Effects of Glutathione, as the Dextran Conjugate, on Acetaminophen-Induced Hepatotoxicity

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Glutathione was covalently attached to dextran (T-40) by the CNBr activation method. In mice given a lethal dose of acetaminophen, the 30-d survival rate increased progressively with coadministration of the conjugate, whereas little improvement was found when free glutathione was given. The dextran conjugate of glutathione maintained the serum transaminase activities at lower levels after acetaminophen administration, giving effective protection against acetaminophen hepatotoxicity.

Keywords glutathione; dextran; conjugate; cyanogen bromide; acetaminophen; hepatotoxicity; survival rate

Acetaminophen (APAP) is a widely used mild analgesic which causes hepatic necrosis and death in both humans and experimental animals when large doses are administered. APAP causes a dose-dependent depletion of hepatic glutathione (GSH).[1,2] Evidence has accumulated which suggests that an active intermediate metabolite of APAP, *N*-acetyl-*p*-benzoquinoneimine, is a hepatotoxic entity which is normally conjugated by GSH.[3-5] When GSH is depleted, covalent binding of the active intermediate to liver cell macromolecules initiates cell damage.

N-Acetylcysteine and other sulfhydryl donors such as cysteine, methionine and cysteamine have been used as effective antidotes protecting against the hepatotoxicity of APAP. It has been suggested that these antidotes except cysteamine share a common mechanism of action in protecting against the toxicity, namely facilitation of GSH synthesis.[6,7]

Since GSH is a naturally occurring sulfhydryl compound apparently involved in detoxication of the reactive intermediate metabolite, it would be an excellent antidote against APAP poisoning. However, extracellular GSH can not permeate into the liver and has a very short half-life due to its rapid renal degradation into the constituent amino acids.[8-10] Although an interorgan shift of the constituent amino acids from the kidney to the liver enables hepatic resynthesis of GSH, administration of free GSH does not lead to a stable increase in the liver GSH.

The circulatory half-lives of proteins have been increased by conjugation to soluble polymers.[11-14] Conjugated enzymes have been shown to exhibit reduced immunogenicity compared with the native enzymes and also enhanced resistance to proteolysis and heat denaturation. Melton *et al.*[15,16] have shown that the covalent attachment of the therapeutic enzyme carboxypeptidase G_2 to soluble dextrans resulted in a marked increase in plasma persistence in mice. They also reported that pronounced uptake of both CNBr-activated dextran and dextran–enzyme conjugate by the liver occurred at similar rates.

The aim of the present study was to establish an intrahepatic delivery system for GSH based on a dextran conjugate of GSH that is expected to be more stable in the systemic circulation and more effectively transported into hepatic cells than is GSH itself; thus, we also examined the influence of the conjugate on APAP-induced liver necrosis in mice.

Experimental

Materials Dextran (T-40, $M_w=43900$, $M_n=26200$) was purchased from Pharmacia Fine Chemicals Co., Sweden. All other chemicals and reagents were of the highest grades commercially available.

Conjugation Method GSH was covalently attached to dextran by the cyanogen bromide activation method.[17] To a stirred solution of dextran (0.2 g) in water (20 ml), cyanogen bromide was added in three portions (40, 40 and 30 mg). The pH was maintained at 11.0 during this process by addition of 4 M NaOH. At 6 min after the final addition of cyanogen bromide, the pH was adjusted to 6.5 by addition of 0.1 M HCl. Then GSH (0.4 g) was added while the pH was maintained at 6.5, and the coupling reaction was allowed to proceed for 24 h at 4°C. The reaction mixture was washed repeatedly with 0.1 M CH_3COOH and concentrated by filtration through an ultrafilter (UP-20 mounted in UHP-43, Toyo, Japan) under high nitrogen pressure, and then freeze-dried. Sulfhydryl groups of the product, a dextran conjugate of GSH (D-GSH), were determined by the method of Ellman.[18]

Animal Experiments The protective effect of D-GSH on APAP-induced hepatotoxicity was examined as follows. Male ddY mice (20—30 g) were treated intraperitoneally with a lethal dose of APAP (5 mmol/kg) dissolved in 0.2 ml of propylene glycol. Mice were treated intravenously with D-GSH (0.39 mmol/kg in GSH equivalent) or GSH (0.39 mmol/kg) dissolved in 0.2 ml of saline both 2 h before and at the same time as APAP injection. The animals were allowed food and water *ad libitum*, and were housed in standard cages and observed for 30 d. In this experiment, several mice were sacrificed for the determination of serum glutamate pyruvate aminotransferase (SGPT) activity by a kit method (Monotest, Boehringer Mannheim, W. Germany).

In the case of the determination of GSH in the liver, mice were given an intravenous injection of 0.4 ml of a saline solution of D-GSH (0.98 mmol/kg in GSH equivalent) or GSH (0.98 mmol/kg) in the same manner as described earlier. The concentration of GSH in the liver was measured by the method of Hissin and Hilf with use of *o*-phthalaldehyde as a fluorescent reagent.[19] It was confirmed that the determination of GSH is not affected by the presence of D-GSH and no GSH is liberated from the conjugate during the determination process.

Results and Discussion

D-GSH obtained synthetically was a water-soluble white powder containing $10.3 \pm 1.5\%$ w/w of GSH (average of 77 batches) based on the determination of sulfhydryl groups by a slight modification of the method of Ellman[18] with Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)).

The effect of administration of D-GSH on APAP toxicity was examined in mice. Table I shows the percentage of surviving mice in each treatment group. Mice treated with a lethal dose of APAP (5 mmol/kg) exhibited a survival rate of 30% 30 d after the insult. Treatment with intravenous injection of D-GSH markedly increased the 30-d survival rate to 85%. However, no improvement was observed in the mice treated with dextran or GSH.

TABLE I. Survival Rate of Mice Given a Lethal Dose of APAP, Effect of D-GSH

Treatment	Survival after 30 d (%)	$n^{a)}$
APAP	30	27
APAP+D-GSH	85	30
APAP+GSH	45	30
APAP+dextran	35	15

a) Number of mice used in the experiment. Mice were injected intraperitoneally with 0.2 ml of a propylene glycol solution of APAP (5 mmol/kg). Mice were treated by intravenous injection of 0.2 ml of a saline solution of D-GSH (0.39 mmol/kg in GSH equivalent), GSH (0.39 mmol/kg) or dextran (3.4×10^{-5} mol/kg) both 2 h before and at the same time as APAP administration.

Blood samples were collected 6, 24 and 48 h after administration of APAP (5 mmol/kg) for the determination of SGPT activity. Figure 1 shows that SGPT activity was increased after the APAP administration. However, treatment with D-GSH maintained the SGPT activity at significantly lower levels.

Figure 2 shows that the hepatic GSH level was reduced to 15% of the control level 2 h after the administration of APAP. Intravenous administration of D-GSH led to a significant increase in the level of GSH. Although injection of free GSH also had a significant effect on the recovery of the hapatic GSH level at 2 and 4 h, it was relatively small compared to that of D-GSH and was no longer appreciable at 6 and 8 h after the APAP administration.

It was found that D-GSH effectively protected mice from APAP acute poisoning due to its hepatotoxicity, whereas free GSH had no effect (Table I, Fig. 1). Buthionine sulfoximine (BSO), a potent inhibitor of γ-glutamylcysteine synthetase, prevents the intracellular synthesis of GSH from its constituent amino acids.[20] When hepatic GSH in mice was exhausted by the injection of BSO, intravenous administration of D-GSH led to marked increase in the level of GSH, whereas free GSH had no significant effect on it.[21] This suggests that D-GSH is taken into the hepatic cells and liberates GSH by hydrolysis.

Clinical dextran is mainly eliminated by the kidney. Molecules with molecular weights lower than about 15×10^3 pass freely through the glomerular filter. Larger molecules are more restricted in their passage, and dextran with a molecular weight above 50×10^3 is practically not excreted.[22] However, such a dextran is completely eliminated from the systemic circulation by biodegradation. In this case, dextran uptake by the liver is seen, and a continuous, rapid and complete elimination from the parenchymal cells takes place.[23] In experiments where the distribution of dextran in mice was studied by histological techniques, both parenchymal and Kupffer cells were shown to participate in the removal of dextran.[24] Dextran was demonstrated in the cytoplasm of parenchymal cells as soon as 2 h after injection and appeared to be maximal at 12 to 24 h. In contrast, Kupffer cells accumulated dextran from 4 h after injection with the amount reaching a maximum at 24 h. Dextran was found to persist much longer in Kupffer cells than in parenchymal cells.[24]

The mechanism of the enhanced anti-hepatotoxicity of GSH afforded by the dextran carrier is not known so far. It is postulated that the effect may be due to protection against rapid renal degradation and hepatic uptake of the conjugate by endocytosis, followed by liberation of GSH (Fig. 2).

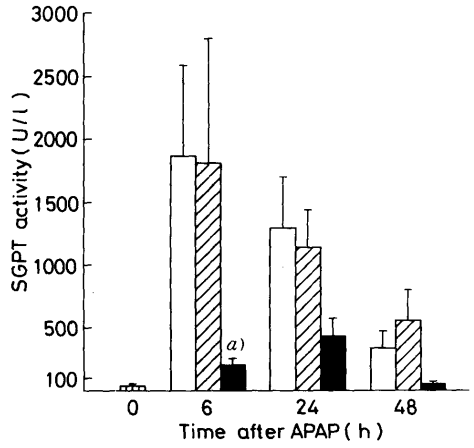

Fig. 1. Time Course of SGPT Activity Following Administration of APAP; Effect of D-GSH

Mice were treated by intravenous injection of 0.2 ml of a saline solution of GSH (0.39 mmol/kg) or D-GSH (0.39 mmol/kg in GSH equivalent) both 2 h before and at the same time as APAP administration (5 mmol/kg, i.p.). Each value represents the mean ± S.E. of 7—14 mice. *a*) $p < 0.05$ when compared with the value of APAP. The Cochran-Cox test was used. ▦, control; □, APAP; ▨, +GSH; ■, +D-GSH.

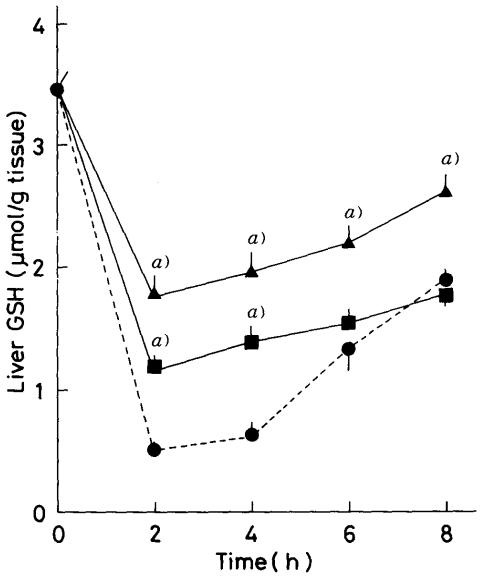

Fig. 2. Effect of D-GSH and GSH on the Hepatic GSH Contents in Mice Treated with APAP

Mice were injected intraperitoneally with 0.2 ml of a propylene glycol solution of APAP (5 mmol/kg). Mice were then treated by intravenous injection of 0.4 ml of a saline solution of D-GSH (▲, 0.98 mmol/kg in GSH equivalent), GSH (■, 0.98 mmol/kg) or saline (●, control) both 2 h before and at the same time as APAP administration. Each value represents the mean ± S.E. of 10 mice. *a*) $p < 0.05$ when compared with the control. The Cochran-Cox test was used.

Although a number of other carriers are currently undergoing experimental trials, application of dextran as a plasma expander makes it clinically acceptable and its high coupling capacity is favorable. The present findings may provide a clinically effective means of augmenting hepatic free thiol content in the event of GSH depletion due to toxic insult.

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