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International Journal of Pharmaceutics 105 (1994) 19–29

international journal of pharmaceutics

Pharmacokinetics of succinylated proteins and dextran sulfate in mice: Implications for hepatic targeting of protein drugs by direct succinylation via scavenger receptors

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(Received 23 June 1993; Modified version received 26 August 1993; Accepted 30 September 1993)

Abstract

The disposition characteristics of three types of ^{111}In-labeled succinylated proteins, succinylated superoxide dismutase (Suc-SOD; Mol. Wt 34 000), bovine serum albumin (Suc-BSA; Mol. Wt 70 000) and uricase (Suc-UC; Mol. Wt 130 000) and [^{14}C]dextran sulfate (DS; Mol. Wt 8000) after intravenous injection were studied in mice. At a dose of 1 mg/kg, [^{111}In]Suc-BSA and [^{111}In]Suc-UC were taken up by the liver to a great extent whereas [^{111}In]Suc-SOD was rapidly excreted into the urine without marked hepatic uptake. [^{111}In]Suc-BSA was preferentially localized in non-parenchymal cells of the liver. Rapid urinary excretion was observed for [^{14}C]DS, however, it accumulated in the liver. Hepatic uptake of [^{111}In]Suc-BSA and [^{14}C]DS was suppressed at a higher dose (100 mg/kg), suggesting a saturable uptake process. Pharmacokinetic analysis revealed that hepatic uptake clearances of [^{111}In]Suc-BSA, [^{111}In]Suc-UC and [^{14}C]DS were 15.8-, 7.2- and 10.4-fold higher than that of [^{111}In]Suc-SOD, respectively, at 1 mg/kg. Hepatic uptake of [^{111}In]Suc-BSA was significantly inhibited by simultaneous administration of excess amounts of other negatively charged macromolecules, such as maleylated BSA (Mal-BSA), heparin, DS and polyinosinic acid (poly[I]), typical ligands for scavenger receptors. On the other hand, native BSA, cationized BSA (Cat-BSA), galactosylated and mannosylated BSA (Gal- and Man-BSA), carboxymethyl dextran (CM-dex) and polycytidylic acid (poly[C]) were not effective inhibitors. Thus, this is the first report that protein drugs can be targeted to liver non-parenchymal cells by direct succinylation through scavenger receptors in vivo. In addition, the importance of molecular weight or total number of anionic charges per protein molecule was suggested.

Key words: Succinylated superoxide dismutase; Succinylated bovine serum albumin; Succinylated uricase; Dextran sulfate; Scavenger receptor; Liver non-parenchymal cell; Pharmacokinetic analysis; Hepatic targeting

1. Introduction

In a series of investigations, we have systematically studied the pharmacokinetic properties of macromolecules in relation to their physicochem-

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SSDI 0378-5173(93)E0303-8

ical characteristics, such as molecular weight and electric charge, and developed various kinds of macromolecular carrier systems for small drugs and proteins (Takakura et al., 1987, 1989a,b, 1990; Fujita et al., 1990, 1992a,b; Noguchi et al., 1991, 1992). In particular, these studies have demonstrated the importance of electric charge in the disposition of macromolecules with molecular weight of more than about 70000 after intravenous injection. Cationic macromolecules are rapidly taken up mainly by the liver due to electrostatic interaction whereas macromolecules having weak anionic charge, such as bovine serum albumin (BSA) and carboxymethyl dextran (CM-dex), show prolonged plasma retention. Based on our findings, we have designed long-circulating protein drug derivatives by conjugation with CM-dex (Fujita et al., 1990, 1992a,b). Weakly anionic dextrans also have been used as a carrier backbone for the synthesis of anticancer drug-macromolecule conjugates utilizing monoclonal antibody (Noguchi et al., 1991, 1992) and sugar (Nishikawa et al., 1993) recognition systems to minimize non-specific interaction with non-target tissues.

It is well known that liver sinusoidal cells such as Kupffer and/or endothelial cells and various macrophages have the scavenger receptors for polyanionic macromolecules with unusually broad, but circumscribed, ligand binding specificity (Krieger et al., 1993). Although a number of in vitro studies on cellular and molecular biological aspects of scavenger receptors have been performed (Acton et al., 1993; Doi et al., 1993; Pearson et al., 1993), there is a scarcity of in vivo data. In particular, little information is available on the in vivo pharmacokinetic behavior of ligands for the scavenger receptors.

In the present study, we performed pharmacokinetic studies on the disposition characteristics of strongly anionized macromolecules, three types of succinylated proteins with different molecular weights in mice. Dextran sulfate, a candidate for use as a chemotherapeutic agent against human immunodeficiency virus (HIV) (Baba et al., 1988), was also used as a polysaccharide with strong anionic charges. Furthermore, the possibility for hepatic targeting of protein drugs by direct succinylation via scavenger receptors in the liver was discussed.

2. Materials and methods

2.1. Chemicals

BSA (crystalized, Mol. Wt 67000) was purchased from ICN Biomedical, U.S.A. Dextran sulfate (DS; Mol. Wt 8000 and 500000), [*carboxy*-^{14}C]DS (Mol. Wt 8000), polyinosinic acid (poly[I]) and polycytidylic acid (poly[C]) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Recombinant human Cu/Zn superoxide dismutase (SOD; Mol. Wt 32000) and uricase (UC; Mol. Wt 128000) were kindly supplied by Asahi Chemical Industry Co., Shizuoka, Japan and Toyobo Co., Osaka, Japan, respectively. Succinic anhydride and maleic anhydride were from Nacalai Tesque, Kyoto, Japan. [*methoxy*-^{14}C]Inulin (Mol. Wt 5000) was obtained from New England Nuclear (Boston, MA, U.S.A.). ^{111}Indium chloride ([^{111}In]Cl_3) was kindly supplied by Nihon Medi-Physics Co., Takarazuka, Japan. All other chemicals were reagent grade products.

2.2. Synthesis and characterization of succinylated proteins

BSA (500 mg) was dissolved in 25 ml of 0.2 M Tris buffer (pH 8.65) and a 120-fold molar excess of succinic anhydride (90 mg) in 100 μl of $(Me)_2SO$ was added. The mixture was stirred for 1 h at room temperature and purified by gel filtration with Toyopearl HW-55S (2.4 cm $\times$ 65 cm) (Toyo Soda Kogyo, Tokyo, Japan) to remove the unreacted compound. Conjugate fractions were collected, concentrated by ultrafiltration and then lyophilized. Succinylated SOD and UC (Suc-SOD and Suc-UC) were also synthesized according to the same method on smaller scales using less succinic anhydride to maintain their biological activities. The degree of modification was determined by measuring the amount of free amino groups in the modified BSA with trinitrobenzenesulfonate (Habeeb et al., 1966). Isoelectric points of proteins were determined by a

chromatofocusing method using Polybuffer exchanger 94 resin and the Polybuffer 74 elution buffer system (Pharmacia, Uppsala, Sweden). Enzymatic activities of SOD and UC were assayed by the methods of nitroblue tetrazolium reduction using a SOD test kit (Wako Pure Chemical, Osaka, Japan) and uric acid oxidation, respectively.

2.3. Radiolabeling of succinylated proteins

After succinylation, proteins were radiolabeled with ^{111}In using the bifunctional chelating agent diethylenetriaminepentaacetic acid anhydride according to the method of Hnatowich et al. (1982). This method was selected for accurate estimation of organ uptake of succinylated proteins, since ^{111}In has been reported to be accumulated in the organ by exchange into an iron-binding protein (Brown et al., 1987) after intracellular degradation. The ^{111}In-labeled proteins had a specific activity of approx. 1 mCi/mg.

2.4. Synthesis of BSA and dextran derivatives

For the synthesis of maleylated BSA (Mal-BSA), BSA (100 mg) was reacted with an excess amount of recrystallized maleic anhydride in 10 ml of 0.1 M $Na_4P_2O_7$ (pH 9.0). The mixture was stirred for 5 min at 4°C with the pH being maintained at 9.0 and purified by gel filtration with Toyopearl HW-55S (2.4 cm × 65 cm) (Toyo Soda Kogyo, Tokyo Japan). Conjugate fractions were collected, concentrated by ultrafiltration and then lyophilized. Cationized BSA (Cat-BSA), galactosylated and mannosylated BSA (Gal- and Man-BSA), and carboxymethyl dextran (CM-dex) were synthesized according to methods reported previously (Takakura et al., 1990; Nishikawa et al., 1992).

2.5. Procedure of animal experiments

Male ddY mice (20–25 g) were obtained from the Shizuoka Agricultural Co-operative Association for Laboratory Animals, Shizuoka, Japan. Mice were injected into the tail vein with saline containing radiolabeled compound and housed in metabolic cages for subsequent collection of urine samples. For competition experiments, [^{111}In]Suc-BSA (1 mg/kg) was injected into mice simultaneously with various kinds of unlabeled BSA and dextran derivatives and polyribonucleotides at a dose of 20 mg/kg. The injection volume was 0.01 ml/g in all experiments. At an appropriate time period after administration, blood was collected from the vena cava under ether anesthesia and centrifuged at 3000 rpm for 2 min to obtain the plasma sample. Organs including the heart, liver, spleen, intestines, kidney and muscle were excised, rinsed in cold saline, weighed and subjected to assay. The intrahepatic distribution of [^{111}In]Suc-BSA in parenchymal (PC) and non-parenchymal cells (NPC) after intravenous injection was determined in different mice by separation of PC and NPC after collagenase perfusion (Horiuchi et al., 1985b).

2.6. Analytical methods

The ^{111}In radioactivities were directly determined in a well-type NaI scintillation counter (ARC-500, Aloka, Tokyo, Japan). To measure the level of ^{14}C-labeled macromolecules, a tissue sample was placed in a counting vial and 0.7 ml of Soluene-350 (Packard, Downers Grove, IL, U.S.A.) was added. The mixture was heated overnight at 50°C, cooled to room temperature and 0.18 ml of 2 N HCl was added. The radioactivity was determined in a liquid scintillation counter (LSC 900, Aloka, Tokyo, Japan) after adding 5 ml of Clear-Sol I scintillation medium (Nacalai Tesque, Kyoto, Japan).

2.7. Data analysis

Tissue distribution patterns of radiolabeled compounds were evaluated according to the method reported previously (Takakura et al., 1987). The plasma radioactivity concentration, $C_p(t)$, was normalized to percent of dose/ml and analyzed by a biexponential function using the non-linear least-square program MULTI (Yamaoka et al., 1981):

$$C_p(t) = A \exp(-\alpha t) + B \exp(-\beta t) \quad (1)$$

Total body clearance (CL_{total}) was calculated by dividing the injected dose by the area under the plasma concentration-time curve (AUC) extrapolated to infinite time. Under the assumption of negligible efflux, tissue uptake clearance index per unit weight (CLI_i) was calculated from:

$$CLI_i = C_i / AUC_{0-t} \quad (2)$$

where C_i is the concentration of radioactivity in each organ at time t. Then the apparent hepatic uptake clearance (CL_{liver}) was expressed as follows:

$$CL_{liver} = CLI_{liver} \times W \quad (3)$$

where W (g) is the total wet weight of the liver. Urinary excretion clearance (CL_{urine}) was calculated according to Eq. 2 using the accumulated amount excreted in urine.

3. Results

3.1. Synthesis of succinylated protein drugs

The physicochemical properties of native and succinylated proteins are summarized in Table 1. Apparent molecular weights of the succinylated proteins were increased compared with the native proteins. Numbers of modified amino groups were 22, 40 and 13 for Suc-SOD, Suc-BSA and Suc-UC, respectively. At these modification ratios, Suc-SOD and Suc-UC retained approx. 50% of enzymatic activity. All succinylated proteins had pI values of less than 4.0.

Table 1
Physicochemical properties of native and succinylated proteins

Compound	Mol. Wt [a]	Number of NH_2 groups [b]	% remaining enzymatic activity [c]	Isoelectric point [d]
SOD	32000	24.0	100.0	5.0–5.2
Suc-SOD	34000	2.0	55.0	< 4.0
BSA	67000	60.0	–	4.2–4.8
Suc-BSA	70000	20.0	–	< 4.0
UC	128000	98.0	100.0	5.4
Suc-UC	130000	85.0	43.0	< 4.0

[a] The molecular weights of compounds were estimated by HPLC gel-filtration chromatography using a Shim-pack Diol-300 column (Shimadzu, Kyoto, Japan).
[b] The numbers of amino groups were determined by the trinitrobenzensulfonic acid method.
[c] SOD and UC enzymatic activities were assayed by the nitroblue tetrazolium reduction and uric acid oxidation methods, respectively.
[d] Isoelectric points of compounds were confirmed by chromatofocusing using Polybuffer exchanger 94 resin and the Polybuffer 74 elution buffer system (Pharmacia, Uppsala, Sweden).

3.2. Plasma clearance and tissue distribution of ^{111}In-labeled succinylated proteins

Fig. 1 shows plasma concentration and liver accumulation of radioactivity following i.v. injection of ^{111}In-labeled succinylated proteins into

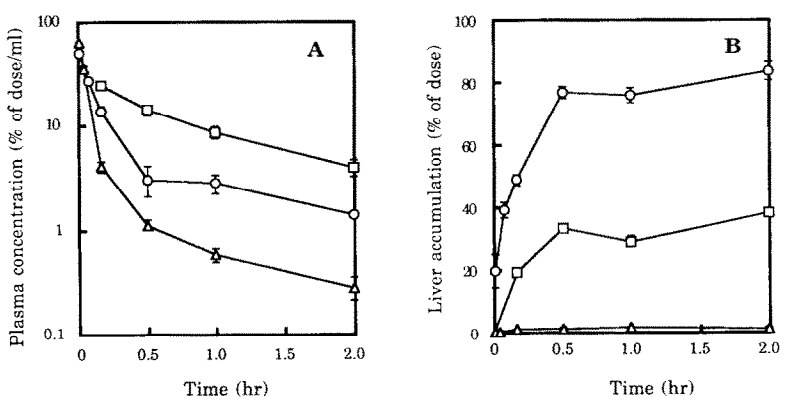

Fig. 1. Plasma concentration (A) and liver accumulation (B) of radioactivity following intravenous injection of ^{111}In-labeled succinylated proteins into mice at a dose of 1 mg/kg. Results are expressed as mean ± SD of three mice. [^{111}In]Suc-SOD (△), [^{111}In]Suc-BSA (○) and [^{111}In]Suc-UC (□).

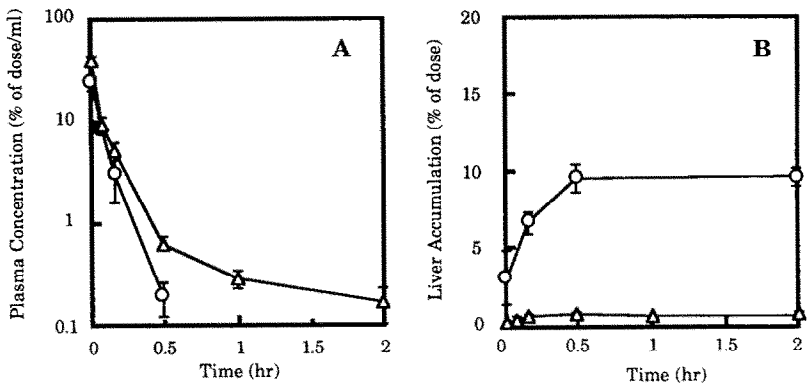

Fig. 2. Plasma concentration (A) and liver accumulation (B) of radioactivity following intravenous injection of [^{14}C]DS (Mol. Wt 8000) into mice. Results are expressed as mean ± SD of three mice. 1 mg/kg dose (○), 100 mg/kg dose (△).

mice at a dose of 1 mg/kg. [^{111}In]Suc-BSA showed the most rapid disappearance from the circulation among succinylated proteins and approx. 80% of dose was accumulated in the liver within 2 h. At 100 mg/kg, hepatic uptake of [^{111}In]Suc-BSA was significantly lower (19.5% of dose at 2 h) (data not shown). In contrast, significant hepatic accumulation was not observed after i.v. injection of [^{111}In]Suc-SOD at 1 mg/kg although it was rapidly cleared from the blood circulation. In this case, approx. 60% of the dose was excreted in the urine within 2 h. [^{111}In]Suc-UC showed the slowest disappearance rate from the bloodstream, however, more than 30% of dose was accumulated in the liver. Marked accumulation of radioactivity was not observed in any other organs than the liver after injection of all types of ^{111}In-labeled succinylated proteins.

3.3. Plasma clearance and tissue distribution of [^{14}C]DS and [^{14}C]inulin

Fig. 2 shows the plasma concentration- and liver accumulation-time profiles of [^{14}C]DS at 1 and 100 mg/kg. At 1 mg/kg, [^{14}C]DS was rapidly cleared from the circulation and about 45% of

Table 2
Pharmacokinetic parameters of native and succinylated proteins, DS, and inulin after intravenous injection to mice

Compound	Dose (mg/kg)	AUC (% of dose h ml^{-1})	Clearance (μl/h)			Tissue uptake clearance index (μl h^{-1} mg^{-1})		
			CL_{total}	CL_{liver}	CL_{urine}	CLI_{liver}	CLI_{kidney}	CLI_{spleen}
[^{111}In]SOD	1	9.5	10534	42.7	1234	26.2	23686	20.7
[^{111}In]Suc-SOD	1	6.1	16433	238	12083	242	10423	34.3
[^{111}In]BSA	1	1430	69.7	15.8	7.1	15.1	28.0	7.5
	100	764	131	20.4	161	18.5	21.2	15.6
[^{111}In]Suc-BSA	1	18.1	5510	3763	393	3350	442	82.2
	100	140	713	245	103	205	265	63.1
[^{111}In]UC	1	215	465	192	69.5	200	269	76.9
[^{111}In]Suc-UC	1	22.8	3601	1384	337	1527	1733	320
[^{14}C]DS	1	4.0	25300	2470	12300	1420	2250	486
	100	5.4	18500	553	10900	419	1000	140
[^{14}C]Inulin	100	3.5	28850	154	26770	168	700	60.1

the dose was excreted in the urine within 30 min. Accumulation of radioactivity in the liver amounted to about 10% (Fig. 2B). At 100 mg/kg, dramatic change was not observed in the plasma elimination profile with a slight increase in the extent of urinary excretion (57%) whereas hepatic uptake of [^{14}C]DS was decreased to about 1%. After injection of [^{14}C]inulin, approx. 93% was recovered in the urine within 1 h while only a trace amount was detected in the liver (data not shown).

3.4. Pharmacokinetic analysis of [^{111}In]Suc-BSA and [^{14}C]DS

Table 2 summarizes AUC, CL_{total}, CL_{liver}, CL_{urine} and CLI_i values for three organs of ^{111}In-labeled native and succinylated proteins and [^{14}C]DS. Native [^{111}In]SOD, [^{111}In]BSA, [^{111}In]UC and [^{14}C]inulin had low CL_{liver} values. In contrast, CL_{liver} values for [^{111}In]Suc-BSA, [^{111}In]Suc-UC and [^{14}C]DS were high while that for [^{111}In]Suc-SOD was considerably smaller. CL_{liver} values of [^{111}In]Suc-BSA, [^{111}In]Suc-UC and [^{14}C]DS were 15.8-, 7.2- and 10.4-fold higher than that of [^{111}In]Suc-SOD, respectively, at 1 mg/kg. [^{111}In]Suc-SOD and [^{14}C]DS had large CL_{urine} values, which were comparable to that for [^{14}C]inulin, a marker of glomerular filtration rate.

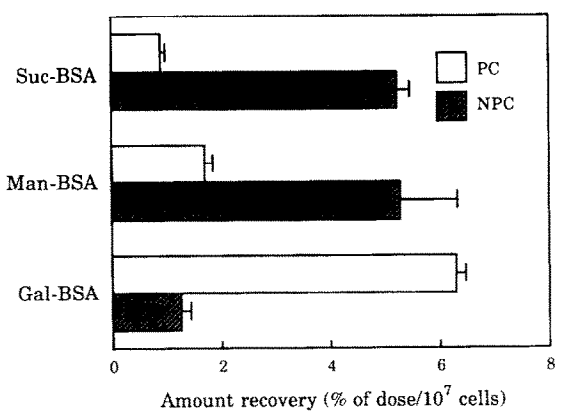

Fig. 3. Cellular localization of [^{111}In]Suc-BSA at 30 min after intravenous injection at a dose of 1 mg/kg. Data for Man-BSA and Gal-BSA are taken from published results (Nishikawa et al., 1992).

3.5. Cellular localization of [^{111}In]Suc-BSA in the liver

Fig. 3 shows the intrahepatic distribution of [^{111}In]Suc-BSA in parenchymal (PC) and non-parencymal (NPC) cells at 30 min after i.v. injection at a dose of 1 mg/kg together with data for [^{111}In]Gal-BSA and [^{111}In]Man-BSA, which have been shown to be taken up selectively by PC and NPC, respectively, via sugar receptor-mediated endocytosis. Similarly to [^{111}In]Man-BSA, [^{111}In]-Suc-BSA was accumulated preferentially in NPC.

3.6. Competition of hepatic uptake of [^{111}In]Suc-BSA by coadministration with BSA and dextran derivatives

To determine whether [^{111}In]Suc-BSA is taken up by the liver via the scavenger receptors, we performed competition studies using various BSA and dextran derivatives and polyribonucleotides. Strongly negatively charged macromolecules, such as Suc-BSA, Mal-BSA, heparin, DS (Mol. Wt 8000 and 500 000) and poly[I] significantly blocked the hepatic uptake of [^{111}In]Suc-BSA. However, Cat-BSA, Gal-BSA, Man-BSA, native BSA and CM-Dex did not show significant inhibition. Coadministration of poly[C] slightly enhanced [^{111}In]Suc-BSA accumulation in the liver.

4. Discussion

High-affinity ligands for scavenger receptors include various types of polyanions, such as acetylated low density lipoprotein (acetyl-LDL), Mal-BSA, poly[I], DS and bacterial lipopolysaccharides (Krieger et al., 1993). However, many polyanions involving poly(D-glutamic) acid, poly[C] and chondroitin sulfate are not ligands (Krieger et al., 1993). Regarding chemically modified albumins, Mal-BSA and formaldehyde-treated albumin (Horiuchi et al., 1985a,b; Jansen et al., 1991) are ligands for scavenger receptors while acetylated albumin is not (Brown et al., 1980). Although Franssen et al. (1993) indicated that succinylated human serum albumin could be a ligand for scavenger receptors, succinylated al-

bumin and other proteins have not been well characterized as a ligand for scavenger receptors.

The present study demonstrated that Suc-BSA was rapidly cleared by the liver NPC after i.v. injection in mice (Fig. 1 and 3). Hepatic uptake was saturated at a higher dose and inhibited by simultaneous injection with typical and reliable ligands for scavenger receptors, Mal-BSA and DS (Fig. 4). The difference in the inhibitory potency of DS with molecular weights of 8000 and 500 000 may be ascribed to the difference in pharmacokinetic properties and/or affinity to the scavenger receptors. Although heparin has been considered not to be a ligand for scavenger receptors (Brown et al., 1980), a recent report by Stehle et al. (1992) has demonstrated that low molecular weight heparin is taken up by the liver via scavenger receptor-mediated uptake. The polyribonucleotides, poly[C] and poly[I], showed contrasting effects, which corresponds to their affinity to scavenger receptors (Krieger et al., 1993). The reason for increased uptake of Suc-BSA by poly[C] co-injection is unknown at present. Thus, the present study has demonstrated that Suc-BSA is taken up by the liver NPC via scavenger receptor-mediated endocytosis in vivo.

Although the scavenger receptor-mediated endocytic pathway has been utilized for small drugs (Mukhopadhyay et al., 1989, 1992), no approaches have been reported on protein drug delivery via the scavenger receptors. We modified SOD and UC, model protein drugs, into succinylated forms aiming at targeting to the liver NPC through scavenger receptor-mediated endocytosis. The NPC are known to be an important source of reactive oxygen species, which cause hepatic injury (Jaeschke et al., 1992). Therefore, targeting of antioxidant enzymes, such as SOD and catalase, to the liver NPC would be useful from the therapeutic viewpoint of hepatic diseases mediated by reactive oxygen species. Succinylation of the amino group in protein resulted in the anionization of the molecule through the replacement of a positively charged amino group by a negatively charged carboxyl group. However, this procedure appeared to be a useful method

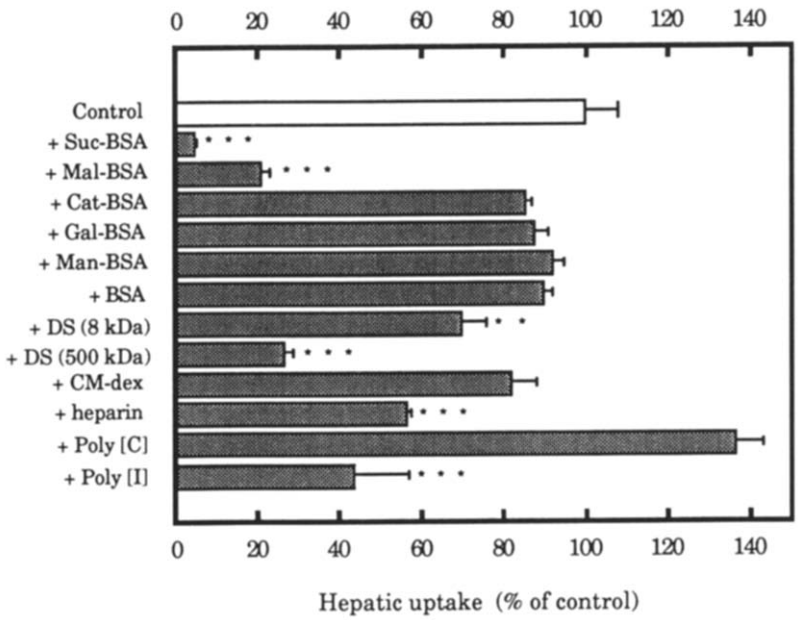

Fig. 4. Competition of hepatic uptake of [^{111}In]Suc-BSA by coadministration with BSA and dextran derivatives and polyribonucleotides after intravenous injection into mice. [^{111}In]Suc-BSA (1 mg/kg) was injected with unlabeled competitive macromolecules (20 mg/kg) and hepatic accumulation was determined at 30 min after intravenous injection. ** $p < 0.01$, *** $p < 0.001$.

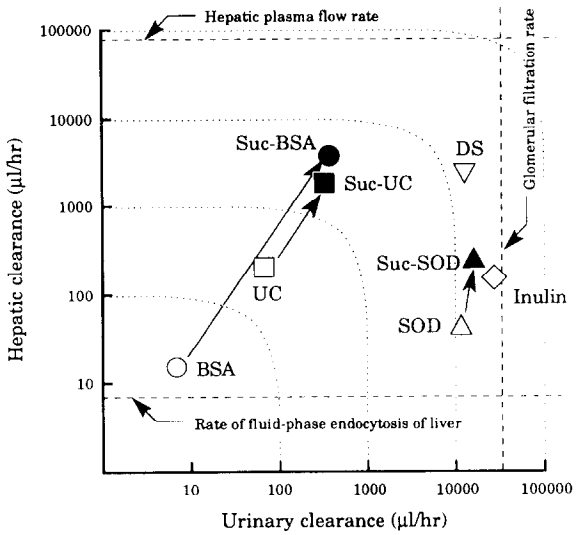

Fig. 5. Hepatic and urinary clearances of native and succinylated proteins, DS and inulin after intravenous injection into mice. Urinary clearances of SOD and Suc-SOD are the sum of urinary and kidney uptake clearances.

for anionization since the proteins used in this study retained satisfactory enzymatic activities (Table 1).

In Fig. 5, the CL_{liver} and CL_{urine} values of three succinylated proteins are shown together with their native counterparts to show the effects of succinylation on their disposition characteristics. Data for DS and inulin are also plotted for comparison. The pharmacokinetic analysis revealed that hepatic uptake clearances of [^{111}In]-Suc-BSA (Mol. Wt 70000), [^{111}In]Suc-UC (Mol. Wt 130000) and [^{14}C]DS (Mol. Wt 8000) were comparable and significantly greater than that of [^{111}In]Suc-SOD (Mol. Wt 34000). In our in vitro studies using cultured mouse peritoneal macrophages, similar results were obtained, i.e., [^{111}In]Suc-BSA was rapidly taken up by the cells while no significant uptake was observed for [^{111}In]Suc-SOD (unpublished results). These results suggested the importance of molecular weight or total number of anionic charges per succinylated protein molecule in recognition by the scavenger receptors. Proteins with molecular weights of more than 70000 could be targeted to the liver via direct succinylation. However, further studies are required on the uptake mechanism of succinylated proteins since some structural requirements might be involved.

On the other hand, a sulfated polysaccharide, [^{14}C]DS, had a large CL_{liver} value regardless of its low molecular weight (8000), which is in contrast to our finding that carboxylated CM-dex (Mol. Wt 70000) had a very small CL_{liver} value (Takakura et al., 1990). Low molecular weight heparin, which is taken up by the liver through a scavenger receptor (Stehle et al., 1992), is also a sulfated polysaccharide. The above suggested that sulfated polysaccharides are ligands for scavenger receptors irrespective of molecular weight, however, chondroitin sulfate is not a ligand (Brown et al., 1980). In addition, hyaluronic acid, a non-sulfated polysaccharide, has been reported to be rapidly taken up by the liver endothelial cells via receptor-mediated endocytosis (Smedsrod et al., 1990). The relationship between the uptake mechanisms of succinylated proteins and polyanionic polysaccharides remains to be elucidated.

At present, macrophage scavenger receptors are the most well-characterized scavenger receptors, trimetric membrane glycoproteins (Doi et al., 1993; Krieger et al., 1993). The macrophage scavenger receptors are expressed on Kupffer cells, liver macrophages. On the other hand, liver sinusoidal endothelial cells have scavenger receptors for acetyl-LDL (Blomhoff et al., 1984; Pitas et al., 1985) and formaldehyde-treated albumin (Jansen et al., 1991) in vivo but both ligands are reportedly recognized by different receptors (Horiuchi et al., 1985a). Recent studies have suggested the existence of various scavenger receptors on Kupffer and liver endothelial cells (Ottnad et al., 1990; Van Berkel et al., 1991; Bickel and Freeman; 1992). A histochemical study has shown that succinylated albumin is mainly located in endothelial cells in the liver (Franssen et al., 1993). It would be of interest to determine quantitatively which type of NPC takes up Suc-BSA by a scavenger receptor-mediated mechanism in the liver.

Acylation of proteins and polyamino acids with dicarboxylic anhydrides including succinylation is one of the common procedures for drug conjugation. For example, succinic anhydride (Trouet et al., 1982; Kaneo et al., 1990), glutaric anhydride

(Roos et al., 1984; Kaneo et al., 1990) and *cis*-aconitic anhydride (Shen and Ryser, 1981) have been employed as spacers to synthesize anticancer drug-macromolecule conjugates. In particular, *cis*-aconityl derivatives of daunorubicin (Diener et al., 1986; Dillman et al., 1988; Durrant et al., 1989) and doxorubicin (Yang and Reisfeld, 1988) have been preferably used by many investigators for the synthesis of lysosomotropic monoclonal antibody-drug conjugates since the *cis*-aconityl linkage is acid-labile. Although the final drug-macromolecule conjugates would have more negative charges than the original protein depending on the degree of modification, little attention has been paid to this aspect. On the other hand, Mal-BSA, an acylated protein with maleic anhydride, has been employed as a carrier of methotrexate (Mukhopadhyay et al., 1989) and daunorubicin (Mukhopadhyay et al., 1992) for scavenger receptor-mediated drug delivery to macrophages, however, their pharmacokinetics have not been characterized. The present study would provide useful information not only for protein drug delivery research but also for studies on macromolecule-drug conjugates.

Thus, the present study has demonstrated that protein drugs can be targeted to the liver nonparenchymal cells by direct succinylation through the scavenger receptors in vivo. The strategy would be a novel method for protein drug delivery in addition to macromolecular conjugation and direct glycosylation (Fujita et al., 1990, 1992a,b).

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