence, a worsening of the disease. This result is especially true in the case of nephrotic syndrome, where the infused HSA is not only rapidly eliminated from the intravascular to the

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ABBREVIATIONS: HSA, human serum albumin; BMH, 1,6-bis(maleimido)hexane; ^{111}In, Indium-111; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; DTPA, diethylenetriaminepentaacetic acid; $t_{1/2}$, half-time; AUC, area under the concentration-time curve; CL, clearance; V_{dss}, steady-state volume of distribution; V_d, distribution volumes; ID, injection of dose; HSA-FeP, albumin-heme; FcRn, Fc receptor; rHSA, recombinant HSA.

extravascular compartment but is also excreted in the urine (Pulimood and Park, 2000). Therefore, it is necessary to administer a conventional HSA preparation frequently to maintain albumin concentration, and this strategy leads to a dramatic increase in the cost of treatment.

To overcome the need to administer the preparation frequently, some investigators have attempted to increase the molecular size of HSA by genetic or chemical dimerization (Andersson, 1970; Matsushita et al., 2006) to prevent its leakage into the extravascular space. The reason for this attempt is that increasing the molecular size is widely recognized as the best strategy for inhibiting the capillary permeability of a plasma expander, such as dextran or hydroxylethyl starch, in a clinical setting. However, the blood retention of an HSA dimer is comparable or only slightly increased compared to an HSA monomer, and the dimer has not been tested under high-permeability conditions (Matsushita et al., 2006). In addition, some disulfide-linked ^{34}Cys HSA dimers, prepared by connecting the ^{34}Cys of two albumin molecules, have low stability (Andersson, 1970) and are structurally modified compared to native HSA (Sollenne et al., 1981). As a result, they are not ideal candidates for use in clinical applications as a plasma expander. Because of this lack of suitability, it would be highly desirable to develop a new type of HSA preparation that has good blood retention under high-permeability conditions as well as normal conditions. Komatsu et al. (2004a) recently reported on the preparation of a new type of HSA dimer, in which two HSA molecules are cross-linked with 1,6-bis(maleimido)hexane (BMH). This HSA dimer is prepared by specifically linking the ^{34}Cys of two molecules using BMH. Because BMH is sufficiently long (16.1 Å) to permit the HSA to maintain its flexibility and hydrophobicity, the structural properties of native HSA can be preserved. In fact, the dimer is almost identical to HSA in terms of ligand-binding capacity and blood compatibility. Because of this similarity, the cross-linked HSA dimer has the potential for serving as a substitute for HSA for patients with high blood vessel permeability and glomerular permeability. Because blood retention is one of the most important factors for the function of a plasma expander, it is noteworthy that little information regarding the pharmacokinetic properties, especially blood retention, of the HSA dimer is available compared to the HSA monomer. Even though it is anticipated that the HSA dimer would be administered under high-permeability conditions, such as nephrotic conditions, data that demonstrate the effect of increased molecular size on the blood retention of the HSA dimer under similar clinical conditions is not available.

The purpose of this study was to clarify the pharmacokinetic properties of the HSA dimer under nephrotic conditions and to verify the potential of the HSA dimer as a versatile plasma expander. To accomplish these objectives, we carried out the pharmacokinetic studies using an Indium-111 (^{111}In)-labeled HSA monomer and the cross-linked HSA dimer in normal and nephrotic rats induced by treatment with doxorubicin (Adriamycin; Wako Pure Chemical Industries, Osaka, Japan).

Materials and Methods

Chemicals. An HSA (Albrec, 25% wt.) was provided by NIPRO (Osaka, Japan). Ethanol and dithiothreitol were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) and used without further purification. 1,6-Bis(maleimido)hexane was purchased from Pierce Biotechnology (Rockford, IL). $^{111}InCl_3$ (74 MBq/ml in 0.02 N HCl) was donated by Nihon Medi-Physics (Takarazuka, Japan). All other chemicals were of the highest grade commercially available, and all solutions were prepared using deionized distilled water. The HSA dimer was synthesized according to procedures we have reported previously (Komatsu et al., 2004a).

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting. The HSA dimer was analyzed via SDS-polyacrylamide gel electrophoresis (PAGE) using 10% polyacrylamide gel and detected by staining with Coomassie Blue R-250. Molecular masses were indicated for the following: bovine serum albumin (66 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). Western blotting was performed using a 10% polyacrylamide gel and a rabbit anti-HSA polyclonal antibody as the primary antibody followed by an anti-rabbit secondary antibody conjugated to horseradish peroxidase. Proteins were detected by using a using an ECL system (ECL Advance Western Blotting Detection Kit; GE Healthcare Bio-Sciences, Little Chalfont, Buckinghamshire, UK) with LAS-4000EPUVmini (Fujifilm, Tokyo, Japan).

Circular Dichroism Spectroscopy. The secondary and tertiary structures of the HSA dimer were examined by recording far- and near-circular-dichroism (CD) spectra. The protein concentration was 8 μM, as determined by the Bradford method (Bradford, 1976), and the buffer used was 50 mM sodium phosphate, pH 7.0, at 25°C. Far- and near-UV intrinsic spectra were recorded from 200 to 250 and 250 to 350 nm, respectively, using a Jasco J-720 spectropolarimeter (Jasco, Tokyo, Japan).

Proteins Labeling with ^{111}In. For the pharmacokinetic experiments, the HSA monomer and dimer were radiolabeled with ^{111}In using the bifunctional chelating agent diethylenetriaminepentaacetic acid (DTPA) anhydride according to the method of Hnatowich et al. (1982). In general, each sample (5 mg) was dissolved in 1 ml of 0.1 M HEPES buffer, pH 7.0, and mixed with 15 mM DTPA anhydride in 10 ml of dimethyl sulfoxide. The mixture was stirred for 60 min at room temperature, and the radiolabeled product was purified by gel filtration on a Sephadex G-25 column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) to remove unreacted DTPA. Fractions containing the sample were collected and concentrated by ultrafiltration at 4°C. A 20-ml aliquot of an $^{111}InCl_3$ solution (74 MBq/ml) was then added to 20 ml of 0.1 M citrate buffer, pH 5.5, and 60 ml of a DTPA-coupled derivative solution was added to the mixture. After 30 min, the mixture was applied to a PD-10 column (GE Healthcare) and eluted with 0.1 M citrate buffer, pH 5.5. Fractions containing the derivatives were collected and concentrated by ultrafiltration at 4°C. The specific activity of ^{111}In-labeled HSA monomer and dimer were 1×10^{10} cpm/ng protein.

Animals. All animal experiments were performed according to the guidelines, principles, and procedures for the care and use of laboratory animals of Kumamoto University (Kumamoto, Japan). All male Sprague-Dawley rats were purchased from Kyudou Co. (Kumamoto, Japan). All animals were maintained under conventional housing conditions, with food and water ad libitum, in a temperature-controlled room with a 12-h dark/light cycle. The animals were acclimated for 1 week before the experiments.

Preparation of Nephrotic Rat. Nephrotic rats were induced according to the method described previously by Bertani et al. (1982), with minor modifications. Male Sprague-Dawley rats were administered doxorubicin as a single injection (9 mg/kg) through the tail vain under ether anesthesia. At 2 weeks after the administration of doxorubicin, blood was collected from the tail vein and plasma was obtained by centrifugation (at 6000*g* for 5 min). In addition, urine was collected for 48 h in a metabolic cage. Plasma albumin concentrations were assayed by the general bromcresol green method using a Wako Albumin B Test (Wako Pure Chemical Industries) as described previously (Doumas et al., 1971), and urinary protein concentration was determined by the Bradford method (Bradford, 1976). According to a previous study, the rats, for which the urinary protein level was more than 180 mg/day, were used as a nephrosis rat model (Bertani et al., 1982). The body weights of normal and nephrotic rats were 196 ± 18 and 179 ± 11 g, respectively (n = 12, no significant differences).

The Pharmacokinetic Experimental Protocol. Just after ^{111}In-labeled HSA monomer and dimer were prepared, the pharmacokinetic study was performed, and the samples were assayed for radioactivity immediately after collection. Both samples were mixed with unlabeled protein to adjust the protein concentration before use in pharmacokinetic experiments (1×10^7 cpm/mg protein). Normal and nephrotic rats were anesthetized using ether and received a single injection of ^{111}In-labeled protein (1 mg/kg, 1×10^7 cpm/kg) via the tail vein. At each time point (3 min, 30 min, and 1, 3, 6, 9, 12, 18, 24, and 48 h) after an injection of the ^{111}In-labeled protein, a 100-μl aliquot of blood was collected from the tail vein, and plasma was obtained by centrifugation (6000*g*, 5 min). At 48 h after the injection of the ^{111}In-labeled protein, the rats were sacrificed and the organs were collected and rinsed with saline.

Urine was collected at fixed intervals in a metabolic cage. The levels of ^{111}In in the plasma and excised organs were determined using a gamma counter (ARC-5000; Aloka, Tokyo, Japan).

Data Analysis. Pharmacokinetic analyses after the administration of ^{111}In-labeled HSA monomer or dimer were carried out using a two-compartment model, and pharmacokinetic parameters were estimated by curve fitting. Pharmacokinetic parameters were calculated by fitting using MULTI, a normal least-squares program (Yamaoka et al., 1981). The two-compartment model can be described by the following equation: $C = A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t)$, where C is the percentage of dose/ml and t is time after administration of radiolabeled proteins. A, B, α, and β are coefficients or exponents in the model equation. As reported previously (Matsushita et al., 2006; Taguchi et al., 2009), the area under the concentration-time curve (AUC), distribution volumes (V_{dss}), and plasma clearance (CL) values were calculated using the following equations: $AUC = A/\alpha + B/\beta$, $V_{dss} = V_1 + V_2$, and CL = dose/AUC, where V_1 and V_2 are central and peripheral distribution volumes. The half-lives of the HSAs were determined as β-phase elimination within a 48-h period. The renal CL was calculated as radioactivity accumulation in urine until 48 h/AUC from 0 to 48 h. Data are shown as the mean ± S.D. for the indicated number of animals. Significant differences among each group were determined using the two-tail unpaired Student's t test. A probability value of $p < 0.05$ was considered to indicate statistical significance.

Results

SDS-PAGE and Western Blotting of HSA Dimer. SDS-PAGE and Western blotting were initially carried out to evaluate the efficiency of HSA dimer synthesis. As shown in Fig. 1A, a single band was detected at approximately 130 kDa, and the molecular mass of the HSA dimer was approximately double that of HSA monomer. A Western blot analysis showed that the HSA dimer and monomer were both recognized by an anti-HSA polyclonal antibody (Fig. 1B), indicating the recognition site of the HSA dimer to the polyclonal antibody against native HSA was preserved.

Structural Characteristics of HSA Dimer. To confirm the structural characteristics of the HSA dimer, near- and far-CD spectroscopy analyses were carried out. The near- and far-CD spectra of the HSA dimer showed the same minima and shape as those of HSA monomer

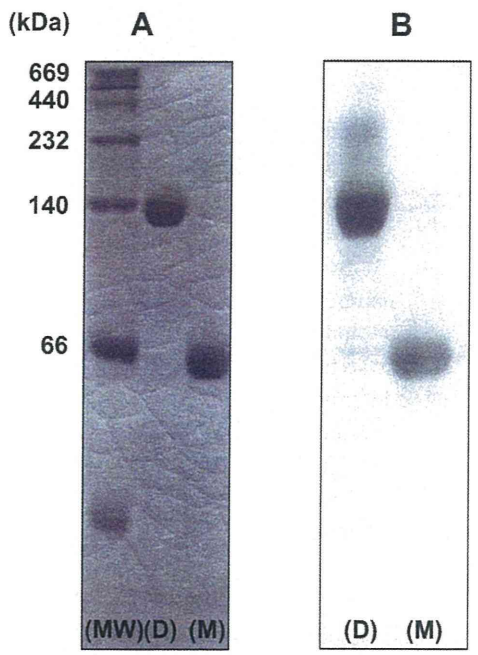

FIG. 1. SDS-PAGE (A) and Western blot analysis (B) of the HSA dimer and monomer. MW, molecular mass; D, HSA dimer; M, HSA monomer.

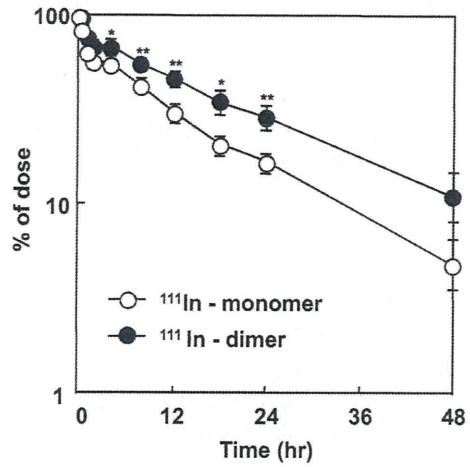

FIG. 2. Relative plasma concentration of ^{111}In-HSA monomer and dimer after intravenous administration to normal rats. ^{111}In-HSA monomer (open circles) and ^{111}In-HSA dimer (closed circles) were injected at a dose of 1 mg/kg. Each data point represents the mean ± S.D. ($n = 6$). *, $p < 0.05$ and **, $p < 0.01$ versus ^{111}In-HSA monomer.

(data not shown). These results suggest the structural characteristics of native HSA are preserved in the HSA dimer.

Pharmacokinetics of HSA Monomer and Dimer in Normal Rats. Fig. 2 shows the time course for the plasma concentration of the ^{111}In-HSA monomer and dimer that was injected into normal rats at a dose of 1 mg/kg, and Table 1 lists the pharmacokinetic parameters obtained using the two-compartment model. The half-time ($t_{1/2}$) of the HSA dimer was 1.3 times longer than that of the HSA monomer (17.2 ± 2.5 and 13.3 ± 1.8 h, $p < 0.05$, for the HSA dimer and monomer, respectively). This result is consistent with our previously reported results on ^{125}I-labeled variants (Komatsu et al., 2004a). Accompanied by the decrease of CL and V_{dss} (CL, 0.59 ± 0.1 and 0.91 ± 0.1 ml/h, for HSA dimer and monomer, respectively, $p < 0.05$; V_{dss}, 13.9 ± 0.3 and 15.5 ± 0.2 ml, $p < 0.05$, for HSA dimer and monomer, respectively), the AUC and $t_{1/2}$ were also significantly increased in the case of the HSA dimer compared to the HSA monomer (AUC, 170 ± 17 and 110 ± 6.0 h · % of dose/ml, $p < 0.05$ and $t_{1/2}$, 17.2 ± 2.5 and 13.3 ± 1.8 h, $p < 0.05$, for the HSA dimer and monomer, respectively).

Figure 3 shows the tissue distribution of the ^{111}In-HSA monomer and dimer [percentage of injection of dose (ID)]at 48 h after administration. Both the HSA monomer and dimer were highly distributed in kidney, liver, skin, and muscle. The accumulation of the HSA dimer in skin and muscle was significantly suppressed compared to that of the HSA monomer (Fig. 3). Furthermore, the urinary excretion of HSA labeled with ^{111}In was also estimated.

TABLE 1

Pharmacokinetic parameters after administration of ^{111}In-rHSA monomer and dimer after intravenous administration to normal rats

All rats received a single injection of ^{111}In-rHSA monomer or dimer at a dose of 1 mg/kg. At each time after the injection of ^{111}In-rHSA monomer or dimer, a blood sample was collected from the tail vein and plasma was obtained. Each parameter was calculated by MULTI using a two-compartment model. Each value represents the mean ± S.D. ($n = 6$).

	^{111}In-Monomer	^{111}In-Dimer
$t_{1/2}$ (h)	13.3 ± 1.8	17.2 ± 2.5*
AUC (h · % of dose/ml)	110 ± 6.0	170 ± 17*
CL (ml/h)	0.91 ± 0.1	0.59 ± 0.1*
V_{dss} (ml)	15.5 ± 0.2	13.9 ± 0.3*
Renal CL (ml/h)	0.052 ± 0.003	0.026 ± 0.002**

* $p < 0.05$ and ** $p < 0.01$ versus ^{111}In-monomer.