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THERAPEUTIC EFFECTS OF SUPEROXIDE DISMUTASE DERIVATIVES MODIFIED WITH MONO- OR POLYSACCHARIDES ON HEPATIC INJURY INDUCED BY ISCHEMIA/REPERFUSION

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SUMMARY: Therapeutic effects of four types of recombinant superoxide dismutase (SOD) derivatives, conjugates with polysaccharides, carboxymethyl (SOD-CMD) and diethylaminoethyl (SOD-DEAED) dextrans and galactosylated (Gal-SOD) and mannosylated (Man-SOD) derivatives, on hepatic ischemia/reperfusion injury were studied in rats. Hepatic injury induced by transient occlusion and subsequent reflow of hepatic blood was evaluated by the analysis of biliary excretion of bromosulfophthalein (BSP) injected intravenously. At a dose of 10000 units/kg, native SOD and SOD-DEAE did not show any significant effect and SOD-CMD showed slight effect. On the other hand, Gal-SOD and Man-SOD, targeted to the liver parenchymal and nonparenchymal cells, respectively, by a receptor-mediated endocytosis, exhibited superior inhibitory effects. These results demonstrated that these glycosylated SOD derivatives were useful for the prevention of hepatic ischemia/reperfusion injury.

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, leads to the use of an antioxidant enzyme such as SOD as therapeutic agents (1). 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 defense mechanism against tissue injury mediated by reactive oxygen species. However, the experimental and therapeutic potentials of SOD are limited because SOD is rapidly cleared by glomerular filtration

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Abbreviations: SOD, superoxide dismutase; PEG, polyethylene glycol; CMD, carboxymethyl dextran; DEAED, diethylaminoethyl dextran; SOD-CMD, SOD-carboxymethyl dextran conjugate; SOD-DEAED, SOD-diethylaminoethyl dextran conjugate; Gal-SOD, galactosylated SOD; Man-SOD, mannosylated SOD; BSP, bromosulfophthalein.

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in the kidney, leading to a plasma elimination half-life of only 5-10 min after intravenous injection in animal models (2,3).

In our series of investigations, we demonstrated that pharmacokinetic properties of protein drugs could be controlled by chemical modification utilizing sugar moieties (4-10). Based on these findings, we developed human recombinant SOD derivatives modified with mono- and polysaccharides in a previous study (11). Pharmacokinetic analysis revealed that targeted delivery of SOD to the liver could be achieved by such chemical modifications. The present report describes the pharmacological effects of SOD derivatives modified with mono- and poly- saccharides on liver injury induced by a transient occlusion followed by reperfusion of the portal vein and hepatic artery.

MATERIALS AND METHODS

Chemicals. Recombinant human SOD (111-Ser) was kindly supplied by Asahi Chemical Industry Co., Shizuoka, Japan. Dextran with an average molecular weight of about 10 kDa was purchased from Pharmacia, Uppsala, Sweden and derivatised to anionic (CMD) and cationic (DEAED) forms as reported (11). D-Galactose and D-mannose were obtained from Wako Pure Chemical, Osaka, Japan. Sulfobromophthalein sodium hydrate (BSP) was purchased from Aldrich Chem. Co., WI, USA. All other chemicals were of the finest grade available.

Synthesis of SOD Derivatives. Four types of SOD derivatives were synthesized as reported previously (11). In brief, two types of SOD-polysaccharide conjugates, SOD-CMD and SOD-DEAED, were synthesized by conjugating SOD with CMD and DEAED oxidized by sodium periodate in 50 mM borate buffer (pH 10.0) for 24 hr at 4 °C in the dark, followed by reduction with 1 mM sodium borohydride for 2 hr at 4 °C. The obtained compounds were purified by gel-filtration chromatography using Sephadex G-75 column . Gal-SOD and Man-SOD were synthesized by reacting SOD with 2-imino-2-methoxyethyl 1-thioglycoside prepared by the method of Lee *et al.* (12) in 50 mM borate buffer (pH 10.0) for 5 hr at room temperature. The reaction mixture was concentrated by ultrafiltration and applied to Sephadex G-25 column equilibrated with 0.1 M acetate buffer (pH 6.0) to separate the coupled product from the unreacted compound. Purity of glycosylated SODs was confirmed by affinity chromatography with Con A-Sepharose (Man-SOD) or agarose-peanut lectin (Gal-SOD). A polyethylene glycol conjugate (SOD-PEG) synthesized by the method reported previously (11) was used for comparison. The physicochemical properties of SOD derivatives are summarized in Table 1.

Table 1. Physicochemical Properties of SOD and SOD Derivatives

Compound	Number of NH_2-groups [a]	Apparent Molecular Weight [b]	Remained Enzymatic Activity [c]	Electric Charge at pH 7.4 [d]
SOD	24.0	32000	100.0	(−)
SOD-CMD	18.0	150000	50.0	(−)
SOD-DEAED	15.0	150000	55.0	(+)
Gal-SOD	2.7	35000	79.4	(−)
Man-SOD	3.8	34000	65.6	(−)

a : Determined by TNBS method.
b : Estimated by HPLC gel filtration chromatography.
c : Determined by nitroblue tetrazolium reduction method.
d : Determined by a batch method using a DEAE-Sephadex A-50 anion exchanger and CM-Sephadex C-50 cation exchanger.

Hepatic Ischemia/Reperfusion Experiment. Hepatic ischemia/reperfusion experiment was carried out according to the method of Kawamoto *et al.* (13). Male Wistar rats (180 - 220 g) maintained on standard rat foods and water *ad libitum* were anesthetized with pentobarbital sodium (50 mg/kg). Five minutes after injection of 0.2 ml of saline (control), SOD, or SOD derivatives (10000 units/kg) into the right femoral vein, hepatic ischemia was elicited by occluding the portal vein and the hepatic artery. After 20 min of occlusion, portal and hepatic arterial blood was allowed to reflow through the liver. After 60 min of reperfusion, BSP dissolved in 0.2 ml of saline was bolusly injected into the right femoral vein. At an appropriate time period after reperfusion, bile samples were collected from the common bile duct during 12 consecutive 5 min collection periods for 60 min. BSP levels in bile was determined spectrophotometrically at 580 nm in 0.1 M NaOH.

Data Analysis. The biliary excretion rate-time curves of BSP after intravenous injection were analyzed based on statistical moment theory (14,15) in order to evaluate hepatic injury quantitatively. Moments are defined as follows:

$$Fa=\int_0^\infty (dXb/dt)dt$$

$$\bar{t}a=\int_0^\infty t(dXb/dt)dt/Fa\ ,$$

where t is the time, and dXb/dt is biliary excretion rate of BSP. The value of dXb/dt is normalized with the injected dose and expressed as % of dose/min. Fa and $\bar{t}a$ are the biliary recovery ratio and biliary mean excretion time of BSP, respectively. The moments are calculated by numeral integration using a linear trapezoidal formula and extrapolation to infinite time based on a monoexponential equation.

RESULTS

The rate of bile flow markedly decreased immediately after reperfusion but it recovered gradually with time after reperfusion and returned to normal level within 60 min. Pretreatment with SOD and its derivatives did not significantly affect the change in bile flow (data not shown).

Fig. 1 shows biliary excretion profiles of BSP during the reflow period after 20 min occlusion of hepatic vessels in rats with sham operation and saline pretreatment (Fig. 1A) and in rats with SOD and its derivatives pretreatment (Fig. 1B and C). From these biliary excretion curves, moment parameters were obtained as summarized in Table 2. In sham-operated group, the biliary level of BSP reached its maximum at 10 min following BSP administration and total biliary BSP recovery of 94 % of dose was obtained. The value of $\bar{t}a$, biliary mean biliary excretion time, was 15.2 min. Transient occlusion followed by reperfusion resulted in a significant decrease in the BSP recovery in the bile (78 %) and increase in $\bar{t}a$ (18.2 min), indicating hepatic injury by ischemia/reperfusion.

Intravenous injection of SOD prior to the occlusion showed no significant effect on both parameters. On the other hand, administration of monosaccharide-modified SODs, Gal-SOD and Man-SOD, inhibited the impairment of biliary excretion of BSP to a great extent. In particular, Fa and $\bar{t}a$ values for Man-SOD treated group were similar to those for sham-operated group. SOD-DEAED failed to recover the biliary excretion of BSP while SOD-CMD showed slight inhibitory effect on impaired biliary excretion of BSP induced by ischemia/reperfusion. SOD-PEG improved Fa value but the ta value was similar to that obtained in rats having occlusion without SOD treatment.

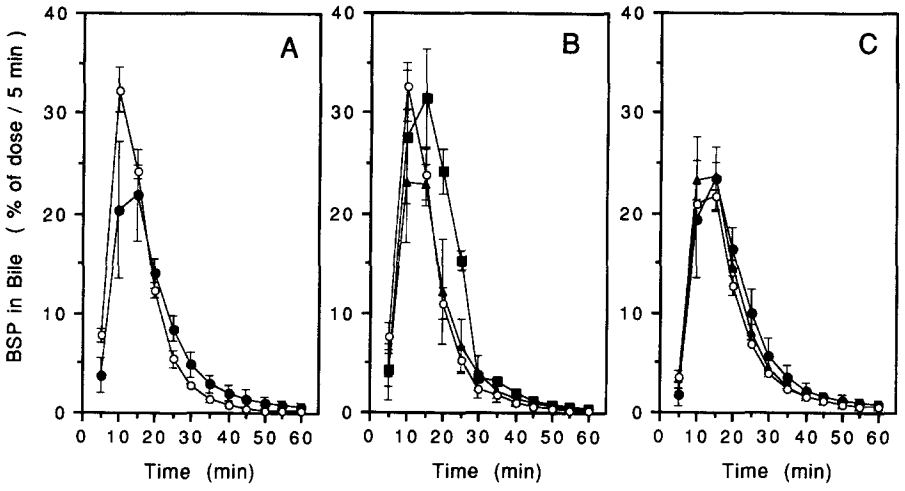

Fig. 1. Biliary Excretion of BSP during Rat Liver Ischemia/Reperfusion Experiment

A. Sham operation (○); Occlusion + saline (●)
B. SOD (▲); Gal-SOD (○); Man-SOD (■)
C. SOD-CMD (▲); SOD-DEAED (○); SOD-PEG (●)
Results are expressed as the mean ± S.D. for at least three rats.

DISCUSSION

The purpose of the present study was to clarify the pharmacological efficacy of SOD derivatives with various pharmacokinetical characteristics (11) on hepatic injury induced by ischemia/reperfusion. Monosaccharide-modified SODs, Gal-SOD and Man-SOD, are derivatives

Table 2. Moment Parameters for Biliary Excretion of BSP after i.v. Injection of SOD Derivatives in Rat Liver Ischemia/reperfusion Experiment

Treatment	Fa (% of dose)	P vs. Sham	P vs. Occlusion	$\bar{t}a$ (min)	P vs. Sham	P vs. Occlusion
Sham operation	93.9 ± 4.7	—	<0.01	15.2 ± 0.5	—	<0.01
Occlusion +saline	78.2 ± 3.2	<0.01	—	18.2 ± 1.4	<0.01	—
Occlusion +SOD	81.8 ± 4.0	<0.01	n.s.	18.0 ± 2.0	<0.05	n.s.
Occlusion +Gal-SOD	91.0 ± 7.5	n.s.	<0.01	15.6 ± 1.3	n.s.	<0.01
Occlusion +Man-SOD	94.6 ± 6.1	n.s.	<0.01	15.5 ± 1.1	n.s.	<0.02
Occlusion +SOD-CMD	86.3 ± 4.2	<0.05	<0.01	17.4 ± 1.2	<0.01	n.s.
Occlusion +SOD-DEAED	81.7 ± 2.0	<0.01	n.s.	20.9 ± 4.1	n.s.	n.s.
Occlusion +SOD-PEG	89.3 ± 2.6	n.s.	<0.01	19.4 ± 3.5	<0.05	n.s.

Results are expressed as the mean ± S.D. for at least three rats.
n.s.; Not significant

designed for intracellular targeting and are effectively internalized by a receptor-mediated endocytosis in the liver parenchymal and nonparenchymal cells, respectively. On the other hand, SOD-CMD is a long-circulating type which shows no significant tissue interaction in addition to the restricted urinary excretion owing to its large molecular size. SOD-DEAED targets the cell-surface of especially the liver due to electrostatic interaction.

We used the published method (13) for the pharmacological study. The degree of injury induced by ischemia/reperfusion was smaller than that reported by the authors (13) probably because fed animals were used in the present study (16). In contrast to native SOD, Gal-SOD and Man-SOD exhibited marked protection effects against hepatic injury after ischemia/reperfusion. Approximately 50 % of injected dose of these glycosylated derivatives were estimated to be targeted to the liver whereas less than 1 % would be taken up by the liver in the case of native SOD (11). Although two polysaccharide conjugates had no significant effect, SOD-PEG which had a longer plasma half-life than SOD-CMD (11) was slightly effective in preventing the injury. Ineffectiveness of SOD-DEAED may be explained by its small accumulation in the liver cells (11) due to slow endocytosis compared with monosaccharide-modified SODs (9).

While Gal-SOD and Man-SOD are effectively targeted to the liver, they are suspected to be inactivated inside the cells by the lysosomal degradation after endocytosis (17,18). However, the derivatives did show superior effects suggesting that they retained enzymatic activities in the cells and protected the cellular damage induced by ischemia/reperfusion. The mechanism of inhibition of the injury by Gal-SOD and Man-SOD is unclear, but it might be postulated that missorting of these glycosylated SODs occur which lead to their escape from regular degradation pathways after internalization (19). Another possibility is that both derivatives are highly resistant to lysosomal degradation. It is recently study reported that the enzymatic activity of unmodified SOD was marginally affected by incubation in the presence of a purified lysosome extract (20). Chemical modification of the enzyme with mannose and galactose might enhance its stability against lysosomal digestion.

Although the mechanism of formation of oxgen-derived free radicals in hepatic injury induced by ischemia/reperfusion remains controversial, a great deal of evidence suggested the involvement of superoxide anions (13,21,22). However, it is uncertain as to which type of cell, endothelial, Kupffer or parenchymal cells in the liver or neutrophiles in the vasculature, is the main source of superoxide anion. Our results may offer some implications. The therapeutic effect of Gal-SOD which is taken up by hepatocytes can be ascribed to its protection of the cells from the damage mediated by superoxide anions. A higher activity of Man-SOD suggested that superoxide anions from the non-parenchymal cells, *i.e.*, endothelial and/or Kupffer cells, played significant roles in mediating or contributing to ischemia/reperfusion injury.

Thus, the present study demonstrated that two types of glycosylated SOD derivatives were useful for the prevention of hepatic ischemia/reperfusion injury. The findings also would give useful basic information on the mechanism of tissue injury mediated by reactive oxygen species and also on that of its protection by SOD derivative.

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