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Targeted Delivery of Human Recombinant Superoxide Dismutase by Chemical Modification with Mono- and Polysaccharide Derivatives

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ABSTRACT

Four types of superoxide dismutase (SOD) derivatives such as SOD-carboxymethyl dextran conjugate, SOD-diethylaminoethyl dextran conjugate, galactosylated SOD and mannosylated SOD were synthesized and their potential for selective targeting to organs or cells was evaluated in mice by pharmacokinetic analysis. All SOD derivatives retained 50 to 80% of the original enzymatic activity and were stable during incubation with mouse serum retaining enzymatic activity greater than 80% for 3 hr. After intravenous injection, native SOD was rapidly excreted into urine and no significant accumulation was observed in the organs except the kidney. SOD-carboxymethyl dextran conjugate gave a long plasma half-life because of impaired glomerular filtration and tissue interaction. By contrast, galactosylated-SOD and mannosylated-SOD were very rapidly eliminated from the circulation and taken up by parenchymal and nonparenchymal cells of the liver, respectively, via receptor-mediated endocytosis. These uptake processes were nonlinear and hepatic uptake clearance decreased as the dose increased, although almost complete extraction was obtained at a dose of 0.1 mg/kg. Furthermore, the accumulation in kidney of both glycosylated SODs was drastically decreased due to reduced renal proximal tubular reabsorption and also enhanced hepatic clearance. SOD-diethylaminoethyl dextran conjugate also rapidly disappeared from plasma and distributed into liver, but its accumulation occurred due to electrostatic interaction and was nonspecific in cellular distribution. These results suggest the possibility of controlling the *in vivo* fate of SOD at a cellular level by chemical modification utilizing sugar moieties with varied physicochemical and/or biological characteristics.

The toxicity of reactive oxygen species, whose production is amplified by pathological events including neutrophil activation, hyperoxia, metabolism of redox-active drugs, radiation exposure and ischemia, suggests use of antioxidant enzymes such as SOD as therapeutic agents (Fridovich, 1983). Thus, controlled manipulation of cellular SOD, an enzyme capable of eliminating superoxide anion, which exists in the upper stream of reactive oxygen metabolism cascade, can help as a defense mechanism against tissue injury mediated by reactive oxygen species. In practice, SOD is used as a therapeutic agent for rheumatoid arthritis in humans (Menander-Huber and Huber, 1977). However, the experimental and therapeutic potentials of SOD are limited because SOD is rapidly cleared by glomerular filtration in the kidney, leading to a plasma elimination half-life of only 5 to 10 min following i.v. injection in animal models (Pyatak *et al.*, 1980; Odlind *et al.*, 1988).

Among the various approaches to solving the problems inherent to protein and peptide drugs such as a short plasma half-life, chemical modification utilizing moieties with various physicochemical or biological characteristics is the most promising (Fuertges and Abuchowski 1990; Takakura *et al.*, 1989a,b). In particular, modification of proteins with carbohydrates seems to be attractive due to their ability to add various characteristics to proteins and because of their high biocompatibilities.

On the other hand, many biologically active proteins are known to be glycoproteins whose carbohydrate structures are important to their *in vivo* behaviors and biological activities. Therefore, from the standpoint of physiological function(s) of a carbohydrate residue and reconstitution of biological potential of glycoproteins from carbohydrate-free recombinant pro-

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ABBREVIATIONS: SOD, superoxide dismutase; DTPA, diethylenetriaminepentaacetic acid; CMD, carboxymethyl dextran; SOD-CMD, SOD-carboxymethyl dextran conjugate; DEAED, diethylaminoethyl dextran; SOD-DEAED, SOD-diethylaminoethyl dextran conjugate; Gal-SOD, galactosylated SOD; Man-SOD, mannosylated SOD; Gal-BSA, galactosylated bovine serum albumin; Man-BSA, mannosylated bovine serum albumin; BSA, bovine serum albumin; CL_{total}, total body clearance; CL_{liver}, hepatic uptake clearance; CL_{urine}, urinary excretion clearance; CLI_i, tissue uptake clearance index per unit weight; CLI_{liver}, liver uptake clearance index; CLI_{kidney}, kidney uptake clearance index; CLI_{spleen}, spleen uptake clearance index; PEG, polyethylene glycol.

teins, chemical modification with carbohydrates would be important.

In this study, we synthesized four types of recombinant SOD derivatives modified with sugar moieties and investigated their potentials in site-specific delivery of the enzyme. Tissue distribution properties of SOD derivatives were determined in mice using ^{111}In-labeled compounds and tissue uptake clearance indices were calculated for a quantitative evaluation.

Materials and Methods

Chemicals

Recombinant human SOD (111-Ser) was kindly supplied by Toyo Jyozo Co., Shizuoka, Japan. Dextran with an average MW of 10 kDa was purchased from Pharmacia, Uppsala, Sweden. D-Galactose and D-mannose were obtained from Wako Pure Chemical, Osaka, Japan. DTPA anhydride was obtained from Dojindo Laboratory, Kumamoto, Japan. ^{111}Indium chloride ([^{111}In]$InCl_3$) was kindly supplied from Nihon Medi-Physics Co., Takarazuka, Japan. All other chemicals were of the finest grade available.

Synthesis of SOD Derivatives

SOD-polysaccharide conjugates. Two types of SOD-polysaccharide conjugates, an anionic CMD conjugate (SOD-CMD) and a cationic DEAED conjugate (SOD-DEAED) were synthesized according to the method reported previously (Fujita *et al.*, 1990; Yasuda *et al.*, 1990). In brief, CMD and DEAED were synthesized by reacting monochloracetic acid and diethylaminoethyl chloride, respectively, with dextran under alkaline conditions at 80°C. These dextran derivatives were oxidized by sodium periodate, conjugated with SOD in 50 mM borate buffer (pH 10.0) for 24 hr at 4°C in the dark, and reduced by 1 mM sodium borohydride for 2 hr at 4°C. The obtained compounds were purified by gel-filtration chromatography using Sephadex G-75 column (1 × 40 cm).

Glyc-SOD. Introduction of galactose and mannose residues to SOD was carried out according to the method of Lee *et al.* (1976). Cyanomethyl 1-thioglycoside (220 mg, 0.94 mmol) was treated with 0.01 M sodium methoxide at room temperature. After 24 hr, the solvent was evaporated in vacuo and the resultant syrup of 2-imino-2-methoxyethyl-1-thioglycoside was added to SOD (300 mg, 9.4 μmol) in 15 ml 50 mM borate buffer (pH 10.0). After 5 hr at room temperature, the reaction mixture was concentrated by ultrafiltration and applied to Sephadex G-25 column (1 × 30 cm) equilibrated with 0.1 M acetate buffer (pH 6.0) to separate the coupled product from the unreacted compound. Purity of the products was confirmed by affinity chromatography with Con A-Sepharose (Man-SOD) or agarose-peanut lectin (Gal-SOD). Gal-BSA and Man-BSA were synthesized by the same procedure as described above.

Analytical Methods

Protein concentration was determined by the method of Lowry *et al.* (1951) using BSA fraction V as a standard in the range of 0 to 200 μg/ml. The degree of modification of amino groups was determined by measuring the amount of free amino groups with trinitrobenzene sulfonic acid using glycine as a standard (Habeeb, 1966). Enzymatic activity of SOD was determined by nitroblue tetrazolium reduction method using a SOD test kit (Wako Pure Chemical, Osaka, Japan). The MW of SOD derivatives was estimated by high-performance liquid chromatography (LC-6A, Shimadzu, Japan) using a Shim-pack Diol-300 column (inside diameter, 7.5 mm × 50 cm) eluted with 20 mM phosphate buffer (pH 7.0) containing 0.2 M sodium sulfate. The apparent MW of SOD derivatives were determined by the calibration curve obtained from the marker proteins (Gel Filtration Kit, Pharmacia, Uppsala, Sweden). The physicochemical properties of SOD derivatives tested in this study are summarized in table 1.

TABLE 1

Physicochemical properties of SOD and SOD derivatives used in this study

Compound	No. of NH_2 Groups[a]	Apparent MW[b]	Retained Enzymatic Activity[c]	Electric Charge[d] (pH 7.4)
SOD	24.0	32,000	100.0	−
SOD-CMD	18.0	150,000	50.0	−
SOD-DEAED	15.0	150,000	55.0	+
Gal-SOD	2.7	35,000	79.4	−
Man-SOD	3.8	34,000	65.6	−

[a] Numbers of amino group were determined by trinitrobenzene sulfonic acid method.

[b] MW of SOD derivatives were estimated by high-performance liquid chromatography using Shim-pack Diol-300 column (Shimadzu, Kyoto, Japan).

[c] SOD enzymatic activity was assayed by nitroblue tetrazolium reduction method.

[d] Net electric charge of SOD derivatives was confirmed by a batch method using a DEAE-Sephadex A-50 anion exchanger and CM-Sephadex C-50 cation exchanger.

Stability of SOD Derivatives in Mouse Serum

One hundred units of SOD derivatives were incubated at 37°C in 1 ml of phosphate buffered saline (pH 7.4) containing mouse serum (20%). Aliquots of the incubation medium were taken at various time intervals and assayed for remaining SOD enzymatic activity.

Radiolabeling of SOD Derivatives

^{111}In labeling of SOD derivatives was performed using the bifunctional chelating agent DTPA anhydride by the method of Hnatowich *et al.* (1982). In brief, SOD derivatives equivalent to 10 mg SOD were dissolved in 1 ml of 0.1 M 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid buffer (pH 7.0) and the twice molar of DTPA anhydride in 40 μl of Me_2SO was added. The mixture was then stirred for 30 min at room temperature and purified by gel-filtration chromatography using PD-10 column (Pharmacia, Uppsala, Sweden) to separate the unreacted DTPA. Forty microliters of $^{111}InCl_3$ solution (74 MBq/ml) was added to 40 μl of 1 M sodium acetate buffer (pH 6.0), and then 80 μl of DTPA-coupled conjugate solution was added to the mixture. After 30 min, the mixture was purified by gel-filtration chromatography using PD-10 column eluted with 0.1 M acetate buffer (pH 6.0), and conjugate fractions were collected and concentrated by ultrafiltration. The radiochemical purities of all compounds were determined to be over 98% by gel-filtration chromatography using Sephadex G-75 column (2 × 40 cm).

Procedure of Animal Experiment

Male ddY mice (20–25 g) were injected into the tail vein with 0.15 M sodium chloride solution containing radiolabeled SOD or SOD derivatives and housed in metabolic cages for subsequent collection of urine samples. For the competitive experiment, ^{111}In-labeled Gal- or Man-SOD (0.1 mg/kg) was injected into mice simultaneously with cold Gal-BSA or Man-BSA at a dose of 20 mg/kg. 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 such as the heart, liver, spleen, intestines, kidney and muscle were excised, rinsed in cold saline, weighed and subjected to assay. The ^{111}In radioactivities of organ samples were counted with a well NaI-scintillation counter (ARC-500, Aloka, Japan). Plasma volume of each organ was determined from the distribution data of [^{111}In]BSA at 10 min after i.v. injection and used for the correction of the tissue concentration. Cellular localization of SOD derivatives in the liver was determined in different mice by fractioning parenchymal and nonparenchymal cells after collagenase perfusion.

Data Analysis

Tissue distribution patterns of SOD derivatives were evaluated according to the method reported previously (Takakura *et al.*, 1987). The plasma ^{111}In-radioactivity concentration, $C_p(t)$, was normalized to % of

dose/ml and analyzed by a biexponential function using the nonlinear least-square program MULTI (Yamaoka *et al.*, 1981):

$$Cp(t) = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

CL_{total} was calculated by dividing the injected dose by the area under the plasma concentration-time curve extrapolated to infinite time. Under the assumption of negligible efflux, CLI_i was calculated from,

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

where C_i was the concentration of ^{111}In-radioactivity in each organ at time, t. Then the apparent CL_{liver} was expressed as follows:

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

where W (g) is the total wet weight of the liver. CL_{urine} was estimated from Eq. (2) using the cumulative amount of SOD derivative excreted in urine.

Results

Physicochemical characteristics of SOD derivatives. Four types of SOD derivatives listed in table 1 were prepared and tested. The covalent attachment of dextran derivatives to SOD increases its molecular size but the molecular sizes of the glycosylated SODs were essentially unchanged. The enzymatic activities of SOD-polysaccharide conjugates and glycosylated SODs were retained at about 50 and 70%, respectively. The net electric charge of SOD derivatives except SOD-DEAED was negative, similar to that of SOD itself.

Stability of SOD derivatives in mouse serum. SOD was stable in mouse serum and 87% of the original activity remained at 3 hr after the start of the incubation (data not shown). All SOD derivatives were also stable in mouse serum and kept their initial activities at similar extents. After 3 hr incubation, incubation medium was subjected to gel-filtration chromatography with Sephadex G-75 to examine metabolic degradation, but no degradation products were detected in any test samples.

Plasma clearance and tissue distribution of [^{111}In]SOD derivatives. Radioactivity concentrations of [^{111}In]SOD in plasma, liver, and kidney following i.v. injection into mice at 0.1 and 10 mg/kg are shown in fig. 1 A, B and C. [^{111}In]SOD was rapidly cleared from blood circulation and accumulated in the kidney at both doses. However, the normalized amount of radioactivity in the kidney was lower at 10 mg/kg (fig. 1C). The concentration of [^{111}In]SOD in the liver was negligible at both doses (fig. 1B).

In fig. 2 A, B and C, [^{111}In]SOD-CMD showed a prolonged plasma retention and reduced renal accumulation and a slight increase in hepatic uptake. On the other hand, [^{111}In]SOD-DEAED was rapidly cleared from plasma and accumulated in the liver. Reflecting the increased accumulation in the liver, the concentration in the kidney was markedly decreased.

More rapid disappearance from the blood circulation and liver accumulation were observed after i.v. injection of [^{111}In]Gal-SOD and [^{111}In]Man-SOD (fig. 3 A, B and C). In both compounds, hepatic uptake was nonlinear and the percent amount of radioactivity in the liver at 1 mg/kg dose was about 20% lower than that at 0.1 mg/kg. Due to high accumulation in the liver, [^{111}In]Gal-SOD and [^{111}In]Man-SOD were not taken up by the kidney.

In this experiment, urine samples were collected for 2 hr ([^{111}In]SOD, [^{111}In]Gal-SOD and [^{111}In]Man-SOD) or 24 hr ([^{111}In]SOD-CMD and [^{111}In]SOD-DEAED) after i.v. administration. At 0.1 mg/kg dose, the amount of radioactivity of [^{111}In]SOD, [^{111}In]Gal-SOD and [^{111}In]Man-SOD excreted in urine within 2 hr were 11.7 ± 4.0%, 3.8 ± 0.5% and 7.2 ± 2.0%, respectively (mean ± S.D.; N = 3). However, at 10 mg/kg, the amount of [^{111}In]SOD excreted in urine was increased to 36.7 ± 4.4%. The radioactivities of [^{111}In]SOD-CMD and [^{111}In]SOD-DEAED excreted in urine within 24 hr were 21.5 ± 2.5% and 19.5 ± 4.1% of dose, respectively.

Cellular distribution of SOD derivatives in the liver. Cellular distributions of ^{111}In-labeled SOD-DEAED, Gal-SOD and Man-SOD after i.v. injection at a dose of 0.1 mg/kg are shown in fig. 4. Although all three compounds significantly accumulated in the liver, the cellular localization patterns were rather different: [^{111}In]Gal-SOD predominantly accumulated in the parenchymal cells in the liver, whereas [^{111}In]Man-SOD was recovered in the nonparenchymal cell fraction after collagenase perfusion. [^{111}In]SOD-DEAED was recovered in both fractions, probably in proportion to their total cell surface area.

Competition of hepatic uptake of glycosylated SOD by co-administration with glycosylated BSA. The liver accumulation of Gal-SOD or Man-SOD (0.1 mg/kg) after single administration or co-administration with Gal-BSA and Man-BSA (20 mg/kg) is shown in fig. 5. When [^{111}In]Gal-SOD was administrated with Gal-BSA, the liver accumulation of [^{111}In]Gal-SOD was reduced from 71.2 to 11.5% of dose at 30 min after injection. Similarly, the uptake of [^{111}In]Man-SOD by the liver was significantly inhibited by co-administration with Man-BSA.

Pharmacokinetic analysis of SOD derivatives. Areas under the curve, CL_{total}, CL_{liver}, CL_{urine} and CLI_i for six organs of ^{111}In-labeled SOD and SOD derivatives are summarized in table 2. Native [^{111}In]SOD has large CL_{urine} and CLI_{kidney} values, but other CLI values were low. These results suggested that the elimination of [^{111}In]SOD from blood circulation occurred by extensive glomerular filtration and subsequent tubular reab-

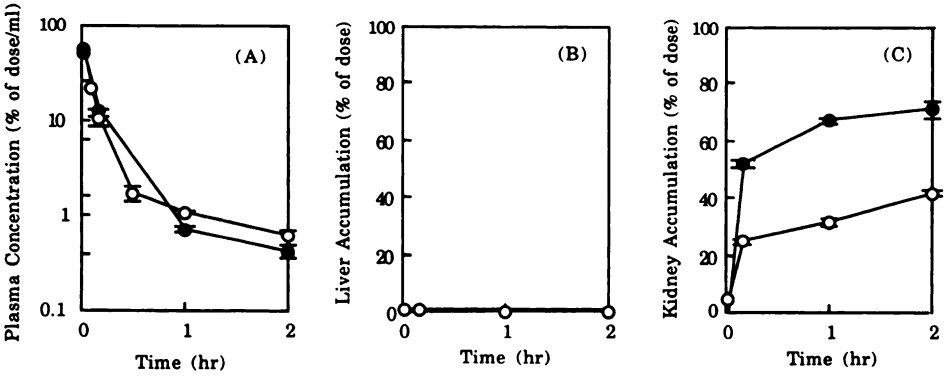

Fig. 1. A, Plasma concentration; B, liver accumulation C, kidney accumulation of radioactivity of [^{111}In]SOD following i.v. injection into mice. Results are expressed as mean ± S.D., N = 3. ●, 0.1 mg/kg dose; ○, 10 mg/kg dose.

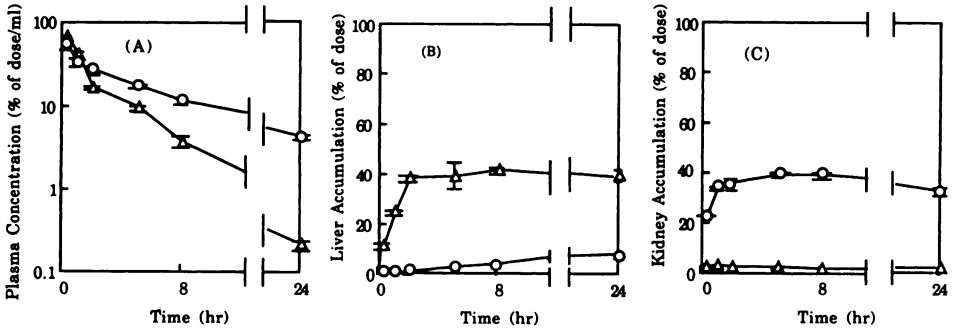

Fig. 2. A, Plasma concentration; B, liver accumulation and C, kidney accumulation of radioactivity of [^{111}In]SOD-polysaccharide conjugates following i.v. injection into mice at 0.1 mg/kg. Results are expressed as mean ± S.D., N = 3. ○, [^{111}In]SOD-CMD; △, [^{111}In]SOD-DEAED.

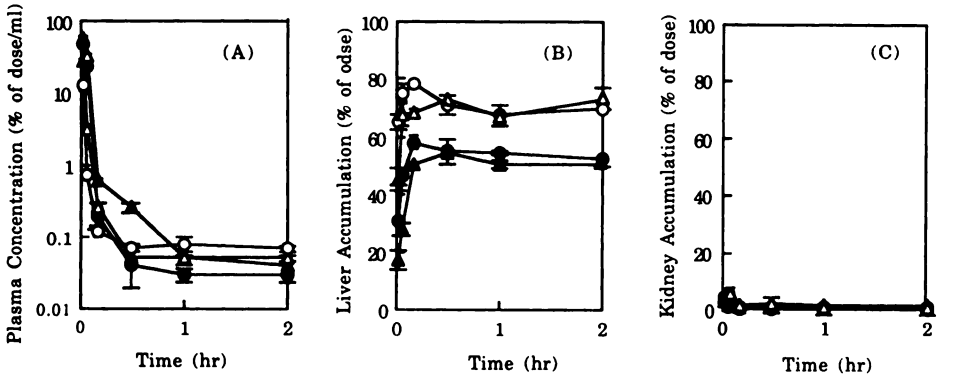

Fig. 3. A, Plasma concentration; B, liver accumulation and C, kidney accumulation of radioactivity of [^{111}In]glycosylated SOD derivatives following i.v. injection into mice. Results are expressed as mean ± S.D., N = 3. ○, [^{111}In]Gal-SOD (0.1 mg/kg); ●, 1 mg/kg; △, [^{111}In]Man-SOD (0.1 mg/kg); (▲), 1 mg/kg.

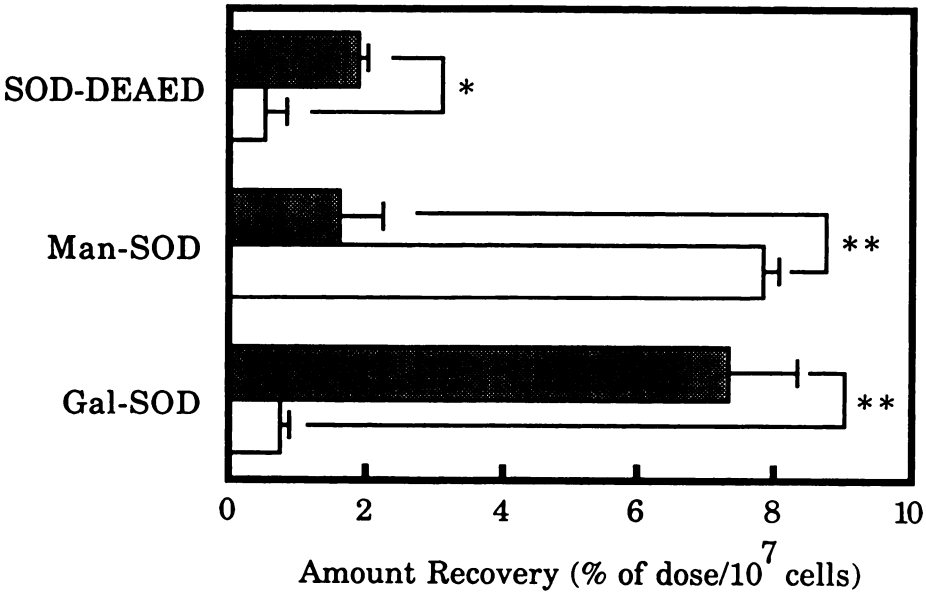

Fig. 4. Cellular localization of ^{111}In-labeled glycosylated SOD derivatives and SOD-DEAED after i.v. injection in mice at a dose of 0.1 mg/kg. □, Parenchymal cells; □, nonparenchymal cells. Results are expressed as mean ± S.D., N = 3. * $P < .01$; ** $P < .001$.

sorption in the kidney. In contrast, [^{111}In]SOD-CMD showed suppressed CLI_{kidney} which resulted in very small CL_{total}. [^{111}In]SOD-DEAED showed a relatively high CL_{liver}. [^{111}In]Gal-SOD showed a large CL_{liver} value at 0.1 mg/kg which approximately corresponds to the hepatic plasma flow rate. [^{111}In]Man-SOD showed a large CLI_{spleen} as well as CLI_{liver}. At 1 mg/kg, however, the CLI values for the liver and spleen of glycosylated SOD were decreased.

Discussion

We have previously studied the *in vivo* pharmacokinetic properties of several model proteins modified with mono- or polysaccharide derivatives such as glycosylated albumin (Nishikawa *et al.*, 1992) and soybean trypsin inhibitor and uricase conjugated with dextran derivatives (Takakura *et al.*, 1989a,b; Yasuda *et al.*, 1990; Fujita *et al.*, 1990, 1991). These investigations demonstrated that the pharmacokinetic behavior of proteins can be controlled by chemical modification with sugar moieties having appropriate physicochemical or biological properties. The purpose of the present study was to develop SOD derivatives useful for targeted delivery based on these findings.

Primarily, an appropriate synthetic method, which can retain the enzymatic activity of SOD, should be selected in order to accomplish the development of SOD derivatives applicable to therapeutics. For polysaccharide modification, periodate oxidation method was used based on our previous studies (Takakura *et al.*, 1989a; Yasuda *et al.*, 1990; Fujita *et al.*, 1990). In the contrast, monosaccharide derivatives were prepared by the

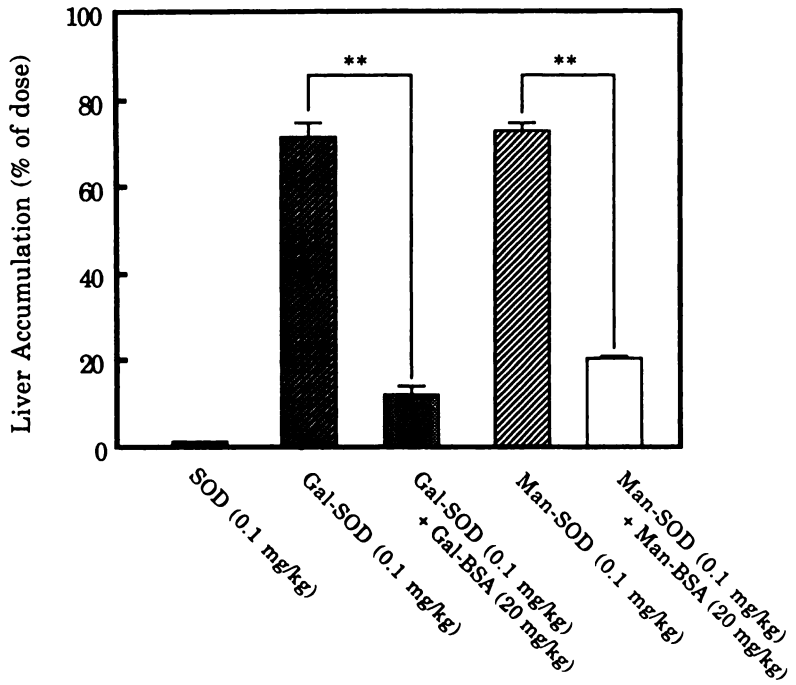

Fig. 5. Competition of hepatic uptake of ^{111}In-labeled glycosylated SOD by co-administration with glycosylated BSA after i.v. injection in mice. Glycosylated SOD (0.1 mg/kg) was injected with cold glycosylated BSAs (20 mg/kg) and liver accumulation was determined at 30 min after injection, ** $P < .001$.

TABLE 2

AUCs, clearances, and tissue uptake clearance indices of ^{111}In-labeled SOD and SOD derivatives

Compound	Dose	AUC[a]	Clearance[b]			Tissue uptake clearance index[b]			
			CL_{total}	CL_{liver}	CL_{urine}	CLI_{liver}	CLI_{spleen}	CLI_{kidney}	CLI_{muscle}
	mg/kg	% dose hr/ml		μl/hr			μl/hr/g		
SOD	0.1	9.49	10,500	42.3	1230.0	26.2	20.7	23,700.0	31.0
	10.0	9.81	10,200	52.1	3750.0	33.5	14.9	21,500.0	54.2
SOD-CMD	0.1	315.0	317	21.8	68.4	21.0	17.4	344.0	1.9
SOD-DEAED	0.1	34.8	2,870	1,440.0	561.0	1,440.0	216.0	46.3	12.0
Gal-SOD	0.1	1.05	95,200	95,000.0	5630.0	78,300.0	54.0	596.0	158.0
	1.0	3.67	28,600	16,300.0	658.0	12,300.0	33.0	354.0	72.8
Man-SOD	0.1	1.67	72,900	53,400.0	3400.0	37,100.0	9790.0	1,280.0	67.3
	1.0	5.06	19,800	11,900.0	5210.0	8,360.0	4010.0	861.0	97.5

[a] AUC, area under curve; calculated by fitting the plasma concentration curve to a biexponential equation using the nonlinear least squares program MULTI (Yamaoka et al., 1981).

[b] Calculated at 0 to 2 hr (SOD, glycosylated SOD and SOD-DEAED) or 0 to 24 hr (SOD-CMD).

method of Lee *et al.* (1976) since the method was reported to maintain biological activities. A satisfactory level of remaining enzymatic activities of all SOD derivatives were obtained by employing these synthetic methods (see table 1).

The present study demonstrated that the *in vivo* fate of human recombinant SOD can be controlled by chemical modification with various sugars. We evaluated pharmacokinetic characteristics of SOD and SOD derivatives after i.v. injection using ^{111}In-labeled compounds since ^{111}In is reported to be accumulated in the organ by the exchange into an iron-binding protein (Brown *et al.*, 1987) after intracellular degradation of proteins. The radioactivity was retained for a relatively long time even in the liver and this enabled us to estimate the net organ uptake clearance. However, at the later phase of experiment, the radioactivity of the liver gradually decreased (data not shown), suggesting a slight release of ^{111}In from the tissue. Therefore, the pharmacokinetic analysis was performed at a relatively early period when the plasma concentration had become less than 1% of dose/ml, but the efflux of ^{111}In-radioactivity from the liver was negligible. In these cases, parameters representing whole body disposition and tissue uptake rates have general pharmacokinetic significance. In addition, SOD is sufficiently stable in plasma, as shown in the stability study, and we obtained plasma concentration-time profiles of enzymatic activity of SOD almost identical to those of radioactivity determination after i.v. injection in mice. Consequently, it is likely that disposition of SOD derivatives was accurately reflected by ^{111}In-radioactivity counting.

The CL_{liver} and CL_{urine} of the four SOD derivatives together with those of polyethylene glycol-conjugated SOD (PEG-SOD) for comparing their basic *in vivo* disposition characteristics are shown in fig. 6. SOD itself is characterized by a large CL_{urine} since it is a small protein having a MW of 32 kDa which is susceptible to glomerular filtration (Taylor and Granger, 1984). The CL_{liver} was markedly increased by conjugating with DEAED, but CL_{urine} was also reduced in this case. In contrast, conjugation of CMD to SOD reduced both CL_{liver} and CL_{urine} and thus resulted in prolonged plasma retention of enzymatic activity. Similar results were obtained for PEG-SOD, as reported by several investigators (Pyatak *et al.*, 1980; Fuertges and Abuchowski, 1990). On the other hand, by introducing monosaccharides to SOD (Gal-SOD and Man-SOD), the value of CL_{liver} was 100 to 1000 times increased without significant change in CL_{urine}. Their CL_{liver} values at a dose of 0.1 mg/kg

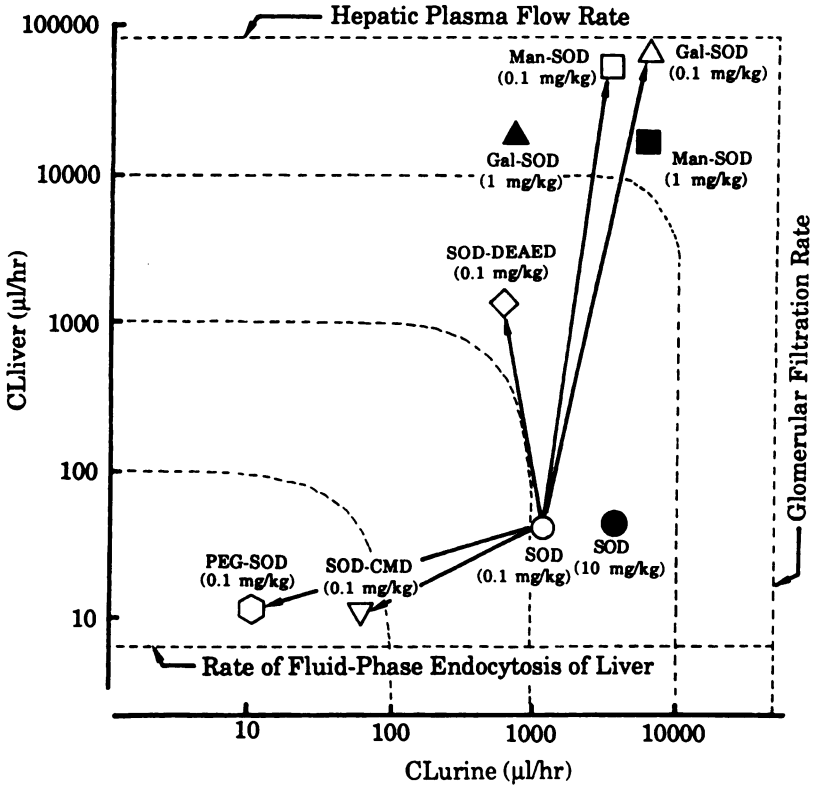

Fig. 6. Hepatic and urinary clearances of SOD derivatives in mice. Hepatic plasma flow rate (Gerlowski and Jain, 1983), rate of fluid-phase endocytosis of liver (Munniksma *et al.*, 1980) and glomerular filtration rate (Dedrick, 1973) were calculated assuming the weight of a mouse to be 25 g.

were nearly equal to the hepatic plasma flow rate, suggesting almost complete uptake of Gal-SOD and Man-SOD in the liver.

In addition to the altered pharmacokinetic properties of SOD derivatives, interesting results were obtained in renal disposition of native SOD in which marked accumulation of radioactivity was observed after i.v. injection. We have demonstrated that this accumulation of [^{111}In]SOD occurred via proximal tubular reabsorption after glomerular filtration in the rat kidney perfusion experiment (unpublished data). The ratios of accumulated amount of [^{111}In]SOD in the kidney to total amount recovered in the kidney and urine were 0.64 (0.1 mg/kg) and 0.24 (10 mg/kg) after i.v. injection in mice (see fig. 1) suggesting saturation in the reabsorption process.

In contrast, [^{111}In]Gal-SOD and [^{111}In]Man-SOD were not accumulated in the kidney. The values of CLI_{kidney} for monosaccharide derivatives were 1 to 2 orders of magnitude smaller than that for [^{111}In]SOD, suggesting that monosaccharide modification drastically reduced the tubular reabsorption of the protein. A recent study reported that glycosylated albumin was virtually excluded from reabsorption process in the proximal tubule using a micropuncture technique (Kowluru *et al.*, 1992). We have also confirmed that the renal tubular reabsorption of [^{111}In]Gal-SOD and [^{111}In]Man-SOD was dramatically suppressed in comparison with that of native [^{111}In]SOD using rat *in situ* isolated perfusion kidney system (unpublished data).

The detailed mechanism of cellular toxicity mediated by reactive oxygen species including superoxide anion is unknown. Therefore, an ideal cellular delivery mode of SOD has not been established although various approaches have been reported for improved therapeutic efficacy of SOD. It is generally known that oxidative stress occurring inside the cell may be minimized, since most cells are highly enriched with antioxidative system. From this reason, maintenance of high extracellular level of SOD is considered to be important. Thus, conjugation of PEG (PEG-SOD) and polystyrene-co-maleic acid to SOD reported to result in prolonged plasma or extracellular space level of SOD and subsequent improved effect (Pyatak *et al.*, 1980; Inoue *et al.*, 1989; Watanabe *et al.*, 1989; Fuertges *et al.*, 1990). In addition, SOD having a C-terminal heparin-binding domain has been genetically constructed and proved to bind the surface of endothelial cells and show inhibitory effects against oxygen toxicity (Inoue *et al.*, 1990).

On the other hand, several reports showed the importance of intracellular level of SOD in protecting cells from oxygen-mediated toxicity (Freeman *et al.*, 1983; Kyle *et al.*, 1988; Beckman *et al.*, 1988; Liu *et al.*, 1989). Augmentation of cellular uptake and pharmacological activities of SOD have been demonstrated through liposomal entrapment and PEG conjugation in cultured endothelial cells. However, the intracellular fate of enzyme was not shown in these studies.

Generally, many glycoproteins are known to bind specific receptors on the cell surface and be internalized. In the present study, two receptor systems, the asialoglycoprotein receptor on hepatocyte (Ashwell and Morell, 1974; Kawasaki and Ashwell, 1976) and the mannose/N-acetylglucosamine receptor on Kupffer and endothelial cells in the liver (Brown *et al.*, 1978; Schlesinger *et al.*, 1978; Magnusson and Berg, 1989) were evaluated as targets for site-specific delivery of enzymes. The advantages of these systems arise from: 1), high affinity of the receptor to the ligand; 2), rapid recycling of the receptor molecules (Magnusson and Berg, 1989) and 3), specific expression of receptors on a certain cell type. After i.v. injection, [^{111}In]Gal-SOD accumulated in the hepatic parenchymal cells while [^{111}In]Man-SOD was taken up by the nonparenchymal cells in the liver and spleen (see fig. 4). Furthermore, specificity of the attached sugar moieties was also confirmed by the competition experiments using glycosylated BSA derivatives (see fig. 5). Thus, receptor-mediated endocytosis was observed in glycosylated

SODs as well as albumin derivatives (Nishikawa *et al.*, 1992) suggesting the generality of this approach in a delivery of enzymes to intracellular space.

It is noteworthy that small proteins like SOD, which undergo rapid glomerular filtration, can be targeted to these cells by modifying with monosaccharides without a significant increase in MW. It is also an interesting issue to clarify the relationship between the receptor-mediated endocytosis and the molecular size of glycoproteins. In our preliminary study, we showed that ^{111}In-labeled glycosylated IgGs (MW: 150 kDa) synthesized by the same method as glycosylated SODs were rapidly accumulated in the liver after i.v. injection in mice. Therefore, it was suggested that proteins with a relatively wide range of MW (35–150 kDa) can be recognized and endocytosed by asialoglycoprotein receptor or mannose/N-acetylglucosamine receptor of the liver.

In contrast, [^{111}In]SOD-DEAED demonstrated extensive hepatic uptake presumably due to electrostatic interaction with both parenchymal and nonparenchymal cells depending on the percentages of their surface area ratio (73:27) (Nishida *et al.*, 1991). We have elucidated hepatic disposition profiles of cationized and glycosylated BSA in details by a computer simulation in a previous paper (Nishida *et al.*, 1992). Slow internalization of cationized BSA adsorbed on the cell surface compared with glycosylated BSA was observed there and SOD-DEAED is expected to be retained on the surface of the liver cells for a considerably long period.

Immunological properties of SOD derivatives are very important, especially in the case of clinical application. Fiume *et al.* (1982, 1986) demonstrated that a drug-conjugated neoglycoprotein, lactosaminated albumin, was not immunogenic when prepared with homologous albumin. Dextran conjugation was also reported to reduce immunogenicity of proteins (Yasuda *et al.*, 1990). Although further studies are required on the immunogenicity/antigenicity of SOD derivatives, chemical modification of human recombinant SOD with mono- or polysaccharides might be advantageous in the aspect.

In conclusion, we demonstrated that targeted delivery of recombinant SOD can be achieved by chemical modification utilizing sugar moieties. The derivatives developed in this study are classified as follows: 1), long-circulating type (SOD-CMD); 2), cell-surface targeting type (SOD-DEAED); 3), intracellular targeting type (Gal-SOD for hepatocyte and Man-SOD for resident macrophages and endothelial cells in the liver and spleen). These compounds would be useful for not only therapeutics of superoxide-related diseases but also for basic analysis of relationship between cellular localization and biological effects of SOD. In particular, Gal-SOD and Man-SOD seem to be unique and effective, although they might be rapidly inactivated inside the cell due to the lysosomal degradation pathways for glycoproteins. We have started pharmacological studies of these SOD derivatives using a rat hepatic ischemia-reperfusion model and promising therapeutic effects were obtained for Gal-SOD and Man-SOD. The therapeutic effectiveness of all types of SOD derivatives will be described in a future report.

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