Pharmacokinetic Analysis of in Vivo Disposition of Succinylated Proteins Targeted to Liver Nonparenchymal Cells via Scavenger Receptors: Importance of Molecular Size and Negative Charge Density for in Vivo Recognition by Receptors

YASUOMI YAMASAKI, KAZUYA SUMIMOTO, MAKIYA NISHIKAWA, FUMIYOSHI YAMASHITA, KIYOSHI YAMAOKA, MITSURU HASHIDA, and YOSHINOBU TAKAKURA
Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan (Y.Y., K.S., K.Y., Y.T.); Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan (M.N., F.Y., M.H.)

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ABSTRACT
In vivo disposition characteristics of succinylated (Suc-) proteins were studied after intravenous injection in mice in relation to their molecular characteristics as negatively charged macromolecules. Recombinant superoxide dismutase (SOD; molecular mass, 32 kDa), bovine serum albumin (BSA; molecular mass, 67 kDa), and bovine IgG (molecular mass, 150 kDa) were used to produce succinylated derivatives with different degrees of modification. 111In-labeled Suc-SODs were rapidly excreted into the urine with no significant hepatic uptake. In contrast, 111In-Suc-BSA and Suc-IgG were significantly taken up by liver nonparenchymal cells via scavenger receptors (SRs) according to the degree of succinylation and the dose injected. Interestingly, highly succinylated BSAs exhibited significant accumulation in the kidney at higher doses when the hepatic uptake was saturated. Pharmacokinetic analysis demonstrated that the hepatic uptake of succinylated proteins depended on the molecular size and the estimated surface density of succinylated amino residues. Further analysis based on a physiological pharmacokinetic model, involving a saturable process with Michaelis-Menten kinetics, revealed that the surface density of negative charges was correlated with the affinity of larger succinylated proteins for the hepatic SRs. Thus, the present study has provided useful basic information for a therapeutic strategy and the molecular design of succinylated proteins for use as drug carriers and therapeutic agents per se for SR-mediated targeting in vivo.

Site-specific drug delivery is a very important strategy for the optimization of drug therapy in terms of efficacy and safety since the pharmacological action of the drug of interest can be targeted to a specific site in the body. Among a variety of site-specific drug delivery methods, the use of a carrier system that is recognized by specific receptors on the target cells is one of the most powerful tools for targeted delivery of a variety of therapeutic agents, including chemotherapeutic compounds, protein drugs, antisense oligonucleotides, and genes (Takakura and Hashida, 1996; Wang and Low, 1998; Smith and Wu, 1999).

Scavenger receptors (SRs), which can recognize anionic macromolecules with unusually broad but circumscribed ligand specificity, are expressed on liver nonparenchymal cells (endothelial and Kupffer cells) and various macrophages (Linehan et al., 2000). The ligands for the SRs involve negatively charged proteins, such as maleylated and succinylated albumins, modified low-density lipoproteins, and polynucleotides (Terpstra et al., 2000). In particular, negatively charged albumins have been used as drug carriers via SRs. Successful receptor-mediated delivery to macrophages in vitro has been achieved with low-molecular-weight antitumor agents conjugated with maleylated albumin (Mukhopadhyay et al., 1992, 1995; Basu et al., 1994). Recently, maleylated albumin has also been used as a targeting carrier for a photosensitizer (Nagae et al., 1998) and a macrophage-activating peptide (Srividy et al., 2000a,b) via SRs. In addition, SR-mediated endocytosis has been used to deliver proteins as antigens. Several studies have shown that the immunological characteristics of proteins can be modulated by maleylation (Abraham et al., 1995, 1997; Singh et al., 1998; Bansal et al., 1999; Nicoletti et al., 1999). In addition, succinylated or other negatively charged albumins are interesting compounds since they exhibit antiviral effects (Jansen

ABBREVIATIONS: SR, scavenger receptor; SOD, superoxide dismutase; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; poly I, polyinosinic acid; poly C, polycytidylic acid; PAGE, polyacrylamide gel electrophoresis; DTPA, diethylenetriaminepentaacetic acid; Suc-, succinylated; PI, propidium iodide; VWF, von Willebrand factor; AUC, area under the curve; CL, clearance; PP, plasma pool; EC, sinusoidal and Disse spaces; IC, intracellular space; SRA, class AI/AII SRs.
et al., 1993; Kuipers et al., 1997). Moreover, we have recently demonstrated that targeted delivery to liver nonparenchymal cells and improved therapeutic effects of catalase can be achieved by direct succinylation of the enzyme (Yabe et al., 1999).

In spite of the therapeutic potential of the delivery approach based on an SR-mediated mechanism, the relationship between the physicochemical characteristics of negatively charged proteins, such as the molecular weight and number of negative charges, and their in vivo pharmacokinetic profiles are not yet fully understood. Therefore, the purpose of the present study was to clarify this relationship to establish a strategy for the rational design of negatively charged proteins as drug carriers and for therapeutic purposes.

Three kinds of model proteins, recombinant superoxide dismutase (SOD), bovine serum albumin (BSA), and bovine IgG were selected and used to prepare succinylated derivatives with different degrees of modification. The pharmacokinetic characteristics of these succinylated proteins were studied after systemic administration of various doses to mice. The in vivo distribution properties of the succinylated proteins were analyzed and discussed in relation to their molecular characteristics as negatively charged proteins.

Materials and Methods

Animals. Male ddY mice (25–27 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions.

Chemicals. BSA, IgG, fluorescein isothiocyanate (FITC), polyinosinic acid (poly I) and polycytidylic acid (poly C) were purchased from Sigma Chemical (St. Louis, MO). Rabbit anti-human von Willebrand factor antisera and rhodamine conjugated donkey anti-rabbit IgG were purchased from DAKO Japan (Kyoto, Japan) and Chemicon International, Inc. (Temecula, CA), respectively. Recombinant human SOD was supplied by Asahi Kasei (Tokyo, Japan). Succinic anhydride was obtained from Nacalai Tesque (Kyoto, Japan). [111In]Indium chloride was supplied by Nihon Medi-Physics (Takarazuka, Japan). All other chemicals were obtained commercially as reagent-grade products.

Synthesis and Characterization of Succinylated Proteins. Succinylated proteins with various degrees of modification were synthesized by reacting different amounts of succinic anhydride with the ε-NH₂ group of the lysine residues of proteins according to a previously described method (Takakura et al., 1994). In brief, each protein was dissolved in 0.2 M Tris buffer, pH 8.65, and an appropriate amount of succinic anhydride was added. The mixture was stirred for 12 h at room temperature. The succinylated proteins were then washed by dialysis, concentrated by ultrafiltration, and lyophilized. The number of free amino acid groups was determined by trinitrobenzene sulfonyc acid using glycine as a standard to estimate the degree of modification (Habeeb, 1986). The molecular masses of the succinylated proteins were estimated by SDS-PAGE. Myosin (mol. wt., 220,000), phosphorylase (mol. wt., 97,400), BSA (mol. wt., 66,000), ovalbumin (mol. wt., 46,000), carbonic anhydrase (mol. wt., 30,000), trypsin inhibitor (mol. wt., 21,500), and lysozyme (mol. wt., 14,300) were used as molecular weight markers. The homogeneity of synthesized proteins was ascertained from the results of SDS-PAGE. The bands of succinylated proteins were very sharp and shifted upward with increasing the degree of succinylation (data not shown). The SDS-PAGE analysis also showed that polymerization of synthesized proteins was negligible. The apparent surface density of the succinylated amino groups was determined by dividing the number of succinylated amino groups by the accessible surface area of the succinylated protein calculated from the following equation: 

\[ \text{Accessible surface area (m²)} = \frac{\text{molecular mass}^{0.73}}{\text{molar mass}} \]

The electrophotometric mobility of the succinylated proteins was determined using a laser electrophoresis-zeta potential analyzer (LEZAJ-500T, Otsuka Electronics).

Radiolabeling and Fluorescein-Labeling of Succinylated Proteins. For the in vivo disposition experiments, proteins were radiolabeled with [111In] using the bifunctional chelating agent DTPA anhydride according to the method of Hnatowich et al. (1982). Briefly, each succinylated protein (5 mg) was dissolved in 1 ml of 0.1 M HEPES buffer, pH 7.0, and mixed with 2- or 3-fold molar amounts of DTPA anhydride in 20 μl of dimethyl sulfoxide. The mixture was stirred for 1 h at room temperature and purified by gel filtration using a Sephadex G-25 column to remove the unreacted DTPA. The protein derivative fractions were collected and concentrated by ultrafiltration. Then, 20 μl of [111In]Cl₃ solution (74 MBq/ml) was added to 20 μl of 1 M sodium acetate, and 60 μl of DTPA-coupled derivative solution was added to the mixture. After 30 min, the mixture was purified by gel filtration using a PD-10 column and by eluting with 0.1 M acetate buffer, pH 6.0. The derivative fractions were collected and concentrated by ultrafiltration. This radiolabeling method is suitable for examining the distribution phase of macromolecules from plasma to various tissues because any radioactive metabolites produced after cellular uptake are retained within the cells where the uptake takes place (Duncan and Welch, 1993; Arano et al., 1994). For confocal microscopic studies, Suc₄₅-BSA was labeled with FITC by the method of Monsigny et al. (1984). In brief, 1 μmol of Suc₄₅-BSA was dissolved in 10 ml of 0.1 M sodium carbonate buffer, pH 9.5, and then 3 μmol of FITC was added to the solution, followed by stirring for 5 h at room temperature. The mixture was purified by gel filtration using a PD-10 column, eluting with n-butanol/water (5:95), and dialysis and then concentrated by ultrafiltration and lyophilized.

In Vivo Disposition Experiments. [111In]-labeled succinylated proteins were injected into the tail vein of male ddY mice at doses of 0.1, 1, 10, and 20 mg/kg. At appropriate times after administration, blood was collected from the vena cava under ether anesthesia, and the mice were sacrificed. Heparin sulfate was used as an anticoagulant. Plasma was obtained from the blood by centrifugation. The kidney, spleen, liver, lung, and heart were removed, rinsed with saline, and weighed. The amount of [111In] radioactivity in urine was also determined by collecting the excreted urine and that remaining in the bladder. The radioactivity in each sample was counted using a well-type NaI-scintillation counter (ARC-500, Aloka, Tokyo, Japan). Contamination by plasma in each tissue sample was corrected using the distribution data for [111In]-BSA after intravenous injection, assuming that BSA was not taken up by the tissue during the 10-min period.

For competition experiments, [111In]-labeled Suc₄₅-BSA (0.1 mg/kg) was injected into mice 1 min after injection of poly I, poly C, or Suc₄₅-BSA at the dose of 10 mg/kg. Ten minutes after injection of [111In]-labeled Suc₄₅-BSA, the liver and kidney were collected and subjected to radioactivity counting as described.

Confocal Microscopic Studies. To examine the cellular localization in the liver and kidney, FITC-Suc₄₅-BSA was administered intravenously to mice at a dose of 20 mg/kg. At 10 min after administration, mice were sacrificed; saline was infused via the portal vein to remove blood, and the liver and kidney were excised. Slices 5 μm thick were made, fixed with 20% formalin buffer. Slices were treated with RNase to avoid staining the cell components, except for the nucleus, and dyed with propidium iodide (PI) to visualize the nucleus. Liver and kidney endothelial cells were stained by a rabbit anti-human von Willebrand factor (vWF) antisera at 1:200, followed by a donkey anti-rabbit IgG rhodamine-conjugated second antibody. The slices were scanned with a confocal laser microscope (MRC-1024; Bio-Rad, Hercules, CA).

Calculation of AUC and Clearances. The plasma [111In] radioactivity concentrations were normalized with respect to the percent-
age of the dose per milliliter and analyzed using the nonlinear least-square program MULTI (Yamaoka et al., 1981). The tissue distribution patterns were evaluated using tissue uptake clearances according to the integration plot analysis. The tissue accumulation at time \( t \) was proportional to the \( \text{AUC}_{0\rightarrow t} \). By dividing the tissue accumulation at time \( t \) (\( X_t \)) and the \( \text{AUC}_{0\rightarrow t} \) by the plasma concentration \( (C_t) \), \( C_t\text{tissue} \) was obtained from the slope of the plot of \( X_t/C_t \) versus \( \text{AUC}_{0\rightarrow t}/C_t \).

**Pharmacokinetic Analysis Based on a Physiological Model.** The time-courses of the plasma concentrations and liver accumulations of \(^{111}\text{In}\)-labeled succinylated proteins were analyzed based on the physiological model shown in Fig. 1 (Nishikawa et al., 1995). In this model, the body is represented by three compartments [i.e., the plasma pool (PP), the sinusoidal and Disse spaces in the liver (EC), and the intracellular space in the liver (IC)]. The PP and EC compartments have apparent volumes of distribution \( V_p \) and \( V_l \), respectively. The IC compartment represents all the plasma spaces within the blood vessels of tissues, except for the liver; it is connected with the EC by hepatic plasma flow. The uptake of succinylated protein from EC to IC is expressed as a saturable process exhibiting Michaelis-Menten kinetics, with a maximum rate of uptake, \( V_{\text{max},l} \) (nanomoles per hour), and a Michaelis constant, \( K_{\text{m},l} \) (nanomolar). Extrahepatic elimination from PP is assumed to be a saturable process represented by \( V_{\text{max},p} \) or \( K_{\text{m},p} \). At time 0, the injected substance is assumed to be distributed in the PP and EC compartments at the same concentration. Mass balance equations for the concentration of succinylated proteins in PP and EC and the amount of succinylated proteins in IC are expressed as:

\[
\frac{dC_p}{dt} = \left( QC_i - QC_p - \frac{V_{\text{max},p}}{K_{\text{m},p} + C_p} C_p \right)/V_p
\]

(1)

\[
\frac{dC_i}{dt} = \left( QC_p - QC_i - \frac{V_{\text{max},l}}{K_{\text{m},l} + C_i} C_i \right)/V_l
\]

(2)

\[\text{Plasma Pool} \quad C_p \quad V_p \quad K_{\text{m},p}, V_{\text{max},p} \quad \text{Extrahepatic elimination} \]

\[\text{Sinusoidal and Disse Spaces} \quad C_i \quad V_l \quad K_{\text{m},l}, V_{\text{max},l} \]

\[\text{Intracellular Space} \quad X_l \]

\[\frac{dX_l}{dt} = \frac{V_{\text{max},l}}{K_{\text{m},l} + C_l} C_l \]

(3)

where \( C_p \) and \( C_i \) are the concentrations of succinylated proteins in PP and EC, respectively, \( X_l \) is the amount accumulated in IC, and \( Q \) is the hepatic plasma flow rate. The values of \( V_p, V_l, \) and \( Q \) were assumed to be 1.5 ml, 0.15 ml, and 85 ml/h, respectively (Gerlowski and Jain 1983).

To estimate the effect of the pharmacokinetic parameters on the relationship between the injected dose and tissue uptake clearance, a computer simulation was performed. Some values were substituted for the \( K_{\text{m},p}, K_{\text{m},l}, V_{\text{max},l}, \) and \( V_{\text{max},p} \) of eqs. 1 to 3; these equations were solved by the Runge-Kutta-Gill method, and then the tissue uptake clearance was calculated.

To determine the parameters, these differential equations were simultaneously fitted to the experimental plasma concentration and liver accumulation data using the nonlinear least-squares method MULTI (Yamaoka et al., 1981) associated with the Runge-Kutta-Gill method MULTI (RUNGE) (Yamaoka and Nakagawa, 1983) on the M382 mainframe computer of Kyoto University Data Processing Center. The damping Gauss-Newton method was used as an algorithm for the nonlinear least-squares method. The plasma concentration and liver accumulation data were weighted reciprocally (i.e., \( 1/C_p^2 \) and \( 1/X_l \)).

**Results**

**Physicochemical Characteristics of Succinylated Proteins.** The physicochemical properties of the succinylated proteins are summarized in Table 1. In each protein, the number of succinylated amino groups depended on the amount of reacted succinic anhydride. Succinylation slightly increased the molecular weight determined by SDS-PAGE. The calculated surface density of the succinylated amino groups correlated with the electrical mobility determined by the laser electrophoresis-\( \zeta \)-potential analyzer (\( r^2 = 0.7652 \)).

**Distribution of \(^{111}\text{In}\)-Succinylated Proteins after Intravenous Injection.** Figure 2 shows the time course of the plasma concentrations, liver accumulation, and kidney accumulation of \(^{111}\text{In}\)-Suc-SODs (A–C), \(^{111}\text{In}\)-Suc-BSAs (D–I), and \(^{111}\text{In}\)-Suc-IgGs (J–L), with different degrees of succinylation after intravenous injection in mice together with those of unmodified proteins. \(^{111}\text{In}\)-SOD, \(^{111}\text{In}\)-Suc9-SOD, and \(^{111}\text{In}\)-Suc22-SOD rapidly disappeared from the plasma circulation in a similar manner but showed no significant accumulation in the liver after administration (Fig. 2, A–C). These profiles were independent of the injected dose. However, a marked difference was observed in their renal excretion, a major elimination pathway for small proteins. Unmodified SOD and Suc3-SOD exhibited significant accumulation in the kidney, which decreased with a concomitant increase in urinary excretion (data not shown) as the dose increased. However, Suc22-SOD was mainly recovered in the urine (30% of dose) with minimal renal uptake (approximately 15% of the dose), regardless of the injected dose.

On the other hand, Suc-BSAs showed distinct biodistribution profiles according to the degree of succinylation and the administered dose (Fig. 2, E–I). \(^{111}\text{In}\)-Suc320-BSA remained in the blood circulation for a long time, and only a little was taken up by the liver. This profile was similar to that of \(^{111}\text{In}\)-BSA (Fig. 2D). With an increase in the degree of succinylation, the elimination rate from the plasma circulation and the amount of Suc-BSA taken up by the liver increased, and Suc46-BSA, Suc46-BSA, and Suc54-BSA all showed sim-
The hepatic uptake seemed to be saturable since increasing the dose reduced the amount and the rate of liver accumulation. Furthermore, it was shown that highly succinylated BSAs (Suc40-BSA, Suc46-BSA, and Suc54-BSA) were taken up by kidney, especially at higher doses, although BSA did not undergo glomerular filtration under a normal physiological condition.

A similar degree of succinylation-dependent hepatic uptake was observed for the larger protein IgG (Fig. 2, J and L). 111In-Suc22-IgG was taken up by the liver at a lower dose, but the rate was slower than that of highly succinylated BSA. On the other hand, 111In-IgG and 111In-Suc22-IgG were retained in the plasma circulation for a long time after intravenous injection, without any significant accumulation in the liver. No marked renal uptake was observed for native IgG and these IgG derivatives. No significant radioactivity was recovered in any tissues other than the liver and kidney after administration of all types of succinylated protein.

**Confocal Microscopic Studies.** Figure 3 shows the confocal microscopic images of mouse liver (A and C) and kidney (B and D) 10 min after injection of FITC-Suc54-BSA (20 mg/kg). The images in Fig. 3, A and B, are stained by PI, and the images shown in Fig. 3, C and D, are stained immunohistochemically by anti-vWF antibody. In Fig. 3A, FITC derived from Suc54-BSA was localized in the cells with smaller nuclei along with the sinusoid not in the parenchymal cells with larger nuclei, indicating that FITC-Suc54-BSA was selectively internalized by liver nonparenchymal cells, including endothelial and/or Kupffer cells. Immunohistochemical staining with endothelial cell-specific anti-vWF antibody showed that vWF positive cells are the major contributors for the uptake (Fig. 3C). On the other hand, vWF positive cells in the kidney were not responsible for the renal uptake of FITC-Suc54-BSA (Fig. 3B and 3D). It seemed that strong fluorescein staining was observed in the luminal side of the proximal renal tubule, suggesting FITC-Suc54-BSA underwent glomerular filtration and was taken up by the tubular cells, although the BSA derivatives cannot pass through the glomerular capillary wall due to size restriction.

**Competition of Hepatic and Renal Uptake of 111In-labeled Suc54-BSA by Preadministration of Poly I, Poly C, or Suc54-BSA.** To determine whether 111In-labeled succinylated proteins are taken up by the liver and by the kidney via specific receptors, competition studies were performed using poly I, poly C, and Suc54-BSA (Fig. 4). Hepatic uptake of 111In-Suc54-BSA was significantly inhibited by poly I and Suc54-BSA but not by poly C (Fig. 4A), indicating that succinylated proteins could be taken up by the liver via SRs. On the other hand, renal uptake of 111In-Suc54-BSA was not obviously inhibited by poly I and poly C, and rather Suc54-BSA increased the amount of Suc54-BSA in the kidney (Fig. 4B). These results show that renal uptake of succinylated proteins was independent of scavenger receptor-mediated endocytosis.

**Calculation of AUC and Organ Uptake Clearances.** For a quantitative comparison between the distribution profiles of the native and succinylated proteins, the clearance values for the total body (CL\text{total}), liver (CL\text{liver}), kidney (CL\text{kidney}), and urine (CL\text{urine}) as well as the AUC, were calculated based on the distribution data shown in Fig. 2 and summarized in Table 2. As far as Suc-SOD was concerned, the CL\text{total} was almost independent of the injected dose, and CL\text{kidney} and CL\text{urine} clearances made a major contribution, although the CL\text{liver} was slightly high in the case of Suc22-SOD. These results suggested that glomerular filtration was a key pathway for the elimination of Suc-SOD after intravenous injection. The CL\text{kidney} value of Suc9-SOD was significantly higher than that of Suc22-SOD, suggesting that Suc9-BSA underwent more efficient tubular reabsorption. As the injected dose increased, the CL\text{kidney} decreased and CL\text{urine} increased, indicating that saturation of tubular uptake took place. On the other hand, in the case of Suc-BSA or Suc-IgG, the CL\text{total} and CL\text{liver} (the main contributor to the CL\text{total}) varied depending on the degree of succinylation and the injected dose. At the lowest dose (0.1 mg/kg), Suc46-BSA and Suc54-BSA had a large CL\text{liver} of about 70 ml/h, a value that is close to the hepatic plasma flow rate (85 ml/h) in mice, and the CL\text{liver} value fell with a decrease in the number of succinyllations per BSA molecule (48.0, 9.4, and 0.4 ml/h for Suc40-BSA, Suc9-SOD, and Suc22-IgG, respectively).

### Table 1

<table>
<thead>
<tr>
<th>Amino Residues</th>
<th>Number of Succinylated Residues</th>
<th>Molecular Weight</th>
<th>Surface Density of Succinylated Residues</th>
<th>Electrical Mobility $\times 10^8$</th>
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<tbody>
<tr>
<td>SOD</td>
<td>0</td>
<td>32,000</td>
<td>0</td>
<td>$-0.661 \pm 0.133$</td>
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<tr>
<td>Suc$_{9}$-SOD</td>
<td>9.3</td>
<td>33,000</td>
<td>0.745</td>
<td>$-0.191 \pm 0.257$</td>
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<tr>
<td>Suc$_{22}$-SOD</td>
<td>21.6</td>
<td>34,000</td>
<td>1.683</td>
<td>$-0.582 \pm 0.273$</td>
</tr>
<tr>
<td>BSA</td>
<td>0</td>
<td>67,000</td>
<td>0</td>
<td>$-0.353 \pm 0.114$</td>
</tr>
<tr>
<td>Suc$_{22}$-BSA</td>
<td>19.7</td>
<td>69,000</td>
<td>0.920</td>
<td>$-0.588 \pm 0.541$</td>
</tr>
<tr>
<td>Suc$_{40}$-BSA</td>
<td>28.0</td>
<td>72,300</td>
<td>1.260</td>
<td>$-0.946 \pm 0.192$</td>
</tr>
<tr>
<td>Suc$_{46}$-BSA</td>
<td>39.7</td>
<td>75,900</td>
<td>1.724</td>
<td>$-1.277 \pm 0.557$</td>
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<tr>
<td>Suc$_{54}$-BSA</td>
<td>46.1</td>
<td>79,800</td>
<td>1.930</td>
<td>$-1.672 \pm 0.359$</td>
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<tr>
<td>Suc$_{9}$-BSA</td>
<td>53.6</td>
<td>84,300</td>
<td>2.157</td>
<td>$-1.912 \pm 0.336$</td>
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<tr>
<td>IgG</td>
<td>0</td>
<td>150,000</td>
<td>0</td>
<td>$0.027 \pm 0.024$</td>
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<td>Suc$_{22}$-IgG</td>
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<td>165,000</td>
<td>0.547</td>
<td>$-0.276 \pm 0.046$</td>
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<td>Suc$_{40}$-IgG</td>
<td>49.9</td>
<td>177,000</td>
<td>1.169</td>
<td>$-0.808 \pm 0.081$</td>
</tr>
</tbody>
</table>

*The numbers of succinylated amino groups were determined by the TNBS method.

*The molecular weights of compounds were estimated by SDS-PAGE.

*Surface density of succinylated amino residues was calculated by dividing the number of succinylated amino groups by the surface area of the protein.

*Electrical mobilities were determined using a laser electrophoresis-zeta potential analyzer (LEZA-500T). Results were expressed as mean ± S.D.
BSA, Suc28-BSA, and Suc20-BSA, respectively), suggesting that the degree of succinylation determines the rate of hepatic uptake. On increasing the injected dose, the $CL_{\text{liver}}$ value for each derivative significantly decreased. Regardless of their molecular weight being greater than the threshold of glomerular filtration, Suc-BSAs with a higher degree of succinylation showed relatively large $CL_{\text{kidney}}$ values. At the higher doses (10 and 20 mg/kg), the $CL_{\text{kidney}}$ values for Suc40-BSA and Suc54-BSA were comparable with the $CL_{\text{liver}}$, indicating that renal uptake plays an important role in the disposition of Suc-BSAs under these conditions. Although clearances were very small for Suc22-IgG, the clearance values of Suc50-IgG were similar to those of Suc28-BSA.

Further analysis was carried out to investigate the relationship between the physicochemical properties and disposition characteristics of the succinylated proteins. In Fig. 5, the hepatic uptake clearances of Suc-IgGs, Suc-BSAs, and Suc-SODs at the lowest dose of 0.1 mg/kg were plotted as a function of the estimated surface density of the succinylated amino groups of these proteins to compare the clearances of the succinylated proteins with different molecular weights. The $CL_{\text{liver}}$ for succinylated catalase (molecular mass, 250 kDa), which has been shown to be effectively taken up by the liver nonparenchymal cells in our previous study (Yabe et al., 1999), was also included in Fig. 5. A good correlation was observed between the surface density of succinylated amino groups and the hepatic uptake clearance, up to a density of about $1.5 \times 10^3$ molecules/Å$^2$; the clearances increased in parallel with the density. Above this density, the clearance values for the liver remained almost constant. These results imply that the apparent surface density of the succinylated amino groups of modified proteins could be a determinant for recognition by SRs on liver nonparenchymal cells. The $CL_{\text{liver}}$ value for Suc22-SOD, however, did not fit this correlation. Despite its relatively high surface density of succinylated amino residues ($1.68 \times 10^3$ molecules/Å$^2$), the $CL_{\text{ liver}}$ was low. This result suggests that the molecular weight or size of the succinylated proteins may be another important factor.

**Simulation Studies Based on a Physiological Model.**

Before determination of the pharmacokinetic parameters in the physiological model shown in Fig. 1, computer simulation studies were carried out to ascertain theoretically the effect of the pharmacokinetic parameters in the model on the relationship between the tissue uptake clearance and injected dose. As shown in Fig. 6, when the $K_m$ value increased from 0.1 to 500 μg/ml, the absolute value of the tissue uptake clearance became lower, and the slope of the curve became more gentle with the threshold dose for rapidly decreasing clearance shifting to a lower dose. In contrast, when the $V_{\text{max}}$ value increased from 1 to 200 μg/h, the absolute clearance value decreased, whereas the profile of the clearance as a function of the injected dose remained almost unchanged. These computer simulation results indicate that the dose dependence of the clearance profiles was significantly affected by $K_m$ but not by $V_{\text{max}}$.

Figure 7 shows the relationship between the hepatic up-
take clearances and the injected dose of Suc-IgGs (Fig. 7A) and Suc-BSAs (Fig. 7B) based on the experimental data. The hepatic uptake clearance values decreased, and the slope of the dose-hepatic uptake clearance curve decreased, in parallel with the reduction in the number of succinylated amino groups. Together with the results of the computer simulation study (Fig. 6), these results suggest that the affinity for SRs in the liver depends on the surface density of the succinylated amino groups on Suc-IgGs and Suc-BSAs. Moreover, the slope of Suc20-BSA was almost equal to that of Suc50-IgG, whose surface density of succinylated amino groups was similar to that of Suc20-BSA. These results show that molecular size and the surface density of succinylated amino groups were important for recognition of hepatic SRs.

Pharmacokinetic Analysis of Distribution Profiles of Succinylated Proteins Based on a Physiological Model. Differential eqs. 1 to 3 were simultaneously fitted to the experimental data of the plasma concentrations and liver accumulation of each [111In]-Suc-BSA, and four parameters ($K_{m,l}$, $V_{max,l}$, $K_{m,p}$, and $V_{max,p}$) were estimated (Table 3). The estimated parameters properly describe the distribution profiles of Suc-BSAs (data not shown). Figure 8 illustrates the $K_{m,l}$ and $K_{m,p}$ values of all Suc-BSAs plotted against the surface density of the succinylated amino groups. These parameters, $K_{m,l}$ and $K_{m,p}$, which correspond to the affinity of the succinylated proteins for hepatic uptake and extrahepatic elimination, respectively, correlated with the surface density of the succinylated amino groups (Fig. 8). With an increase in the surface density, $K_{m,l}$ decreased from 2500 nM for Suc20-BSA to 5.3 nM for the Suc54-BSA, and $K_{m,p}$ decreased from 24,000 nM to 230 nM. On the other hand, $V_{max,l}$ and $V_{max,p}$ remained relatively constant. The parameters of Suc50-IgG were also plotted to examine the correlation for Suc-BSAs. These results suggested that the in vivo affinity of the succinylated proteins for SRs was closely related to the surface density of the succinylated amino groups on the modified proteins.

Discussion

In recent years, several new members of the SR family have been cloned on the basis of their ability to recognize...
modified lipoproteins, and the family has been divided into six classes (Terpstra et al., 2000). The distinct, but partly overlapping, binding properties of the SR classes represent a complication in defining their respective activity in terms of ligand uptake. Most SRs can bind a variety of polyanionic ligands, including negatively charged albumins. Since many of the SRs are expressed in sinusoidal endothelial cells and Kupffer cells in the liver, plural SRs may be responsible for the hepatic uptake of succinylated proteins. We and others have shown that highly succinylated albumins are extensively taken up by mouse and rat liver (Franssen et al., 1993; Jansen et al., 1993; Takakura et al., 1994; Furitsu et al., 1997; Kuipers et al., 1997). Although both Kupffer cells and liver endothelial cells were involved in the uptake, the endothelial cells made the greatest contribution. The hepatic uptake of Suc-BSA was significantly inhibited by maleylated BSA, dextran sulfate, and poly I but not by poly C. This inhibition profile was similar to that observed in class AI/AII SRs (SRA), the most well characterized SRs (Krieger, 1992; Gough and Gordon, 2000). For a rational design of succinylated proteins for SR-mediated targeting, it is necessary to understand the quantitative relationship between the physicochemical characteristics and the nonlinear in vivo pharmacokinetics. Although our previous studies suggested the importance of the molecular weight of proteins (Takakura et al., 1994; Furitsu et al., 1997), a detailed analysis of this

### TABLE 2

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remained to be carried out. In the present study, a systematic pharmacokinetic study was performed using a series of succinylated proteins with various molecular weights and degree of modification to achieve this aim.

In this study, we used 111In-labeled succinylated proteins that are more suitable than those labeled with radioiodine to estimate the tissue distribution since the radioactivity remains within the lysosomes and is only slowly released from cells after receptor-mediated endocytosis. This property enables us to quantitatively determine the tissue distribution by counting radioactivity. However, the amount of 111In radioactivity in tissues gradually decreased in some cases, especially the hepatic accumulation of highly succinylated proteins at lower doses (Fig. 2). Therefore, we used the distribution data showing no obvious decrease in radioactivity to calculate the organ uptake clearances and to fit the differential equations to the experimental data.

The present study has demonstrated that succinylated proteins are selectively taken up by liver nonparenchymal cells via SRs according to the degree of succinylation and the injected dose. Confocal microscopic studies, including immunohistochemical studies, showed a significant contribution of the liver nonparenchymal cells, especially endothelial cells, to the hepatic uptake of Suc-BSA (Fig. 3). Furthermore, involvement of the SR was confirmed by the competition experiments using poly I, a typical ligand for the receptor (Fig. 4). The hepatic uptake clearance correlated well with the estimated surface density of the succinylated amino groups, suggesting that a negative charge density is a critical factor for recognition by SRs. Further pharmacokinetic analysis showed that the affinity corresponded well to the negative charge density. Suc22-SOD, however, failed to be taken up by the liver regardless of its high negative-charge density. Taken together, these results suggest that not only the negative-charge density, but also the molecular weight or size is important. These findings provide useful guidelines for the development of targeted delivery systems using succinylated proteins. To design a molecule for efficient SR-mediated hepatic targeting, a protein larger than BSA, having a succinylated amino group density of about $1.5 \times 10^3$ molecules/Å$^2$, should be used. Higher densities than this will not dramatically increase targeting efficacy. This information will be important when succinylation is applied to biologically active proteins, such as enzymes. Since unnecessary chemical modification sometimes impairs the activity, an appropriate m-

![Graph of hepatic uptake clearance vs. dose](image_url)

**Fig. 5.** Estimated surface density of succinylated amino groups $\times 10^3$ (molecules/Å$^2$). Hepatic (A) and renal (B) uptake clearances of 111In-labeled Succ IgG (○), Succ-BSA (▲), Succ-SOD (●), and succinylated catalase (□) in mice after intravenous injection at a dose of 0.1 mg/kg.

![Graph of tissue uptake clearance vs. dose](image_url)

**Fig. 6.** Simulation of the effect of pharmacokinetic parameters [i.e., Michaelis constant (A) and maximum rate (B)] on the relationship between the tissue uptake clearance and injected dose.

(A) Michaelis constant

(B) Maximum rate

--- $K_m = 0.1 \mu g/mL$
--- $K_m = 1$
--- $K_m = 10$
--- $K_m = 70$
--- $K_m = 500$

--- $V_{max} = 1 \mu g/hr$
--- $V_{max} = 7$
--- $V_{max} = 50$
--- $V_{max} = 100$
--- $V_{max} = 200$
imum degree of modification should be selected. Retrospec-
tively, these considerations are supported by our previous
successful approach using Suc-catalase (Fig. 5A) (molecular
mass, 250 kDa; $1.5 \times 10^3$ succinylated molecules/Å$^2$), with
a relatively high remaining enzymatic activity. This compound
has been shown to be effectively targeted to liver nonparen-
chymal cells and has important therapeutic potential in the
management of hepatic injuries induced by ischemia/reperfu-
sion (Yabe et al., 1999).

Previous studies have demonstrated that a charged colla-
gen-like domain containing a lysine cluster of the SRA forms
a positively charged groove that specifically interacts with
negatively charged ligands (Doi et al., 1993). It has also been
suggested that the spatial distribution of the negatively
charged residues or the negative charge density of ligands
plays an important role in electrostatic interactions (Pearson
et al., 1994). Recently, Suzuki et al. (1999) proposed a hy-
thesis that ligand binding to SRs, sufficient to allow cellu-
lar uptake, requires not only a high density of negative
charges but also an increase in the apparent affinity by
numerous interactions between one ligand and multiple SR
molecules. It is likely that larger succinylated proteins offer
a better chance of multiple binding compared with smaller
ones with a higher curvature. The low affinity of Suc-SODs
for SRs might be supported by this hypothesis, that both a
negative charge density and multiple binding would be a
prerequisite for efficient recognition in vivo, assuming that
SRA or other receptors with similar characteristics play a
major role. The detailed molecular mechanisms await further
investigation.

Suc-SOD significantly accumulated in the kidney. This,
however, should be primarily ascribed to tubular reabsorp-
tion after efficient glomerular filtration of this small protein.
Interestingly, the degree of succinylation significantly af-
fected the renal handling of SOD. Efficient and saturable
renal uptake was observed for Suc$_{9}$-SOD, whereas the renal
accumulation of Suc$_{22}$-SOD was significantly lower. This
finding, suggesting the importance of the free amino groups
on protein derivatives in the uptake by renal tubular epithe-
llial cells (Sumpio and Maack, 1982; Christensen et al., 1983),
is in good agreement with our previous observations involv-
ing various chemically modified small proteins (Mihara et al.,
1994).

Unexpectedly, we found marked renal accumulation of
highly succinylated BSA, which is not susceptible to glomeru-
lar filtration due to their size under a normal physiological
condition (Fig. 2), and our confocal microscopic studies re-
vealed that Suc$_{42}$-BSA localized predominantly in the luminal
side of the proximal renal tubule in the kidney (Fig. 3).
This phenomenon was clearly observed only at higher doses
in which the uptake via hepatic SRs was saturated and
plasma concentrations were maintained for long periods.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_{m,l}$</th>
<th>$V_{max,l}$</th>
<th>$K_{m,p}$</th>
<th>$V_{max,p}$</th>
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<tbody>
<tr>
<td></td>
<td>nM</td>
<td>nM hr</td>
<td>nM</td>
<td>nM hr</td>
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<td>Suc$_{50}$-BSA</td>
<td>2501 ± 1736</td>
<td>0.98 ± 0.56</td>
<td>23552 ± 89932</td>
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<td>Suc$_{28}$-BSA</td>
<td>52.16 ± 20.24</td>
<td>0.78 ± 1.65</td>
<td>917.32 ± 516.69</td>
<td>4.75 ± 1.69</td>
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<td>Suc$_{46}$-BSA</td>
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<td>2.99 ± 0.32</td>
<td>675.75 ± 31.48</td>
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<td>Suc$_{32}$-BSA</td>
<td>12.33 ± 0.05</td>
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<td>136.89 ± 39.75</td>
<td>5.46 ± 0.58</td>
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<td>Suc$_{34}$-BSA</td>
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<td>229.13 ± 66.49</td>
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<td>Suc$_{50}$-IgG</td>
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<td>0.84 ± 0.16</td>
<td>278.19 ± 104.26</td>
<td>2.60 ± 0.62</td>
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Fig. 7. Hepatic and renal uptake clearances of $^{111}$In-labeled succiny-
lated proteins after intravenous injection in mice at the doses of 0.1, 1, 10,
and 20 mg/kg. A, IgG (○), Suc$_{22}$-IgG (○), and Suc$_{50}$-IgG (△); B, BSA (○),
Suc$_{22}$-BSA (○), Suc$_{50}$-BSA (○), Suc$_{46}$-BSA (○), Suc$_{32}$-BSA (△),
Suc$_{34}$-BSA (triangle).
These results suggested that, following intravenous injection, the BSA derivative underwent glomerular filtration to a significant extent regardless of its large size, and subsequent uptake by the renal tubular epithelial cells (i.e., reabsorption) occurred in a similar manner to smaller proteins like SOD. It is reasonable that neither poly I nor poly C showed obvious inhibitory effects on renal accumulation of $^{111}$In-Suc54-BSA in the competition experiments (Fig. 4), assuming that the accumulation was ascribed mainly to protein reabsorption. Increased renal uptake of $^{111}$In-Suc54-BSA after an excess dosing of cold Suc-BSA in the same experiment also can be explained.

Although Kuipers et al. (1997) also reported that a certain amount of radioactivity was located in the kidney, the mechanism is unknown. It is postulated that glomerular permeability might be enhanced through an unknown action of highly succinylated protein in the kidney. Although the mechanism for this phenomenon is not clear, we speculate that mesangial cells might play an important role. It has been reported that negatively charged BSA enhances production of nitric oxide (NO) from macrophages (Alford et al., 1998), and following that, glomerular mesangial cell relaxation is enhanced by NO (Stockand and Sansom, 1997). Although further studies are needed, NO may be involved in the phenomenon that causes Suc-BSA to be passed through glomeruli and accumulated in the proximal renal tubule.

In conclusion, the present study has demonstrated that the hepatic uptake of succinylated proteins is determined by the affinity for SRs expressed on the liver nonparenchymal cells, and the affinity depends on the molecular size of the protein and the surface density of the succinylated amino groups of the protein. Furthermore, we have shown that highly succinylated proteins are also accumulated in the kidney probably, due to altered glomerular permeability. Thus, the present study has provided useful basic information for therapeutic strategies and the molecular design of succinylated proteins for use as drug carriers and therapeutic agents for SR-mediated targeting in vivo. Based on the finding, we are currently developing the targeted delivery system of antigen proteins through the SR-mediated endocytosis. To control antigen-specific immune responses by effective delivery of antigen, direct succinylation of the antigen and conjugation of epitope peptide derived from the antigen to Suc-BSA have been used.

**Fig. 8.** Relationship between $K_{m,l}$ (A), $K_{m,p}$ (B), $V_{max,l}$ (C), and $V_{max,p}$ (D) of $^{111}$In-labeled succinylated proteins and the degree of succinylation. Parameters of $^{111}$In-labeled Suc-BSA (●) and $^{111}$In-labeled Suc-IgG (○) were plotted against the estimated surface density of the succinylated amino groups.
In Vivo Disposition of Succinylated Proteins

References


Address correspondence to: Dr. Yoshinobu Takakura, Department of Drug Metabolism and Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan. E-mail: takakura@pharm.kyoto-u.ac.jp