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Targeted protection of hepatocytes from galactosamine toxicity in vivo

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Summary. We present an in vivo model for specific protection of normal hepatocytes from damage by the highly specific hepatotoxin galactosamine. The idea is based on the fact that normal, unlike malignant, hepatocytes possess unique cell-surface receptors that can bind and internalize galactose terminal (asialo)glycoproteins by receptor-mediated endocytosis. A targetable carrier-antagonist conjugate was formed by coupling asialofetuin to the galactosamine antagonist uridine monophosphate. Intravenous injection of the antagonist conjugate resulted in specific uptake by the liver. Rats treated with carrier-antagonist conjugate together with a toxic dose of galactosamine developed significantly less hepatotoxicity than did controls. We conclude that a galactosamine antagonist can be targeted to liver, resulting in specific protection of hepatocytes from galactosamine toxicity in vivo. Because hepatoma cells lack asialoglycoprotein receptor activity, this "targeted rescue" may be of value in the differential protection of normal cells in the treatment of hepatocellular carcinoma.

Introduction

Chemotherapeutic agents currently used to treat hepatocellular carcinoma are typically unsuccessful, largely because they lack organ specificity. The use of galactosamine has been explored because it is a highly selective liver toxin in vitro and in vivo. The selectivity is due to elevated intrahepatic levels of two enzymes of the galactose metabolic pathway, galactokinase and UDP-glucose: galactose-1-*P*-uridyltransferase [2, 4], that allow galactosamine to be metabolized as a galactose analog [13]. This eventually leads to trapping and depletion of intracellular uridine intermediates in hepatocytes and hepatocyte-derived cells [16]. However, use of galactosamine as an antitumor agent is problematic: the high doses of galactosamine required to destroy hepatoma cells are also toxic to normal hepatocytes.

We have previously shown that a galactosamine antagonist can be targeted to hepatocytes, specifically protecting them from galactosamine toxicity in vitro [30]. In the work described below, we present evidence that targeted protection of normal hepatocytes from galactosamine toxicity is also possible in vivo.

Our experiments were based on three facts: (1) galactosamine is a highly specific hepatotoxin that causes a dose-dependent depletion of essential uridine intermediates [16]; (2) galactosamine toxicity can be antagonized by supplemental administration of uridine [5]; and (3) normal hepatocytes possess *unique* receptors that bind and internalize asialoglycoproteins (glycoproteins bearing terminal galactose residues) [1]. The internalized asialoglycoproteins are delivered to lysosomes and degraded [6]. We hypothesized that if a uridine intermediate were coupled to an asialoglycoprotein, the antagonist could be specifically delivered to normal hepatocytes via asialoglycoprotein receptors.

Materials and methods

Female Sprague-Dawley rats (220–270 g) were obtained from Zivic-Miller Laboratories (Allison Park, Pa). D(+)-galactosamine, uridine 5'-monophosphate, insolubilized neuraminidase, and poly L-lysine (mol. wt., 3,600 daltons) were purchased from Sigma Chemical Co. (St. Louis, Mo), bovine fetuin was obtained from Gibco (Grand Island, NY), dextran sulfate was purchased from Pharmacia (Uppsala, Sweden), sodium iodide I 125 was obtained from Amersham Corporation (Chicago, Ill), and 1-ethyl-3-(3-dimethylamino)propyl carbodiimide was purchased from Pierce Chemical Co. (Rockville, Ill).

Preparation of a targetable galactosamine antagonist. The asialoglycoprotein asialofetuin (AsF) was prepared by desialylation of bovine fetuin.

Abbreviations: AsF, asialofetuin; PL, poly L-lysine; UMP, 5'-uridine monophosphate; ALT, alanine aminotransferase; AsOR, asialoorosomucoid

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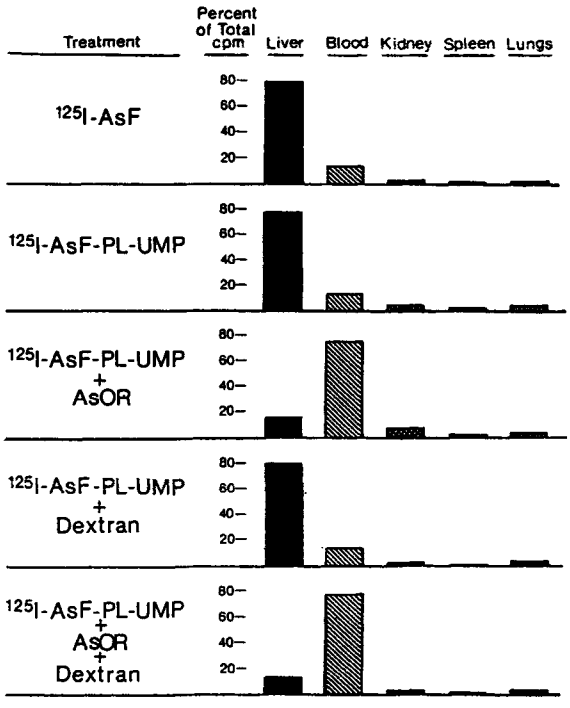

Fig. 1. Organ distribution of injected conjugate. Rats were injected i.v. with radiolabeled AsF or AsF-PL-UMP conjugate; controls received conjugate plus excess AsOR or plus dextran sulphate (for details see Materials and methods). Animals were sacrificed 10 min later and the distribution of radioactivity among organs was determined

using neuraminidase to expose terminal galactose residues by a modification of the method of Oka and Weigel [21]. Analysis of residual protein-bound sialic acid by the method of Warren [27] determined that the fetuin was 94% desialylated. To create a targetable carrier protein with a large capacity to bind antagonist, 55 μM AsF was coupled to 470 μM poly L-lysine (PL) (mol. wt., 3,600 daltons) using 1-ethyl-3-(3-dimethylamino)propyl carbodiimide as previously described [30]. 5'-Uridine monophosphate (UMP) was then coupled to the carrier protein according to the method of Halloran and Parker [9] and purified by column chromatography [30]. The conjugate was stable at 4° C for at least 2 weeks.

Organ distribution of injected conjugate. To determine whether the conjugate retained its ability to be recognized by asialoglycoprotein receptors in vivo, both AsF and the AsF-PL-UMP conjugate were radiolabeled with sodium iodide I 125 using a solid-phase lactoperoxidase method as described by the manufacturer (BioRad). Rats were injected i.v. with sterile saline containing 1 μg sodium iodide I-125-labelled AsF (^{125}I-AsF) or 1 μg labelled conjugate (^{125}I-AsF-PL-UMP) (based on AsF content). To determine whether liver uptake of the conjugate occurred via asialoglycoprotein receptors, a control rat was given 1 μg ^{125}I-AsF-PL-UMP plus an excess (10 mg) of unlabeled asialoorosomucoid (AsOR) to compete for hepatic asialoglycoprotein receptors. To evaluate the extent of nonspecific hepatic uptake of conjugate, 15 mg dextran sulfate, an inhibitor of nonparenchymal "scavenger" receptor activity, was injected i.v. 15 min prior to the conjugate injection according to the method of Van der Sluijs et al. [26]. Another control received both dextran sulfate and excess AsOR. At 10 min after injection of labeled protein, blood was drawn from the retro-orbital plexus and the animals were sacrificed. The distribution of radioactivity among organs was determined by gamma counting and expressed as a percentage of total counts.

To determine the effect of the targeted antagonist on galactosamine toxicity to hepatocytes in vivo, female rats were injected i.p. with 800 mg/kg galactosamine in 2.5 ml sterile saline (pH 7.4). To allow sufficient time for internalization and degradation of the conjugate and release of the antagonist, the targetable antagonist conjugate was injected i.v. (in 5 ml sterile saline) as a pretreatment 2 h prior to the galactosamine injection. The minimal amount of conjugate required to protect hepatocytes was determined by i.v. injection of varying doses. Using the conjugate dose thus determined to be optimal (34 mg/kg), the ability of this antagonist conjugate to prevent galactosamine toxicity was evaluated relative to controls: i.v. injected pretreatments of equal volumes of either sterile saline or saline containing AsF or UMP in molar amounts equivalent to that provided by the conjugate. Blood was withdrawn from the retro-orbital plexus at 24, 42, 48, and 72 h after galactosamine injection. Hepatotoxicity was evaluated by measurement of serum alanine aminotransferase (ALT) levels (Sigma assay kit) according to the manufacturer; all assays were carried out in duplicate and results were expressed as international units per liter (IU/l). Addition of conjugate to ALT standards as well as serum samples demonstrated that the conjugate had no effect on the ALT assays.

A Kruskal-Wallis test was used to evaluate differences among the four groups (6–7 rats per treatment). Once a significant difference among treatments was determined, pair-wise comparisons were evaluated with Wilcoxon-Mann-Whitney tests [31].

Potential toxicity of the conjugate itself was evaluated by injecting conjugate alone at the dose and volume used in the previous experiment. The animal was observed at various intervals, then sacrificed at 42 h (the time of peak galactosamine toxicity and, therefore, of maximal conjugate protection in the previous experiment). Various organs and tissues were removed and prepared for histological examination.

Results

Figure 1 shows the organ distribution of radiolabeled proteins 10 min after i.v. injection. For rats receiving either conjugate or AsF, approximately 80% of the counts were taken up by the liver. The addition of an excess of AsOR led to successful competition with the labeled conjugate for hepatic asialoglycoprotein receptors, resulting in removal by the liver of only 16% of the injected counts. Injection of dextran sulfate, which inhibits nonspecific uptake via nonparenchymal "scavenger" receptors, had no effect on liver uptake of the conjugate, which still accounted for 80% of the injected radioactivity. Administration of both dextran sulfate and excess AsOR had no further effect on liver uptake of the conjugate beyond that of the excess of AsOR alone. These data indicate that neither the PL, the UMP, nor the process of conjugation had altered the recognition of AsF by hepatic asialoglycoprotein receptors in the intact rat.

The behavior of a rat receiving conjugate (34 mg/kg) showed no obvious indications of cardiopulmonary distress or neurological deficits. No abnormalities were revealed by histological examination of the liver, kidney, spleen, heart and surrounding large vessels, lungs and trachea, brain, peripheral nerves and ganglia, adrenal gland, lymph nodes, skeletal muscle, and adipose tissue (data not shown).

Figure 2 compares the effect of the AsF-PL-UMP conjugate (34 mg/kg) to that of saline, AsF, or UMP controls in antagonizing galactosamine hepatotoxicity. Selective uptake by the liver of conjugate in trace amounts, demonstrated above, was also found at this higher dose of conjugate (data not shown). Because ALT values reached their highest levels at 42 h (always decreasing by 72 h), serum ALT values were compared at this peak 42-h time point. A Kruskal-Wallis test determined that there were significant differences among the four groups, with an alpha level of

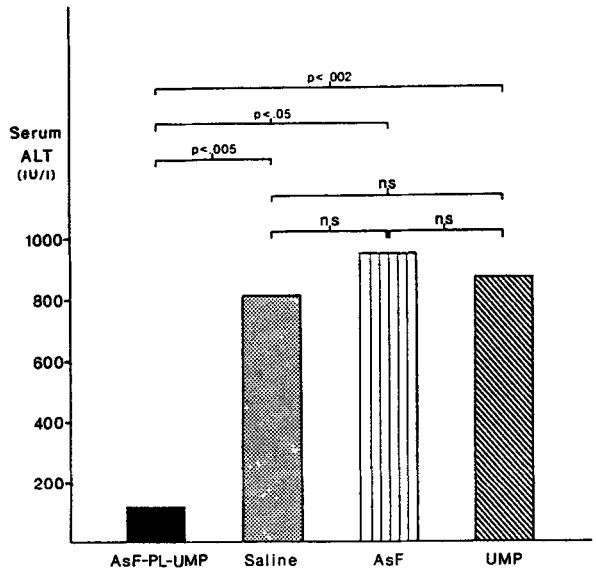

Fig. 2. Effect of antagonist-conjugate pretreatment on galactosamine toxicity: median serum ALT levels at 42 h. Rats were injected i.v. with 5 ml of either saline or saline containing AsF-PL-UMP conjugate, AsF, or UMP in molar amounts equivalent to that provided by the conjugate. After 2 h, each rat was injected i.p. with 800 mg/kg galactosamine (for details see Materials and methods). Significance was tested by Kruskal-Wallis and Wilcoxon-Mann-Whitney tests. (Pretreatment ALT values averaged 38 ± 9 IU/l.

0.01. Pair-wise comparisons (Wilcoxon-Mann-Whitney tests) determined that animals pretreated with AsF-PL-UMP conjugate experienced significantly less hepatotoxicity than did saline controls, as measured by serum ALT levels ($P<0.005$). Animals that received conjugate likewise had significantly lower ALT values than those receiving either AsF alone or UMP alone ($P<0.05$ and $P<0.002$, respectively). There were no significant differences among the three controls.

Discussion

Our data indicate that an AsF-PL-UMP conjugate can be targeted to hepatocytes, resulting in protection of these cells from galactosamine toxicity in vivo. The inhibition of uptake of ^{125}I-AsF-PL-UMP by competition with an excess of AsOR indicates that the targeting of the antagonist was directed by the asialoglycoprotein component of the conjugate. The lack of effect of UMP injected alone in amounts and under conditions identical to these used for the conjugate argues against of intravascular cleavage of UMP from the conjugate as a mechanism of the observed protection by the conjugate. It has previously been shown that uridine intermediates given in sufficient quantities can antagonize galactosamine toxicity [5]. The lack of effect of administration of UMP alone can be explained by the fact that uridine in the form of the conjugate was targeted only to hepatocytes, whereas free UMP could be dispersed by the circulation for uptake throughout the body. Unlike UMP in the form of a conjugate, free UMP provided to the *liver* from the circulation was evidently inadequate to prevent galactosamine toxicity.

Another possible mechanism that could account for the observed targeted antagonism is intracellular release of galactose from the large quantity of asialoglycoproteins present in the conjugate. This could result in competition with galactosamine for the intracellular metabolic enzymes involved in the development of galactosamine toxicity. Our finding that AsF given alone in the same amount as provided by the conjugate failed to prevent galactosamine hepatotoxicity, argues against any significant contribution by this route.

Although the AsF-PL-UMP conjugate was taken up selectively by the liver, the possibility remains that the procedure of conjugation might have imparted the AsF-PL-UMP molecule with an ability to antagonize galactosamine toxicity by some nonspecific mechanism. However, a fetuin-PL-UMP conjugate failed to rescue liver from galactosamine toxicity (data not shown). The glycoprotein fetuin is identical to AsF except that it does not contain terminal galactose residues and is not expected to be recognized by hepatic asialoglycoprotein receptors.

A large number of agents have been shown to ameliorate galactosamine hepatotoxicity in vitro and in vivo, but some exert their initial effect through extrahepatic pathways. Two synergistic mechanisms for galactosamine toxicity have been proposed, both of which are necessary for liver cell death [18]. The first, initiated within the hepatocyte, involves uridylate trapping that eventually leads to a membrane defect, with subsequent influx of Ca^{2+} ions [7, 19, 22]. The second involves a series of extrahepatic events initiated by galactosamine and leading to histaminemia, endotoxemia, leukotriene production, and depression of the reticuloendothelial system [3, 8, 18, 29]. Agents that interfere with either pathway have been found to reduce galactosamine toxicity. A variety of agents that have been shown to interfere with the intrahepatic pathway might serve as effective targeted antagonists directed specifically to hepatocytes. Therefore, although we have chosen uridine as an antagonist, other pyrimidine nucleotide precursors that antagonize galactosamine toxicity [17] may be targeted in a similar fashion. Furthermore, agents that interfere with the effects of intracellular Ca^{2+} accumulation, e.g., calcium chelators, enhancers of subcellular calcium sequestration [19, 20] and inhibitors of Ca^{2+} ion flux across membranes [22] may provide alternative agents for use in a targeted antagonist system.

Although some hepatomas are clearly sensitive to galactosamine toxicity [30], others are more resistant [12, 14], largely due to increased rates of de novo synthesis of uridylates [11, 25, 28]. In such cases, toxicity to hepatomas can be increased by the addition of an agent to block de novo pyrimidine synthesis [14]. Until now, use of galactosamine as a chemotherapeutic agent for hepatomas has been hindered by the fact that normal hepatocytes are at least as sensitive to its toxicity as are hepatoma cells [10, 12, 14, 15]. However, with the exception of only one human hepatoma cell line, Hep G2 [23], malignant hepatocytes display virtually no asialoglycoprotein receptor activity [1, 24]. Therefore, they should be unable to take up the conjugate antagonist. Thus, in the presence of the toxin, malignant cells would receive the full hepatotoxic effect of galactosamine and normal hepatocytes could be specifi-

cally protected. Our targeted antagonist system may therefore enable the use of tumoricidal doses of toxin, which would otherwise be precluded by toxicity to normal cells.

In the future, we plan to test the protective effect of our conjugate on normal hepatocytes in galactosamine-treated rats bearing transplantable Morris 7777 hepatomas, which have been demonstrated to be sensitive to galactosamine toxicity [30]. For other hepatomas more resistant to galactosamine alone, an agent that blocks de novo uridine synthesis may be combined with galactosamine. Because targeted delivery of antagonists would still be possible in these cases, specific protection of normal hepatocytes by our conjugate may also be of value with combinations of antipyrimidines for the treatment of hepatomas.

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References

1. Ashwell G, Morell AG (1974) The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. Adv Enzymol 41: 99
2. Bertoli D, Segal S (1966) Developmental aspects and some characteristics of mammalian galactose 1-phosphate uridyltransferase. J Biol Chem 241: 4023
3. Chojkier M, Fierer J (1985) D-Galactosamine hepatotoxicity is associated with endotoxin sensitivity and mediated by lymphoreticular cells in mice. Gastroenterology 88: 115
4. Cuatrecasas P, Segal S (1965) Mammalian galactokinase: developmental and adaptive characteristics in the rat liver. J Biol Chem 240: 2382
5. Decker K, Keppler D (1974) Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. Rev Physiol Biochem Pharmacol 71: 77
6. Dunn WA, Hubbard AL, Aronson NN Jr (1980) Low temperature selectively inhibits fusion between pinocytic vesicles and lysosomes during heterophagy of ^{125}I-asialofetuin by the perfused rat liver. J Biol Chem 255: 5971
7. Farber JL (1982) Biology of disease: membrane injury and calcium homeostasis in the pathogenesis of coagulative necrosis. Lab Invest 47: 114
8. Grun M, Liehr H, Grun W, Rasenack U, Brunswig D (1974) Influence of liver-RES on toxic liver damage due to galactosamine. Acta Hepato-Gastroenterol 21: 5
9. Halloran MJ, Parker CW (1966) The preparation of nucleotide-protein conjugates: carbodiimides as coupling agents. J Immunol 96: 373
10. Jackson RC, Williams JC, Weber G (1976) Enzyme pattern-directed chemotherapy: synergistic interaction of 3-deazauridine with D-galactosamine. Cancer Treat Rep 60: 835
11. Keppler D (1975) Consequences of uridine triphosphate deficiency in liver and hepatoma cells. *In:* Pathogenesis and mechanisms of liver cell necrosis. University Park, Baltimore, p 87
12. Keppler D (1978) Approaches to the chemotherapy of hepatomas. *In:* Primary liver tumors. MTP, Lancaster, p 485
13. Keppler D, Decker K (1969) Studies on the mechanism of galactosamine hepatitis: accumulation of galactosamine-1-phosphate and its inhibition of UDP-glucose pyrophosphorylase. Eur J Biochem 10: 219
14. Keppler DOR (1977) Uridine triphosphate deficiency, growth inhibition, and death in ascites hepatoma cells induced by a combination of pyrimidine biosynthesis inhibition with uridylate trapping. Cancer Res 37: 911
15. Keppler DOR, Smith DF (1974) Nucleotide contents of ascites hepatoma cells and their changes induced by D-galactosamine. Cancer Res 34: 705
16. Keppler DOR, Rudigier JFM, Bischoff E, Decker KFA (1970) The trapping of uridine phosphates by D-galactosamine, D-glucosamine, and 2-deoxy-D-galactose: a study on the mechanism of galactosamine hepatitis. Eur J Biochem 17: 246
17. Keppler DOR, Pausch J, Decker K (1974) Selective uridine triphosphate deficiency induced by D-galactosamine in liver and reversed by pyrimidine nucleotide precursors. J Biol Chem 249: 211
18. Liehr H, Grun M, Seelig H-P, Seelig R, Reutter W, Heine W-D (1978) On the pathogenesis of galactosamine hepatitis: indications of extrahepatocellular mechanisms responsible for liver cell death. Virchows Arch [Cell Pathol] 26: 331
19. MacDonald JR, Gandolfi AJ, Sipes IG (1985) Structural requirements for cytoprotective agents in galactosamine-induced hepatic necrosis. Toxicol Appl Pharmacol 81: 17
20. MacDonald JR, Thayer KJ, White C (1987) Inhibition of galactosamine cytotoxicity in an in vivo/in vitro hepatocellular toxicity model. Toxicol Appl Pharmacol 89: 269
21. Oka JA, Weigel PH (1983) Recycling of the asialoglycoprotein receptor in isolated rat hepatocytes: dissociation of internalized ligand from receptor occurs in two kinetically and thermally distinguishable compartments. J Biol Chem 258: 10253
22. Schanne FAX, Pfau RG, Farber JL (1980) Galactosamine-induced cell death in primary cultures of rat hepatocytes. Am J Pathol 100: 25
23. Schwartz AL, Fridovich SE, Knowles BB, Lodish HF (1981) Characterization of the asialoglycoprotein receptor in a continuous hepatoma line. J Biol Chem 256: 8878
24. Stockert RJ, Becker FF (1980) Diminished hepatic binding protein for desialylated glycoproteins during chemical hepatocarcinogenesis. Cancer Res 40: 3632
25. Sweeney MJ, Parton JW, Hoffman DH (1974) Biosynthesis of uridine-5'-monophosphate in rat liver and Morris hepatomas. Adv Enzyme Regul 12: 385
26. Van der Sluijs P, Bootsma HP, Postema B, Moolenaar F, Meijer DKF (1986) Drug targeting to the liver with lactosylated albumins: does the glycoprotein target the drug or is the drug targeting the glycoprotein? Hepatology 6: 723
27. Warren L (1959) The thiobarbituric acid assay of sialic acids. J Biol Chem 234: 1971
28. Weber G, Shiotani T, Kizaki H, Tzeng D, Williams JC, Gladstone N (1978) Biochemical strategy of the genome as expressed in regulation of pyrimidine metabolism. Adv Enzyme Regul 16: 3
29. Wendel A, Tiegs G (1987) Leukotriene D4 mediates galactosamine/endotoxin-induced hepatitis in mice. Biochem Pharmacol 36: 1867
30. Wu GY, Keegan-Rogers V, Franklin S, Midford S, Wu CH (1988) Targeted antagonism of galactosamine toxicity in normal rat hepatocytes in vitro. J Biol Chem 263: 4719
31. Zar JH (1984) Biostatistical analysis. Prentice-Hall, Englewood Cliffs, New Jersey