Targeting of superoxide dismutase to the liver results in anti-inflammatory effects in rats with fibrotic livers

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Background/Aims: The rapid clearance from plasma and the limited uptake of superoxide dismutase (SOD) in the liver hampers the effectiveness of this enzyme in liver diseases. We therefore compared the pharmacokinetics and in vivo efficacy of SOD with two modified forms of this protein: SOD coupled to the copolymer DIVEMA and mannosylated-SOD.

Methods: Reactive oxygen scavenging activity of SOD conjugates was tested in livers of bile duct ligated rats. Intrahepatic production of reactive oxygen species (ROS) and neutrophil infiltration were studied immunohistochemically and related to the organ and cellular distribution of radiolabeled SOD conjugates.

Results: Native SOD was rapidly cleared from the circulation and accumulated in renal tubuli. The enzyme had no effect on the intrahepatic ROS production. Covalent attachment of SOD to DIVEMA yielded a polyanionic conjugate with a prolonged elimination half-life compared to native SOD. In contrast to native SOD, DIVEMA-SOD was taken up by the liver via scavenger receptors. Mannosylation of SOD (Man-SOD) resulted in a conjugate that was rapidly cleared from the blood. This Man-SOD was taken up by non-parenchymal liver cells. The pharmacokinetics of SOD and its derivatives were similar in normal and bile duct ligated rats. Efficacy studies with Man-SOD revealed only a slight decrease in intrahepatic ROS production. However, DIVEMA-SOD exhibited a potent inhibitory effect on ROS production in the liver. Nearly complete ROS-scavenging activity was observed in the portal areas.

Conclusions: Considering the prolonged half-life, the increased delivery of SOD to the target cells, and the concomitant increased effectiveness, application of DIVEMA-SOD seems a promising new approach to attenuate intrahepatic inflammatory processes.

Key words: Bile duct ligated rats; DIVEMA-SOD; Kinetics; Man-SOD; Oxide radical scavenging; SOD; Superoxide dismutase.

Reactive oxygen species (ROS) play an important role in the pathogenesis of liver fibrosis (1–3). A large variety of stimuli is supposed to induce this chronic disease. ROS production by macrophages and neutrophils is a prominent early feature in this process (1,4,5). Firstly, ROS stimulate fibrosis by damage to hepatic cells (6,7) which in turn enhance the inflammatory process, but they may also induce activation of endothelial cells (8), Kupffer cells (9) and hepatic stellate cells (2,9), which leads to the production of a cascade of pro-inflammatory cytokines and growth factors within the liver. Even collagen synthesis in hepatic stellate cells, the hallmark of fibrosis, is directly enhanced by oxygen free radicals (5,10,11). Some studies report beneficial effects of oxygen free radical scavengers, such as α-tocopherol (12,13) and N-acetylcysteine (14) in experimental models of fibrosis. However, anti-inflammatory and anti-fibrotic effects of the major oxygen free radical scavenging enzyme SOD have been disappointing in vivo (15,16). Relevant effects have only been found in vitro, which may be related to the very short half-life of oxygen radicals. As a consequence, efficient radical scavengers have to meet three crucial
criteria: they have to be present at the right place, at the right moment and in the right concentration.

Cell-specific delivery of SOD is a promising option to meet all three criteria. Uncoupled SOD is rapidly cleared from the circulation by glomerular filtration (t1/2 is approximately 6 min) (17). Conjugates of SOD and the macromolecule poly-ethylene glycol (PEG) (18,19) have been the subject of many studies. It has been shown that the latter modification prevents the renal clearance and prolongs the plasma half-life of SOD profoundly (20,21). However, coupling of SOD to PEG does not induce an increased uptake of this scavenger in target cells. We have now studied the effects of SOD coupled to the copolymer DIVEMA (22) and mannosylation of this enzyme to facilitate uptake by Kupffer and liver endothelial cells. These compounds were studied in vivo in rats with fibrotic livers, induced by bile duct ligation. It is shown that covalent attachment of SOD to DIVEMA leads to a significant uptake of SOD by non-parenchymal cells of the liver in normal and fibrotic rats, in contrast to native SOD. Moreover, a profound reduction in intrahepatic ROS production in vivo could be demonstrated in fibrotic animals treated with this new compound. Parallel experiments with native SOD and with Man-SOD, which is also preferentially taken up by hepatic sinusoidal cells, favor the applicability of DIVEMA-SOD as a site-specific ROS scavenger with a putative antifibrotic activity.

Materials and Methods

Chemicals

Cu, Zn superoxide diamutase (SOD) isolated from bovine erythrocytes was obtained from Waku Pure Chemicals Industries Ltd, (Japan) with a specific activity of 3650 Units·mg⁻¹. All other compounds were supplied by Merck (Darmstadt, Germany).

Preparation of DIVEMA-SOD and Mon-SOD

DIVEMA-SOD and Man-SOD were conjugated with the copolymer of divinyl ether and maleic anhydride (DIVEMA). Mw 30 kDa and Mw/Mn = 1.4) using an amino group protecting agent, 2,3-dimethylmaleic anhydride (22). The resulting DIVEMA-SOD was purified by FPLC (Superose 12 preparative column, Pharmacia, Uppaala, Sweden). The enzymatic activity of DIVEMA-SOD was measured according to the method of McCord & Fridovich (23), using native SOD as a reference. The SOD content of this conjugate was 18.8% and the activity was 380 U·mg⁻¹.

Man-SOD. Man-SOD was synthesized according to Lee et al. (24). Cyanomethyl-1-thiomannoside was activated with 0.01 M sodium methoxide in methanol at RT. After 74 h the solvent was evaporated and the remaining syrup containing 2-imino-2-methoxyethyl-l-thiomannoside was mixed with 15 ml of 50 mM borate pH 10, containing SOD. After 2 h, the mannosylated SOD was purified and characterized as described (25). The number of mannose residues were 17 mol/mol SOD and the enzymatic activity of this conjugate was 2750 U·mg⁻¹.

Monomeric radio-iodinated probes

The SODs were radio-iodinated by the chloramine T method to a final activity of about 1 μCi·μg⁻¹ protein (26). Before each experiment, free ¹²⁵I was removed by extensive dialysis against phosphate-buffered saline, until the percentage precipitable iodine was at least 95% with trichloroacetic acid at a final concentration of 10%.

Animal studies. pharmacokinetics

Male Wistar rats (outbred strain HsdCpb:WU, Harlan, The Netherlands) weighing 230–260 g were maintained on rat chow (Hope Farms) and tap water ad libitum in a temperature controlled chamber at 24°C with a 12-h light/dark circle. All animal experiments were performed after receiving approval of the local Animal Care and Use Committee of the University of Groningen. Liver fibrosis was induced by ligation of the common bile duct according to standard procedures (27). Three weeks after the obstruction all animals showed severe fibrosis of the liver (28). At this time point the kinetics, the tissue distribution and efficacy of SOD and its conjugates were studied.

The animals were anesthetized with Hypnorm/Valium and the trachea was intubated to facilitate respiration. The carotid artery was cannulated with a polyethylene cannula (ID, 0.5 mm; OD, 1.0 mm) for rapid blood sampling. Three different doses: 4, 10 and 40 mg·kg⁻¹ of SOD, Man SOD and DIVEMA SOD, spiked with 0.5·10⁶cpm ¹²⁵I labeled protein were given as a bolus in 0.4 ml 0.154 M NaCl via the vena venaoralis. Blood samples, 200 μl each, were collected via the cannula in the carotid artery at various time points between 2 and 120 min after administration. After heparinization with 2 μl of 500 IU·ml⁻¹ heparin, the samples were centrifuged for 5 min at 10000 rpm. The clear plasma was treated as follows: 100 μl of plasma was mixed with 100 μl ice cold 20% trichloroacetic acid/0.1% NaI, and stored for 10 min on ice. The samples were centrifuged and the supernatant was pipetted off. The pellet was washed once with 100 μl of 20% TCA/0.1% NaI. The pellet and pooled supernatants were assayed for gamma radiation (RiaStar, Packard Instruments, Meriden, USA).

The amount of radioactivity was converted to the amount of mg drug carrier per ml of rat plasma.

Pharmacokinetic profiling

The plasma concentration versus time data were analyzed by the curve fitting program MW/PHARM (Mediware, Groningen, The Netherlands) (29). It was assumed that the pharmacokinetics of SOD, Man-SOD and DIVEMA-SOD can be described by a single compartment model. The parameters of the model: the volume of distribution V (in ml·kg⁻¹), the plasma clearance Clp (in ml·min⁻¹·mg⁻¹), elimination half-life t½ (in h⁻¹) and the elimination rate kₚ (in h⁻¹) were obtained by a curve-fitting procedure by minimizing the sum of squared deviations (SSQ) between the logarithm of the measured plasma concentrations and the logarithm of the concentration calculated according to the pharmacokinetic model. For each compound the data of all animals (dose and time of administration, and time and measured concentration of plasma samples, n=24–54) were used simultaneously for the estimation of V, Clp, t½ and kₚ. The fitting procedure was repeated several times using two different algorithms (Simplex & Marquardt) for finding the above-mentioned parameters. From the covariance matrix, the standard error (SE) of the parameters was calculated (30). The suitability of the applied model, one-compartment model with first-order elimination was checked by repeating the analysis using an open two-compartment model with first-order elimination. With this model no improved fit could be obtained and therefore the single compartment model was further employed for the pharmacokinetic analysis.

Tissue distribution

Male rats (outbred strain HsdCpb:WU) were anesthetized with Hypnorm/Vetan. Tracer amounts of ¹²⁵I radioiodiated SOD, Man-SOD and DIVEMA-SOD were given intravenously. The animals were sacrificed 10 min after dosing. Tissues were removed and assayed for their radioactivity. Radioactivity was corrected for the amount of blood remaining in the organs. Tissue distribution was also studied after pre-administration of blockers of the scavenger receptor: formaldehyde-treated HSA (Form-HSA) (31), DIVEMA and a blocker of the mannoside receptor: mannan (32).
Immunohistochemical detection of SOD in tissue sections

A dose of 10 mg·kg⁻¹ of SOD, Man-SOD or DIVEMA-SOD was iv injected and after 60 min samples of the liver and spleen were frozen in liquid isopentane (−80°C). Cryostat sections of 4 μm were stained with polyclonal antibodies against Cu,Zn-SOD (generously provided by Dr. Ir. H. W. Verspaget, Leiden, The Netherlands). Staining was performed according to standard indirect immunoperoxidase techniques with AEC in the final step (33).

To identify the cells responsible for the uptake of SOD or SOD derivatives, double stainings were performed. Acetone-fixed cryostat sections (4 μm) were stained with the polyclonal anti-SOD serum and a monoclonal antibody against Kupffer cells (ED2, Serotec, Oxford, UK) or with the anti-SOD serum and a monoclonal antibody against endothelial cells (RECA-1, Serotec). SOD and the designated cell marker were subsequently visualized with respectively peroxidase conjugated goat-anti-rabbit IgG and alkaline phosphatase conjugated rabbit-anti-mouse IgG (both from Dako, Glostrup, Denmark). Routine histochemical analysis of sections stained for SOD and hematoxylin-eosin was performed to assess the binding of SOD derivatives to parenchymal and bile duct epithelial cells.

Fig. 1. Plasma concentration time profiles of SOD (panel A) in normal and BDL rats and tissue distribution profile of a tracer amount of iodinated SOD (Panel B) in BDL rats. Tissues were removed 10 min after dosing. *Liver uptake of SOD is statistically significant (Student's t-test, p<0.05) compared to the uptake of SOD in normal livers. All experiments are the mean values±SD (n=3).

Fig. 2. Plasma concentration time profiles of Man-SOD (panel A) in normal and BDL rats and tissue distribution profile of a tracer amount of iodinated Man-SOD (Panel B) in BDL rats. Tissues were removed 10 min after dosing. All experiments are the mean values±SD (n=3).
Demonstration of activated neutrophils
The presence of neutrophils was demonstrated in air-dried, 4-μm cryostat sections by demonstrating endogenous peroxidase (PO) activity (34). This staining is shown to be quantitatively related to the number of neutrophils, taking advantages of endogenous H2O2 production by activated neutrophils. Sections were incubated for 20 min at 37°C in 0.1 M Tris-HCl buffer pH 7.2 containing 0.5 mg·ml⁻¹ 3,3-diaminobenzidine (DAB, Sigma). H2O2 production by neutrophils and concomitant PO activity will induce oxidation and polymerization of DAB. Control experiments in which catalase was added to the incubation medium (0.01 to 0.1 mg·ml⁻¹) showed that staining was inhibited in a dose-dependent manner, confirming the involvement of endogenous H2O2 production in DAB polymerization. Superoxide anion production by activated PMN was demonstrated in kidney cryostat sections according to the method of Briggs et al. (35), applied at the light microscopical level. This method is based upon the oxidation of Mn²⁺ to Mn³⁺ by O₂⁻ and the subsequent oxidation of DAB by Mn³⁺. Inhibition of staining by addition of SOD (Sigma) to the medium (300 U·ml⁻¹) confirms the demonstration of O₂⁻ production by this method (34). Sections were routinely stained with hematoxylin, and infiltrating cells were evaluated by light microscopic methods.

Efficacy studies
Bile duct obstructed animals were anesthetized as described above. SOD or the SOD-conjugates were iv injected with a dose resulting in an activity of 100 units per ml of plasma at 60 min after administration. The doses were: 50 mg·kg⁻¹ for both Man-SOD and DIVEMA-SOD and 100 mg·kg⁻¹ for SOD. At this time point the animals were sacrificed and the liver, spleen, lungs and kidneys were removed for histochemical evaluation of O₂⁻ radical production.

Results
Dosages of 4, 10 and 40 mg·kg⁻¹ SOD, Man-SOD and DIVEMA-SOD were iv injected in normal and BDL rats. The resulting plasma concentration versus time profiles are shown in the Fig. 1a to 3a. In normal rats a linear correlation between the injected dose and the AUC were observed, which indicates first-order pharmacokinetic conditions. The pharmacokinetics of SOD and DIVEMA-SOD in BDL rats also showed a linear relation between the doses and the AUC.

TABLE 1
Pharmacokinetic parameters of native superoxide dismutase (SOD) and SOD conjugates after iv administration in normal and bile duct ligated (BDL) rats. Mean values±SE

<table>
<thead>
<tr>
<th>Protein</th>
<th>Rat type</th>
<th>Cₚ (ml·min⁻¹·kg⁻¹)</th>
<th>V (ml·kg⁻¹)</th>
<th>t₁/₂ (h)</th>
<th>kₑ (h⁻¹)</th>
<th>Φ</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>Normal</td>
<td>5.06±0.41</td>
<td>75.5±8.3</td>
<td>0.17</td>
<td>4.02±0.21</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>BDL</td>
<td>3.66±0.49</td>
<td>57.6±10.8</td>
<td>0.18</td>
<td>3.81±0.21</td>
<td>39</td>
</tr>
<tr>
<td>Man-SOD</td>
<td>Normal</td>
<td>3.14±0.16</td>
<td>108.0±6.0</td>
<td>0.44</td>
<td>1.58±0.13</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>BDL*</td>
<td>8.38±0.40</td>
<td>144.0±12.0</td>
<td>0.19</td>
<td>3.18±0.21</td>
<td>21</td>
</tr>
<tr>
<td>DIVEMA-SOD</td>
<td>Normal</td>
<td>1.13±0.07</td>
<td>60.9±5.3</td>
<td>0.63</td>
<td>1.11±0.09</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>BDL</td>
<td>0.94±0.04</td>
<td>69.5±3.5</td>
<td>0.86</td>
<td>0.81±0.05</td>
<td>51</td>
</tr>
</tbody>
</table>

Cₚ: plasma clearance; V: volume of distribution; t₁/₂: elimination half-life; kₑ: elimination constant; Φ: degrees of freedom; *kinetic parameters calculated on the lower two doses.
Pharmacokinetic analysis using the curve-fitting program MW/PHARM (29) provided the kinetic parameters as given in Table 1. Using single-compartment analysis, the volume of distribution, elimination half-life, clearance and the elimination rate constant were calculated. The plasma clearance and elimination half-life of SOD were in the same order in normal and BDL rats. In contrast, the kinetics of Man-SOD was significantly altered in BDL rats compared to control animals. At the lower doses of Man-SOD a more rapid elimination was observed. The plasma clearance increased about three times from 3.1 to 8.6.

Fig. 4. Immunohistochemical localization of SOD and SOD conjugates in the liver and kidneys of BDL rats. Panel A and B: SOD in the kidney and liver of BDL rats, respectively. Panel C and D: Man-SOD in the kidney and liver of a BDL rat respectively. Panel E and F: DIVEMA-SOD in the kidney and liver of a BDL rat, respectively. Original magnification 100x.

Fig. 5. Immunohistochemical staining for SOD in livers of DIVEMA-SOD-treated rats. Sections were double-stained for SOD (brown staining) and the Kupffer cell marker ED2 (Fig. A, blue staining) or sections were stained for SOD and the endothelial cell marker RECA-1 (Fig. B blue staining). Arrows indicate double positive cells. It is shown that DIVEMA-SOD accumulates in both endothelial and Kupffer cells. Original magnification 100x.
Oxide radical scavenging by SOD conjugates in vivo

Fig. 6. Demonstration of ROS production in livers of BDL rats 1 h after injection of SOD and DIVEMA-SOD. Panel A: Positive untreated control, 3 weeks BDL rat, Panel B: 3 weeks BDL rat treated with SOD, Panel C: 3 weeks BDL rat treated with DIVEMA-SOD. Original magnification 100×.

ml·min⁻¹·kg⁻¹. Saturation of plasma elimination was observed at the highest dose of 40 mg·kg⁻¹. The tissue distribution at 10 min after iv injection of tracer amounts of ¹²⁵I labeled SODs in normal and BDL rats are given in Fig. 1b, 2b and 3b, respectively.

SOD accumulated in the kidneys in both BDL and control animals. In the BDL rats, inflammatory responses were found in particular in the liver and to a minor extent in the spleen, the lungs and the kidneys. Man-SOD was mainly taken up by the liver. Pre-injection of SOD had no effect on the tissue distribution of this SOD derivative, whereas mannan, a known mannose receptor blocker (36) significantly reduced the hepatic binding and uptake of Man-SOD in normal rats. However, mannan did not have an effect on the liver binding and uptake of Man-SOD in BDL rats. At the same time a higher level of radioactivity was detected in the lungs of the BDL animals compared to the controls. The pulmonary disposition could be blocked by mannan.

Coupling of DIVEMA to SOD yielded a polyanionic conjugate with an average molecular weight of 300 kDa (22). As can be seen from Fig. 3a, coupling of DIVEMA to SOD resulted in a prolonged residence time in the bloodstream. The elimination half-life increased significantly (Student’s t, p<0.01) from 0.17 h for SOD to 0.86 h for DIVEMA-SOD and the clearance showed a 5-fold reduction from 5 ml·min⁻¹·kg⁻¹ to approximately 1 ml·min⁻¹·kg⁻¹. Tissue distribution studies of DIVEMA-SOD showed that this polyanionic compound was mainly taken up by the liver, and that pre-injections of Form-HSA and DIVEMA blocked the liver uptake (Fig. 3b). The liver
uptake of DIVEMA-SOD in BDL rats was about two times lower and could not be blocked by pre-administration of Form-HSA.

From the pharmacokinetic data obtained in BDL rats, we calculated the dose to be given to obtain a SOD activity of 100 units \( \cdot \text{ml}^{-1} \) at 60 min after dosing. The doses were 100, 50 and 50 mg \( \cdot \text{kg}^{-1} \) for SOD, Man-SOD and DIVEMA-SOD, respectively. At 60 min, the rats were sacrificed and the liver, spleen, lungs and kidneys were removed for histochemical evaluation.

Immunohistochemical staining for SOD using polyclonal antibodies revealed no endogenous staining of SOD in liver, kidney, spleen and lung sections of control rats. Rats treated with native SOD displayed strong staining for this protein in the kidney, where SOD was abundantly present in the proximal tubuli (Fig. 4a). No staining was found in the liver (Fig. 4b), lung and spleen. There was no difference between normal and fibrotic animals. Rats treated with Man-SOD showed only minor staining for this protein in the kidney; a faint staining was found within the proximal tubular cells (Fig. 4c). In contrast, Man-SOD was clearly detected in the liver, where staining occurred in a non-parenchymal cellular distribution pattern (Fig. 4d). In the spleen, a moderate staining for SOD was found, whereas the lungs were negative. Again, no differences in staining patterns were observed between normal and diseased animals. Animals that received DIVEMA-SOD showed abundant staining of SOD in the liver (Fig. 4f). Staining corresponded with a non-parenchymal localization, predominantly along the sinusoids. This was also confirmed in double-staining experiments. Double-staining of liver sections from DIVEMA-SOD-treated rats for SOD and the Kupffer cell marker ED2 revealed double-positive cells (Fig. 5a). Also after double-staining for SOD and an endothelial cell marker (RECA-1) double-positive cells were found (Fig. 5b), indicating uptake in both Kupffer cells and endothelial cells. Histochemical examination of liver sections for uptake of SOD derivatives in hepatocytes and bile duct epithelial cells, which are both readily identifiable in hematoxylin-eosin stained tissue, demonstrated no co-localization of SOD in these two cell types.

In kidneys (Fig. 4e), and lungs DIVEMA-SOD could not be detected and a weak staining was found within the extracellular matrix of the spleen. Distribution patterns for DIVEMA-SOD in BDL rats were similar to the distribution pattern in normal rats.

### Discussion

Superoxide anions play an important role in the pathogenesis of inflammatory diseases including liver fibrosis (1–3). During early fibrogenesis Kupffer-, endothelial- and hepatic stellate cells are activated, and mediators derived from these cells sustain autocrine loops which lead to manifest fibrosis. One of these factors is ROS produced by infiltrating neutrophils and Kupffer cells. Also, endothelial cells may contribute to the intrahepatic free oxygen radical production. Released superoxide anions can subsequently induce damage to...

...production was found in normal rats. SOD had no apparent effect in BDL-rats (Fig. 6b). However, a clear reduction in free oxygen radicals was noted in BDL-rats treated with Man-SOD or DIVEMA-SOD: the number of cells positive for ROS production as well as the intensity of the positive cells strongly decreased in the portal areas of the liver. This decline in staining as compared to untreated BDL-rats was most prominent in DIVEMA-SOD-treated BDL-rats (Fig. 6c). Immunohistochemical examination of these livers for influx of neutrophils (HIS 48 positive cells) and Kupffer cells (ED2 positive cells) did not reveal differences between untreated BDL rats and BDL-rats receiving DIVEMA-SOD or Man-SOD. No reduction in ROS production was found in lungs of BDL rats treated with either Man-SOD or DIVEMA-SOD as compared with untreated BDL-rats. In normal rats, administration of Man-SOD or DIVEMA-SOD resulted in a slight increase in the number of positive cells in all organs tested.

**Oxygen free radical scavenging effects of the conjugates in vivo**

Cryostat tissue sections of rats were analyzed for oxygen free radical production by staining for \( \text{O}_2^- \), the presence of \( \text{H}_2\text{O}_2 \) and peroxidase activity. Endogenous \( \text{O}_2^- \) production in normal rats could be found in the red pulpa of the spleen, while occasionally ROS-producing cells were also detectable in the lungs. Livers and kidneys were negative for ROS-producing activity. In contrast, livers and lungs of BDL rats were strongly positive for ROS-producing cells (Fig. 6a). \( \text{O}_2^- \) production activity was demonstrated in the portal areas of BDL rats. Staining for \( \text{H}_2\text{O}_2/\text{PO} \) activity showed a similar distribution pattern but more positive cells were found and staining per cell was more intense. Staining for ROS-production corresponded with the staining for granulocytes using an antibody against polymorphonuclear neutrophils (HIS48), whereas in BDL-rats also some double-staining with ED2, a marker for Kupffer cells, was found (results not shown). The number of ROS-producing cells in cryostat sections of kidneys and spleens was slightly enhanced in BDL-rats as compared to normal rats.

Control rats treated with SOD showed an increase in the number of ROS-producing cells in the liver, the kidney and in the lungs as compared to untreated normal rats. SOD had no apparent effect in BDL-rats (Fig. 6b). However, a clear reduction in free oxygen radicals was noted in BDL-rats treated with Man-SOD or DIVEMA-SOD: the number of cells positive for ROS production as well as the intensity of the positive cells strongly decreased in the portal areas of the liver. This decline in staining as compared to untreated BDL-rats was most prominent in DIVEMA-SOD-treated BDL-rats (Fig. 6c). Immunohistochemical examination of these livers for influx of neutrophils (HIS 48 positive cells) and Kupffer cells (ED2 positive cells) did not reveal differences between untreated BDL rats and BDL-rats receiving DIVEMA-SOD or Man-SOD. No reduction in ROS production was found in lungs of BDL rats treated with either Man-SOD or DIVEMA-SOD as compared with untreated BDL-rats. In normal rats, administration of Man-SOD or DIVEMA-SOD resulted in a slight increase in the number of positive cells in all organs tested.

Discussion

Superoxide anions play an important role in the pathogenesis of inflammatory diseases including liver fibrosis (1–3). During early fibrogenesis Kupffer-, endothelial- and hepatic stellate cells are activated, and mediators derived from these cells sustain autocrine loops which lead to manifest fibrosis. One of these factors is ROS produced by infiltrating neutrophils and Kupffer cells. Also, endothelial cells may contribute to the intrahepatic free oxygen radical production. Released superoxide anions can subsequently induce damage to...
hepatic cells, that in its turn enhances the inflammatory process among others via direct activation of NFκB. This leads to a stimulation of cytokine production by Kupffer cells (9) and collagen production by stellate cells (2).

To study the pathogenic role of superoxide anions during fibrogenesis and to look for therapeutic intervention strategies, we aimed to inhibit intrahepatic free oxygen radical production using targeted forms of SOD.

Despite the abundant presence of superoxide anions in inflammatory conditions, reports on therapeutic interventions with superoxide dismutase in liver diseases are scarce. Protective effects of SOD have been found in vitro and ex vivo during cardiac perfusion experiments (37). In vivo, effects of exogenous SOD only have been found in kidneys (34,38) and after local administration of this enzyme in lungs (39) and paws (40). In vivo, SOD accumulates in the kidneys (40,41) since the low molecular weight protein is glomerular filtrated and is reabsorbed by proximal tubular cells (40). Due to the rapid renal clearance the plasma half-life of SOD is approximately 6 min (Fig. 1a). Other organs accumulate this protein poorly (42) and consequently therapeutic levels are not maintained in plasma or non-renal tissues (Fig. 1b).

Cell-specific delivery of SOD may be an attractive way to achieve therapeutic concentrations in non-renal tissues. Coupling of SOD to polyethylene glycol prevents renal clearance and considerably prolongs the half-life of SOD conjugates in plasma (21). However, there is no specific delivery to other tissues than the kidneys. In contrast, mannosylated (43) and liposome-encapsulated (44) SOD conjugates are specifically delivered to macrophages, whereas negatively charged SOD conjugates have now been shown to be specifically taken up by endothelial cells (41). In view of the important role of these two cell types in the pathogenesis of liver fibrosis, we examined the tissue distribution and effect of SOD substituted with mannose groups and SOD coupled to the polyanion DIVEMA.

The pharmacokinetics and pharmacological effects of these conjugates during liver fibrosis have not been examined before. Previous studies have shown that these compounds retain their radical scavenging capacity in vitro during synthesis (45). The development of histochemical methods to detect in situ free oxygen radical levels (34), combined with the application of specific antibodies against SOD enabled us to relate tissue and cellular distribution of SOD with free oxygen radical scavenging directly in inflamed tissues in vivo.

Assessment of the pharmacokinetic profile of SOD, DIVEMA-SOD revealed a significant prolongation of the half-life. Yet, its plasma half-life was lower as compared with the reported half-life of PEG-SOD. The half-life of DIVEMA-SOD did not significantly change after induction of fibrosis, whereas the clearance of Man-SOD was strongly increased in BDL rats (Table 1). Man-SOD is likely to be predominantly taken up by Kupffer cells, whereas DIVEMA-SOD is a substrate for scavenger receptors and may preferentially accumulate in endothelial cells. Liver fibrosis is characterized by a strong increase in the number of Kupffer cells, whereas the number of endothelial cells is not affected by this disease.

Tissue distribution studies with radiolabelled SOD conjugates showed an accumulation of Man-SOD and DIVEMA-SOD in the liver (Fig. 2b & 3b), in contrast to native SOD that mainly accumulated in kidneys (Fig. 1b). These data confirm other reports on the disposition of native and modified forms of SOD (38,41).

Histochemical results obtained in the present study with polyclonal anti-SOD antibodies support these tissue distribution studies. After iv administration, native SOD was only detectable in renal tubular brushborders, reflecting its glomerular filtration and tubular re-uptake (Fig. 4a). The faint renal and hepatic staining for SOD in animals treated with Man-SOD may imply that either the conjugate is not sufficiently recognized by mannose receptors or after recognition it is rapidly endocytosed and degraded. In view of the high intrahepatic accumulation of radiolabeled Man-SOD in our tissue distribution studies, the latter explanation is more likely.

Microscopical analysis showed that DIVEMA-SOD was abundantly present along the sinusoids of the liver, consistent with binding to the endothelial lining (Fig. 4f). Apparently DIVEMA-SOD is less rapidly internalized than Man-SOD due to partial saturation of the scavenger receptor at the dose range used. From the tissue distribution studies it can be concluded that Man-SOD and DIVEMA-SOD bind to and enter relevant cell types of the liver, in contrast to native SOD. In particular, DIVEMA-SOD is readily detectable in fibrotic rat livers. Double-staining studies on livers of rats treated with DIVEMA-SOD demonstrated that SOD accumulated in both Kupffer and endothelial cells of the liver (Fig. 5). These cells are the relevant target cells for the cell-specific delivery of oxygen free radical scavengers. No uptake was found in hepatocytes and bile duct epithelial cells.

In a subsequent series of experiments we examined the radical scavenging effect of SOD conjugates in bile duct ligated rats. Common bile duct ligation leads to
an influx of inflammatory blood cells, severe intrahepatic inflammatory cell influx and fibrosis within 3 weeks (27). Three weeks after bile duct ligation, ROS production was most prominent, in particular in the portal areas. On the basis of the kinetic parameters determined, we aimed at an equimolar plasma concentration of all three conjugates, 60 min after injection. Consequently, the observed differences in oxygen radical scavenging activity cannot be attributed to differences in plasma levels of SOD.

In animals receiving native SOD, no effects on ROS levels in the fibrotic livers were observed. In contrast, intrahepatic ROS production was largely inhibited 1 h after administration of Man-SOD or DIVEMA-SOD (Fig. 6), while the numbers of infiltrating neutrophils and Kupffer cells were not changed. These results therefore clearly demonstrate the importance of a specific delivery of this enzyme. Studies examining the effects of chronic administration of SOD conjugates on hepatic inflammation and fibrosis are in progress. Taking into account the direct effect of oxygen radicals on hepatic stellate cell activation and collagen production, the application of SOD polymeric conjugates in treatment of liver fibrosis could be an attractive concept for new anti-fibrotic therapies.

Acknowledgements

Antibodies against SOD were kindly provided by Dr. Ir. H. W. Verspaget, University Hospital Leiden, The Netherlands. Part of this research was performed as a result of a fellowship of the first author to Japan, sponsored by the Canon Foundation, Leiden, The Netherlands.

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