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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Abbreviations: HSA, Human serum albumin; $t_{1/2}$, half-life; AUC, area under the concentration-time curve; CL, clearance; $V_d$, distribution volumes; % of ID, % of injection of dose; HSA-FeP, albumin-heme; FcRn, Fc receptor
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ABSTRACT

Human serum albumin (HSA) is clinically used as a plasma expander in patients with hypoalbuminemia, and also can function as a drug carrier. However, the administered HSA is readily eliminated from the blood circulation under pathological conditions, especially the nephrotic syndrome. Here we present data on the pharmacokinetics of a structurally defined HSA dimer, 2 HSA molecules that are crosslinked 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-life ($t_{1/2}$) 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 two-fold longer than that of the HSA monomer. This 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 new plasma expander and a useful drug carrier, but also as an artificial albumin-based oxygen carrier under 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 (Robert I, 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 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 is especially true in the case of the nephrotic syndrome, where the infused HSA is not only rapidly eliminated from the intravascular to extravascular compartment but is also excreted in the urine (Pulimood and Park, 2000). Therefore, it is necessary to administer a conventional HSA preparation frequently, in order to maintain albumin concentration, and this leads to a dramatic increase in the cost of treatment.

To overcome this issue, 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 is that it is widely recognized that increasing the molecular size is 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 \(^{34}\text{Cys}\) HSA dimers, prepared by connecting the \(^{34}\text{Cys}\) of 2 albumin molecules have a 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, it would be highly desirable to develop a new type of HSA preparation, which 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 2 HSA molecules are cross-linked with 1,6-bis(maleimido)hexane (BMH). This HSA dimer is prepared by specifically linking the \(^{34}\text{Cys}\) of 2 molecules using BMH. Since 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, 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. Since, 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 are 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 demonstrates 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 this, we carried out the pharmacokinetic studies using an \textsuperscript{111}In-labeled HSA monomer and the cross-linked HSA dimer in normal and nephrotic rats induced by treatment with adriamycin (doxorubicin).
MATERIALS AND METHODS

Chemicals

A HSA (AlbrecR, 25 wt.%) was provided by NIPRO (Osaka, Japan). Ethanol, dithiothreitol (DTT) was purchased from Kanto Chemical, (Tokyo, Japan) and was used without further purification. 1,6-Bis (maleimido) hexane was purchased from Pierce Biotechnology (Rockford, USA). $^{111}$InCl$_3$ (74 Mbq/mL in 0.02 N HCl) was donated by Nihon MediPhysics (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 our previously reported procedures (Komatsu et al., 2004a).

SDS-PAGE and Western Blotting

The HSA dimer was analyzed via SDS-PAGE, using 10% polyacrylamide gel, and detected by staining with Coomassie blue R-250. Molecular weights were marked as follows, bovine serum albumin (M.W. 66 kDa), lactate dehydrogenase (M.W. 140 kDa), catalase (M.W. 232 kDa), ferritin (M.W. 440 kDa) and thyroglobulin (M.W. 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 using a using an ECL system (ECL Advance Western Blotting Detection Kit; GE Healthcare Bio-sciences Corp.) with LAS-4000EPUVmini (Fujifilm, Tokyo, Japan).

CD Spectroscopy

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

Proteins labeling with $^{111}\text{In}$

For the pharmacokinetic experiments, the HSA monomer and dimer were radiolabeled with $^{111}\text{In}$ using the bifunctional chelating agent diethylenetriaminepentaacetic acid (DTPA) anhydride according to the method of Hnatowich et al. (1982). Typically, each sample (5 mg) was dissolved in 1 mL 0.1 M 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.0, and mixed with 15 mM DTPA anhydride in 10 mL 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 (Amersham Biosciences) to remove unreacted DTPA. Fractions containing the sample were collected and concentrated by ultrafiltration at 4°C. A 20 mL aliquot of an $^{111}\text{InCl}_3$ 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 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 $^{111}\text{In}$-labeled HSA monomer and dimer were $1 \times 10^{10}$ 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. All male SD 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-hr dark/light cycle. The animals were acclimated for one week, prior to the experiments.

**Preparation of Nephrotic Rat**

Nephrotic rats were induced according to the method described by Bertani (1982), with minor modifications. Male SD rats were administrated adriamycin (doxorubicin) as a single injection (9 mg/kg) through the tail vain under ether anesthesia. At 2 weeks after the administration of adriamycin (doxorubicin), blood was collected from tail vein, and plasma was obtained by centrifugation (6000 g, 5 min). In addition, urine was collected for 48 hr in a metabolic cage. Plasma albumin concentrations were assayed by the general bromcresol green (BCG) method using a Wako AlbuminBTest (Doumas et al., 1971), and urinary protein concentration was determined by the method of Bradford (1976). According to previous study, the rats, for which the urinary protein level was over 180 mg/day, were used as a nephrosis rat model (Bertani T 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 preparing $^{111}$In labeled HSA monomer and dimer, 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 ($1\times10^7$ cpm/mg protein). Normal and nephrotic rats were anesthetized using ether and received a single injection of $^{111}$In labeled protein (1 mg/kg, $1\times10^7$ cpm/kg) via the tail vein. At each time point (3 min, 30 min, 1, 3, 6, 9, 12, 18, 24 and 48 hr) 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 hr after the injection of the $^{111}$In labeled protein, the rats were sacrificed, the organs 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 % of dose/mL, $t$ is time after administration of radiolabeled proteins. $A$, $B$ and $\alpha$, $\beta$ are coefficients or exponents in the model equation. As previously reported (Matsushita et al., 2006; Taguchi et al., 2009), AUC, $V_{dss}$ and CL were calculated using the following equations:

$$AUC = \frac{A}{\alpha} + \frac{B}{\beta}$$

$$V_{dss} = V_1 + V_2$$

$$CL = \frac{Dose}{AUC}$$

where AUC is the area under the concentration-time curve, CL is plasma clearance, $V_1$ and $V_2$ are central and peripheral distribution volumes. The half-lives of the HSAs were determined as $\beta$-phase elimination within a 48 hr period. The renal CL was calculated as radioactivity accumulation in urine until 48 hr/AUC from 0 to 48 hr. Data are shown as the means ± SD 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 around 130 kDa, and the molecular weight of the HSA dimer was approximately double that of HSA monomer. A western blot analysis showed that the HSA dimer and the HSA 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 (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

Figure 2 shows the time course for the plasma concentration of the $^{111}$In-HSA monomer and dimer that had been 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-life ($t_{1/2}$) of the HSA dimer was 1.3-times longer than that of the HSA monomer (17.2 ± 2.5 and 13.3 ± 1.8 hr, p<0.05, for the HSA dimer and monomer, respectively). This result is consistent with our previous reported results on $^{125}$I-labeled variants (Komatsu et al., 2004a). Accompanied by the decrease of CL and distribution volumes (Vdss) (CL; 0.59 ± 0.1 and 0.91 ± 0.1 mL/hr, p<0.05, Vdss; 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 with the HSA monomer (AUC; 170 ± 17 and 110 ± 6.0 hr*% of dose/mL, p<0.05, t1/2; 17.2 ± 2.5 and 13.3 ± 1.8 hr, p<0.05, for the HSA dimer and monomer, respectively).

Figure 3 shows the tissue distribution of the 111In-HSA monomer and dimer (% of injection of dose (% of ID)) at 48 hr 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 with that of the HSA monomer (Fig. 3). Furthermore, the urinary excretion of HSA labeled with 111In was also estimated. The radioactivities of 111In at 48 hr after administration of the HSA monomer and dimer were negligible (4.9 ± 0.2, 3.3 ± 1.7 % of ID for the HSA monomer and dimer, respectively, no significant differences).

Pharmacokinetics of HSA Monomer and Dimer in Nephrotic Rats

A pharmacokinetic study of the HSA dimer was also performed in nephrotic rats induced by adriamycin (doxorubicin) treatment. The serum albumin level in nephrotic rats was 3.41 ± 0.2 g/dL (v.s. normal rats; 3.74 ± 0.2 g/dL, no significant differences), and urinary protein was 213 ± 28 mg/day (v.s. normal rats; 8.0 ± 3.4 mg/day, p<0.001). These data are consisted with previous report (Bertani T et al., 1982) and indicate that nephrosis was induced in the adriamycin (doxorubicin) treated rats.

As shown in Fig. 4 and Table 2, the plasma concentration of HSA monomer was rapidly cleared compared to that of the HSA dimer in nephrotic rats (13.5 ± 2.4 and 7.0 ± 1.9 hr, p<0.01, for the HSA dimer and monomer, respectively), while the plasma concentration of the HSA dimer in nephrotic rats was similar to that in normal rats. The CL for the HSA dimer was decreased to one third of the HSA monomer (0.56 ± 0.1 and 1.8 ± 0.4 mL/hr, p<0.05, for the HSA dimer and monomer, respectively). The AUC was also significantly increased (177 ±
15 and 56 ± 6.0 hr\(^*\)\% of dose/mL, \(p<0.01\), for the HSA dimer and monomer, respectively).

As shown in Fig. 5, the HSA monomer and dimer were both mainly distributed in the kidney, liver, skin and muscle, the same as that in normal rats. Among these, there were no significant differences between the accumulation of the HSA monomer and dimer in liver, skin and muscle. Interestingly, the accumulation of the HSA dimer in the kidney was dramatically decreased compared to that of the HSA monomer.

The urinary excretion of \(^{111}\text{In}\) in nephrotic rats were also measured (Fig. 6). The radioactivity of \(^{111}\text{In}\) after administration of the HSA monomer was 75.1 ± 13.0 \% of ID at 48 hr, while the radioactivity after HSA dimer administration was decreased by half compared to that after the administration of the HSA monomer (34.6 ± 20 \% of ID at 48 hr after injection, \(p<0.05\)). In addition, the renal CL of HSA monomer was 1.81 ± 0.46 mL/hr, while that of HSA dimer was 0.20 ± 0.12 mL/hr in nephrotic rats.
DISCUSSION

The major finding of this study is that the HSA dimer has the ability to maintain the blood retention properties as well as that reported for normal conditions, even though under the high permeability conditions, such as nephrosis, it can be expected to function as a novel plasma expander.

In the case of normal rats, $t_{1/2}$ for the HSA dimer ($17.2 \pm 2.5 \text{ hr}$) was 1.3-times higher than that of the HSA monomer ($13.3 \pm 1.8 \text{ hr}$) accompanied by decrease the clearance from circulation. It is generally known that approximately 40% of native albumin exists in the vascular space, and the remaining 60% is present in the extravascular space. This ratio is equilibrated between each space through the vascular endothelium. Our data suggest that the movement of the HSA dimer from the vascular space to the cellular space through the vascular endothelium is suppressed, the molecular weight of the dimer is double that of the monomer (Fig. 1). In fact, in normal rats, the $V_{\text{dss}}$ value for the HSA dimer ($13.9 \pm 0.3 \text{ mL}$) was significantly decreased compared with that for the HSA monomer ($15.5 \pm 0.2 \text{ mL}$). As a result, the accumulation of HSA dimer in skin and muscle were significantly suppressed compared with the corresponding values for the HSA monomer (Fig. 3). This conclusion is supported by the findings reported from Matsushita et al. (2006) who fused 2 molecules of HSA to produce a recombinant HSA dimer using a yeast expression system. They clearly showed that the plasma $t_{1/2}$ of the recombinant HSA dimer was prolonged by 1.1-times due to a decrease in the vascular permeability of the HSA dimer compared to that for the HSA monomer in carrageenin-air-pouch rats. Moreover, Sejrsen et al. (1985) reported that the predominant transcapillary transport mechanism for $^{131}$I-albumin is compatible with transcapillary diffusion through pores with an effective equivalent pore radius of 145 Å. Hence, the extent of extravasation of albumin may be reduced by increasing its molecular size. It has also been shown that albumin, with an estimated radius of 35.5 Å, and water do not
share a common pathway in crossing the endothelial monolayer, suggesting the existence of a large pore pathway for albumin (Dull et al., 1991). Namely, the increase in the molecular size of HSA clearly led to a retardation in extravasation through the vascular endothelium, resulting in a longer lifetime in the blood stream.

In the cases of certain renal injuries or obstacles, such as the nephrosis syndrome, infused HSA is easily filtered by the renal glomerulus. In contrast, the $t_{1/2}$ for the HSA dimer in nephrotic rats ($13.5 \pm 2.4$ hr) was comparable to that for the HSA monomer ($13.3 \pm 1.8$ hr) in normal rats. Thus, the $t_{1/2}$ for the HSA dimer was approximately double that of the HSA monomer in nephrotic rats ($7.0 \pm 1.9$ hr). In general, the glomerular biological membrane has properties that allow for high filtration rates of water and small and mid-sized molecules, but does not allow larger proteins such as HSA to be filtered. These restrictions can be explained by several mechanisms, including charge repulsion in the glomerular basement membrane and barrier depending on their molecular size (Haraldsson et al., 2008). The molecular weight of the HSA dimer is exactly 132,741, and the HSA dimer retains a negative charge as well as the HSA monomer ($pI= 4.8$) (Komatsu et al., 2004a). Therefore, the increased $t_{1/2}$ for the HSA dimer in nephrotic rats is likely to be due to the increased molecular size of the molecule. In fact, several results in this study support this hypothesis: (i) the renal distribution and urinary excretion of the HSA dimer were decreased significantly, compared to those of the HSA monomer (Fig. 5 and 6), (ii) the renal accumulation of the HSA dimer in nephrotic rats was similar to that in normal rats (Fig. 3 and 5) , (iii) after administration of the HSA dimer to nephrotic rats, the reduction in urinary excretion prolonged the $t_{1/2}$ (Fig. 4 and 6). Although the renal CL of HSA monomer was consisted with the systemic CL of HSA monomer in nephrotic rats, the renal CL of HSA dimer was lower than the systemic CL of HSA dimer. This suggested that the increased $t_{1/2}$ of HSA dimer in nephrotic rats was not explained by only suppression of protein leakage across the glomeruli. In the previous reports, bone was
one of the major distribution tissues of albumin (Yedgar et al., 1983), and albumin was a constituent of the organic matrix in bone (Triffitt and Owen, 1973). Therefore, HSA dimer may be distributed and utilized in bone. However, in this study, we could not examine the clearance of HSA in bone. Further study will be needed to achieve this issue.

Very recently, Martini et al. (2008) demonstrated a new type of plasma expander, PEG-HSA (carrying 6 copies of PEG-5000 chains per molecule; molecular weight: 97 kDa), that is effective at reduced plasma concentrations and potentially has a better defined pharmacokinetic profile because of its larger molecular size. Furthermore, they suggested that the PEGylation of HSA possibly results in a more effective plasma volume expander during hemodilution or in resuscitation fluids that are used the treatment of hemorrhagic shock with the advantages of a longer $t_{1/2}$ because of reduced glomerular filtration and movement to the extravascular space. Since the molecular weight of the HSA dimer is approximately 130 kDa, which is larger than the PEG-HSA alluded to above, it has promise as a new plasma volume expander instead of native HSA monomer for patients with hypoalbuminemia, especially the nephrosis syndrome.

As described above, since the HSA dimer shows superior blood retention properties, it would be also expected to serve as a carrier for drug delivery in high permeability conditions. Tsuchida et al. (1997) previously reported on an HSA-based artificial oxygen carrier “albumin-heme (HSA-FeP)”, which is a synthetic heme with a covalently bound proximal base that is incorporated into the hydrophobic cavities of HSA. HSA-FeP reversibly binds and releases $O_2$ under physiological conditions, the same as hemoglobin (Komatsu et al., 1999). An in vivo study using hemorrhagic shocked rats revealed that, the renal cortical $O_2$-tensions and skeletal tissue $O_2$-tensions were increased when HSA-FeP was injected (Tsuchida et al., 2000; Komatsu et al., 2004b). An artificial oxygen carrier is also required to maintain high blood circulation, because it must temporarily function until a blood transfusion
is available or until autologous blood is recovered after a massive hemorrhage. In fact, Komatsu et al. (2004a) demonstrated that the HSA dimer enables a maximum of 16 molecules of FeP to bind and the obtained dimeric HSA-FeP bound oxygen approximately twice higher than blood. The HSA-FeP dimer shows promise as a new type of oxygen carrier that is efficiently retained in circulating blood under nephrotic conditions.

Although this study provides a demonstration of the utility of the HSA dimer as a plasma expander, our model has several limitations with respect to extrapolating it for use in a human clinical setting. Our studies only dealt with the nephrotic syndrome, as induced by adriamycin (doxorubicin). Since the nephrotic syndrome is a multiplex pathology induced by several factors, such as heredity and immunity, it will be necessary to demonstrate the pharmacokinetic properties of the HSA dimer in several nephrotic syndrome model animals. In addition, the dose using in this study (1 mg/kg) is less than a pharmacologic dose in clinical situation. A pharmacokinetic examination using pharmacologic doses of monomer and dimer should be one of the subjects of future investigation. Moreover, recent evidence indicates that the Fc receptor (FcRn) expressed in the kidney reclaimed albumin, thus maintaining the serum concentration of albumin (Sarav et al., 2009). This finding indicates that interactions between albumin and FcRn are important aspects of the pharmacokinetics of albumin. However, it is well established that there is a large species difference in the interaction of albumin and FcRn. In this study, since human albumin was administrated to rats, the interaction between HSA and rat FcRn would likely be negligible. Thus, this interaction is not likely to contribute to the pharmacokinetic data related to the HSA dimer as shown in this study. However, it will be necessary to examine the interactions of the HSA dimer and human FcRn for clinical development.

In conclusion, the cross-linked HSA dimer show potential for use as a new plasma volume expander, since it showed superior blood retention characteristics in various clinical
situations. It’s use would allow us to reduce the multiple administrations required in the case of conventional HSA preparations, and could be quite cost-effective. In addition, the HSA dimer is also predicted to function as a versatile carrier for drug delivery systems, in particular, an albumin based artificial oxygen carrier. Very recently, recombinant HSA has been approved as a serum derived HSA alternative in Japan. As a result, it appears likely that a recombinant HSA dimer could be used as a substitute of HSA preparations, even if in cases of various pathological conditions such as the nephrosis syndrome.
REFERENCES


Footnote

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K.T. and Y.U. contribute equally to this work.
FIGURE LEGENDS

Fig. 1. SDS-PAGE (A) and Western Blot Analysis (B) of the HSA Dimer and Monomer.
MW, Molecular weight; D, HSA-dimer; M, HSA-monomer

Fig. 2. Relative Plasma Concentration of $^{111}$In-HSA Monomer and Dimer after i.v. Administration to Normal Rats
$^{111}$In-HSA monomer (open circle) and $^{111}$In-HSA dimer (closed circle) were injected at a dose of 1 mg/kg. Each data point represents the mean ± SD (n=6). *, $p<0.05$, **, $p<0.01$ vs. $^{111}$In-HSA monomer.

Fig. 3 Tissue Distribution of Radioactivity at 48 hr after the i.v. Administration of $^{111}$In-HSA Monomer or Dimer to Normal Rats
$^{111}$In-HSA monomer (open column) or $^{111}$In-HSA dimer (closed column) were injected at a dose of 1 mg/kg. Each column represents the mean ± SD (n=6). *, $p<0.05$, vs. $^{111}$In-monomer.

Fig. 4. Relative Plasma Concentration of $^{111}$In-HSA Monomer and Dimer after i.v. Administration to Nephrotic Rats
$^{111}$In-HSA monomer (open circle) or $^{111}$In-HSA dimer (closed circle) were injected at a dose of 1 mg/kg. Each point represents the mean ± SD (n=6). **, $p<0.01$ vs. $^{111}$In- HSA monomer.

Fig. 5. Tissue Distribution of Radioactivity at 48 hr after i.v. Administration of $^{111}$In-HSA Monomer or Dimer to Nephrotic Rats
$^{111}$In- HSA monomer (open column) or $^{111}$In- HSA dimer (closed column) were injected at a dose of 1 mg/kg. Each column represents the mean ± SD (n=6). **, $p<0.01$, vs. 

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Fig. 6. Urinary Excretion of $^{111}\text{In}$-rHSA Monomer and $^{111}\text{In}$-rHSA Dimer after i.v. Administration to Nephrotic Rats

$^{111}\text{In}$-HSA monomer (open circle) or $^{111}\text{In}$-HSA dimer (closed circle) were injected at a dose of 1mg/kg. Each point represents the mean ± SD (n=4). *; $p<0.05$, vs. $^{111}\text{In}$-monomer.
Table 1

Pharmacokinetic parameters after administration of $^{111}$In-rHSA monomer and dimer after i.v. 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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$^{111}$In - monomer</th>
<th>$^{111}$In - dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{1/2}$ (hr)</td>
<td>$13.3 \pm 1.8$</td>
<td>$17.2 \pm 2.5$ *</td>
</tr>
<tr>
<td>AUC (hr* % of dose/mL)</td>
<td>$110 \pm 6.0$</td>
<td>$170 \pm 17$ *</td>
</tr>
<tr>
<td>CL (mL/hr)</td>
<td>$0.91 \pm 0.1$</td>
<td>$0.59 \pm 0.1$ *</td>
</tr>
<tr>
<td>Vdss (mL)</td>
<td>$15.5 \pm 0.2$</td>
<td>$13.9 \pm 0.3$ *</td>
</tr>
<tr>
<td>Renal CL (mL/hr)</td>
<td>$0.052 \pm 0.003$</td>
<td>$0.026 \pm 0.002$ **</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD (n=6).

*; p<0.05 **; p<0.01 vs. $^{111}$In-Monomer.
Table 2

Pharmacokinetic parameters after administration of $^{111}$In-rHSA monomer and dimer after i.v. administration to nephrotic model rats

All nephrotic model 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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$^{111}$In - monomer</th>
<th>$^{111}$In - dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{1/2}$ (hr)</td>
<td>7.0 ± 1.9</td>
<td>13.5 ± 2.4 **</td>
</tr>
<tr>
<td>AUC (hr* % of dose/mL)</td>
<td>56 ± 15</td>
<td>177 ± 15 **</td>
</tr>
<tr>
<td>CL (mL/hr)</td>
<td>1.8 ± 0.4</td>
<td>0.56 ± 0.1 *</td>
</tr>
<tr>
<td>Vdss (mL)</td>
<td>15.3 ± 0.7</td>
<td>13.5 ± 0.3</td>
</tr>
<tr>
<td>Renal CL (mL/hr)</td>
<td>1.8± 0.5</td>
<td>0.20 ± 0.12 **</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD (n=6).

*; p<0.05, **; p<0.01 vs. $^{111}$In-monomer.
Fig. 1

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(A) (B)

(MW) (D) (M) (D) (M)

(kDa)

669 440 232 140 66

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Fig. 3

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% of dose

0 6 12 18

kidney
liver
spleen
lung
heart
pancreas
stomach
small intestine
large intestine
muscle
skin
brain
testis

111In - monomer
111In - dimer
Fig. 4

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111In - monomer

111In - dimer

% of dose

Time (hr)
Fig. 5

Taguchi et al.

% of dose

0 6 12 18

kidney
liver
spleen
lung
heart
pancreas
stomach
small intestine
large intestine
muscle
skin
brain
testis

111In - monomer
111 In - dimer

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Fig. 6 Taguchi et al.

The graph shows the percentage of dose remaining over time for two different forms of In: monomer and dimer. The monomer form (open circles) decreases more rapidly than the dimer form (closed circles). The time points are at 0, 12, 24, 36, and 48 hours. The percentage of dose decreases from 100% at time 0 to approximately 0% at 48 hours for the monomer form, while it decreases from 100% to about 50% for the dimer form. The graph includes error bars to indicate variability.