Original

The Effects of D-galactosamine- or Carbon Tetrachloride-Induced Regeneration on Induction of Rat Liver Cell Foci in a Model for Detection of Initiation Activities of Chemicals

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Abstract: The effects of D-galactosamine (D-gal) and carbon tetrachloride (CCl₄) administration on induction of liver cell proliferation in a medium term liver bioassay for detection of initiation activity of 1,2-dimethylhydrazine (DMH) were investigated with special emphasis on kinetics of cell proliferation and cytochrome P450 (CYP) expression. In experiment I, fluctuation of cell proliferation and CYP 2E1, which is one of the enzymes involved in bioactivation of DMH to ultimate form, levels of liver cells after D-gal administration (4000 mg/kg, i.g.) or CCl₄ administration (1 ml/kg, i.g.) was analyzed by bromodeoxyuridine labeling and western blotting, respectively. In experiment II, induction of glutathione S-transferase placental form (GST-P)-positive foci by DMH in a model for detection of initiation activities using D-gal or CCl₄ administration as a toxicity-regeneration dependent proliferative stimulus was evaluated. Although high BrdU labeling indices continued from 36 h to 72 h after D-gal and CCl₄ treatment, cell proliferation after intragastric D-gal administration was very low compared with the CCl₄ case. Decrease of CYP 2E1 apoprotein content in the microsomes was slight after D-gal administration, whereas decrease up to about 40% of the control level was evident from 12 h until 60 h after CCl₄. When the carcinogen was injected 60 h after D-gal administration, there was appreciable increase in the area and numbers of GST-P positive foci, similar to the case with CCl₄ administration. Sensitivities for detection of initiation activity with intragastric D-gal or CCl₄ administration were equal, despite the kinetics of cell proliferation and CYP 2E1 being very different. With CCl₄ administration, initiation depended on fluctuation of CYP 2E1, reversibly in intragastric D-gal treatment, initiation depended on cell kinetics. (*J Toxicol Pathol* 2002; **15**: 13–18)

Key words: D-galactosamine, carbon tetrachloride, initiation, CYP 2E1, rats, liver

Introduction

For detection of initiation activity in the relatively short term, evaluation of induction of glutathione-S transferase placental form (GST-P) positive foci in rat liver has been widely used^{1–3}. Cell proliferation and bioactivation of procarcinogens are important parameters for the initiation process⁴ and induction of liver regeneration is the first step in

induction of GST-P positive foci for detection of initiation activity³. Two-thirds partial hepatectomy has been utilized for induction of liver cell proliferation⁵, but this requires surgical expertise and presents problems, especially when with large numbers of rats. In contrast, chemical hepatectomy achieved with hepatotoxicant administration is very simple. Carbon tetrachloride (CCl₄) has been employed for this purpose^{1,2}, but causes hepatic necrosis in centrilobular areas where expression of cytochrome P450 (CYP) is strongest⁶, so that bioactivation of carcinogens may be effected⁷. D-galactosamine (D-gal) is another hepatotoxicant with a different mechanism of liver injury from CCl₄. A toxic dose induces cell proliferation^{8,9}, therefore, D-gal administration will be utilized as cell

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proliferative stimulus in a model for detection of initiation activities of chemicals. However, the effect of liver regeneration induced by D-gal administration on initiation of carcinogenesis has been unclear.

In the present study, we therefore compared the effects of intragastric D-gal- and CCl₄-induced liver cell proliferation in a medium term liver bioassay for detection of initiation activity of 1,2-dimethylhydrazine (DMH), concentrating attention on cell kinetics and expression of CYP 2E1 which is one of the enzymes involved in bioactivation DMH to ultimate carcinogens¹⁰.

Materials and Methods

Animals

The animals, male F344 rats (Charles River Japan Inc., Atsugi), were housed five per plastic cage on wood chips for bedding under constant conditions (12 h light/dark cycle,

60% humidity at $22 \pm 2^\circ\text{C}$) on Oriental NMF diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*. They were acclimatized for one week before the start of experiments, at which time they were 7 weeks old and weighed 150–170 g.

Chemicals

DMH was purchased from Tokyo Kasei Co. (Tokyo), CCl₄ and D-galactosamine hydrochloride from Wako Pure Chemical Industries Ltd. (Osaka), and bromodeoxyuridine (BrdU) from Sigma Chemical Co. (St. Louis, MO, USA). The diet containing 0.015% of 2-acetylaminofluorene (2-AAF) was purchased from Japan Clea Co., Ltd. (Osaka, Japan).

Experimental protocols (Fig. 1)

In experiment I (Fig. 1A), for the analysis of cell proliferation kinetics of liver cells after D-gal administration (4000 mg/kg, i.g.) or CCl₄ administration (1 ml/kg, i.g.),

Fig. 1. Schematic representation of the protocols for (A) experiment I and (B) experiment II. See Materials and Methods for further details.

groups of rats were given an i.p. injection of BrdU (100 mg/kg) 1 h before sacrifice, 12, 24, 36, 48, 60, 72 h, and 7 days after D-gal administration as in Fig. 1A. Liver slices were fixed in 10% neutral buffered formalin for BrdU. For microsome analysis, liver was frozen by liquid nitrogen and stored at -80°C until prepared for microsome.

In experiment II (Fig. 1B), in order to determine correlates among cell proliferation, kinetics of CYP 2E1 and induction of GST-P-positive foci, rats were divided into six groups as shown in Fig. 1B, given doses of DMH (10 mg/kg wt. i.g.) administered at 12, 24, 36, 48, 60, and 72 h after D-gal administration, respectively. The control group received no DMH administration after D-gal i.g. The CCl_4 -administration group was administered CCl_4 instead of D-gal, and received DMH i.g. 60 h after administration at the high point of susceptibility to induction of GST-P-positive foci reported previously². Subsequently, diet containing 0.015% of 2-AAF was given for the following 2 weeks. Three weeks after DMH administration, all animals received a dose of CCl_4 (0.8 ml/kg body wt. i.g.)⁵. At the end of week 5 the survivors were killed and liver slices were fixed in 10% neutral buffered formalin for immunohistochemical examination of GST-P-positive foci. All animals were handled in accordance with the guidelines for animal experimentation of the Faculty of Agriculture, Gifu University.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analysis for CYP 2E1 protein

Liver microsomes after D-gal or CCl_4 administration were prepared essentially as described by Burke *et al.*¹¹. Briefly, livers obtained in experiment I were transferred into 0.25 M glycerol/50 mM Tris pH 7.4 and homogenized with a glass Potter-Elvehjem type homogenizer with a Teflon pestle. Each liver homogenate was centrifuged at 13,500 g for 20 minutes and the supernatant was re-centrifuged at 105,000 g for 60 minutes¹². The resulting pellet of the microsomal fraction, was washed once in 0.15 M KCl/10 mM Tris pH 7.6, centrifuged and processed for the BioRad DC protein assay (BioRad, Hercules, USA) based on Lowry's method¹³. Microsomal preparations were snap frozen in liquid nitrogen and stored at -80°C until use.

Liver microsomes were separated on a 10% SDS-polyacrylamide gel¹⁴ and blotted on to Hybribond P (Amersham, Buckinghamshire, England) in 20% methanol, 400 mM glycine, 50 mM Tris-HCl pH 8.3 at 4°C for 60 V for 4 h. CYP 2E1 was detected with an antibody kit purchased from Amersham using the ECL detection system as recommended by the manufacturer (Amersham). A positive control for CYP 2E1 was added as supplied by the manufacturer. Quantitation of the results was performed as described by Trautwein *et al.*¹²

Immunohistochemistry and analysis of sections

The avidin-biotin complex method¹⁵ was used to demonstrate GST-P-positive foci using anti-GST-P-IgG

(MBL, Nagoya), BrdU-labeled cells with anti-BrdU IgG (DAKO Japan Co., Kyoto) and expression of CYP 2E1 with anti-CYP 2E1 apoprotein IgG (Chemicon International, Inc., Temecula, CA). The numbers of foci >0.1 mm in diameter and the total areas of liver sections examined were assessed as detailed previously¹⁻³. The generation of BrdU labeling indices were performed as described previously². The significance of differences in the quantitative data from experiment II was statistically evaluated using ANOVA.

Results

Cell proliferation kinetics and changes of CYP 2E1 protein contents Hepatic injury by CCl_4 administration resulted in centri-lobular necrosis (Fig. 2). At 36 h after CCl_4 administration, necrosis was maximal and the histological architecture had regained a normal appearance at one week thereafter. D-gal-administration induced scattered necrosis with infiltration of inflammatory cells at 36 h after administration (Fig. 2) with recovery again within one week. The data for liver cell proliferation kinetics are summarized in Fig. 3A. Label incorporation after CCl_4 administration increased until 60 h. High labeling indices (approximately 20% to 30% of cells positive for BrdU) continued from 36 h to 72 h. There were BrdU-incorporated hepatocytes in the periportal area at high proliferating time. In contrast, cell proliferation after D-gal administration was comparatively low, the mean BrdU index being about 3% of cell positive at 48 h after the treatment, elevation continuing until 72 h. The BrdU-incorporated hepatocytes localized also in the periportal area at 48 h after D-gal treatment.

The data for CYP 2E1 apoprotein content in the microsomal proteins are summarized in Fig. 3B. After CCl_4 administration, CYP 2E1 apoprotein contents decreased up to about 40% of control from 12 h until 60 h after. On the other hand, only slight decrease at 12 hrs and 7 days after D-gal administration, but not to less than 70% of the control level. Immunohistochemically, the CYP 2E1-positive cells was in the centrilobular area of normal rat liver and the pattern of CYP 2E1 positive cells did not change with time after D-gal administration. Contrastively, most hepatocytes were negative 12 to 48 hrs after CCl_4 administration and staining recovered after 7 days.

Induction of GST-P positive foci

Data for the area and numbers of GST-P positive foci per unit area of liver section are summarized in Fig. 4. When given 60 h after D-gal administration, there was appreciable increase of area and numbers of GST-P positive foci. The area and number of GST-P-positive foci at 12, 60 h after D-gal and the group with CCl_4 administration were significantly elevated compared with the controls, and the area of GST-P-positive foci at 60 hr after D-gal was significantly higher than that of the group with CCl_4 administration, however, numbers of GST-P-positive foci were not significantly different between animals at 60 h after D-gal and animals with CCl_4 administration.

Fig. 2. Liver sections from animals treated with CCl₄ (A) or D-gal (B) in experiment I. A, Severe centri-lobular necrosis in a liver at 36 h after CCl₄ i.g. B, Scattered necrosis with infiltration of inflammatory cells (arrows) in a periportal area at 36 h after D-gal i.g. C, central vein. $\times 37$.

Fig. 3. BrdU labeling indices (A) and CYP 2E1 apoprotein contents (B) in the livers of rats at various times after D-gal (closed circles) and CCl₄ (open circles) administration. CYP 2E1 values are relative to the control level as 1. Data are mean $\pm$ SD for 5 animals in each group.

Discussion

In the present study the proliferative period after intragastric D-gal administration was from 48 h to 72 h, similar to the case receiving CCl₄ treatment^{1,2} but the degree of induction of proliferation was very much lower^{1,2}. In spite of this, the numbers of GST-P positive foci were not significantly different as compared with the CCl₄ group. D-gal traps uracil nucleotides by its toxic intermediary

metabolites (UDP-galactosamine and UDP-glucosamine) in rat liver¹⁶ and induces depletion of hepatic UTP, UDP-glucose and UDP-galactose which are essential materials in biosynthesis of macromolecules such as nucleic acids. Consequently, organelle injury and necrosis of hepatocytes occur¹⁷. CCl₄-induced hepatic necrosis is also caused by metabolic intermediates including trichloromethyl radicals and cytochrome P450 associated with bioactivation of carcinogen is reduced⁷. The radicals bind to CYP 2E1 which

Fig. 4. Numbers and areas of GST-P-positive liver cell foci $>0.1\text{mm}$ in diameter in experiment II. Data are mean $\pm$ SD values for 7–11 animals in each group. * $P < 0.05$ as compared with the group without DMH administration (Cont.). * $P < 0.05$ as compared with the CCl_4 administration group.

is in fact responsible for their activation, degrading and inactivating the enzyme (suicide metabolism)⁷ while causing necrosis of the expressing cells. Necrosis induced by D-gal administration has been reported to predominate in the periportal areas¹⁷, and therefore reduction of metabolic enzymes localized in centrilobular areas such as cytochrome P450 is much less. The present quantification by western blotting demonstrated decrease of CYP 2E1 apoprotein content after D-gal administration to be slight (up to approximately 30%). In our study, the centrilobular pattern of CYP 2E1 positive cells did not change with time after D-gal administration. Therefore, necrosis induced by intragastric D-gal administration did not influence CYP 2E1 contents and localization. In contrast, CYP 2E1 apoprotein contents until 60 hr after CCl_4 administration were decreased markedly. For detection for initiation with CCl_4 administration in experiment II, we earlier demonstrated that the 60 h time point is most effective for carcinogen application². Thus, intragastric D-gal administration would be expected to have less suppressive effects on bioactivation of DMH by CYP 2E1 than CCl_4 , counterbalancing the difference regarding proliferation kinetics. However, the route of D-gal administration in this study was intragastric administration similar to CCl_4 treatment, because we wanted to avoid the influence of difference of administering routes. In previous reports of hepatotoxicity, lower dose of D-gal was administered by intraperitoneal injection^{16–18}. Therefore, there will be a need for comparing the difference of administering routes of D-gal hereafter.

The area of GST-P positive foci with DMH timed at 60 h after D-gal administration was significantly higher than the value with CCl_4 administration. Selection pressure on GST-P positive cells by 2-AAF- CCl_4 treatment should be equal, therefore much of the difference in area might be caused by proliferation of GST-P positive cells in the period prior to 2-AAF exposure.

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References

1. Kobayashi K, Mutai M, Goto K, Inada K, Tsukamoto T, Nakanishi H, and Tatematsu M. Effects of carbon tetrachloride administration on initiation of liver cell foci by the non-hepatocarcinogens N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and benzo(a)pyrene (B(a)P). *Cancer Lett* 1997; **118**: 55–60.
2. Sakai H, Tsukamoto T, Yamamoto M, Yanai T, Masegi T, Inada K, Nakanishi H, and Tatematsu M. Summation of initiation activities of low doses of the non-hepatocarcinogen 1,2-dimethylhydrazine in the liver after carbon tetrachloride administration. *Cancer Lett* 2000; **148**: 59–63.
3. Tezuka N, Tada M, Kojima M, Nakanishi H, Mori A, and Tatematsu M. Effects of partial hepatectomy on initiation of liver cell foci by 4-nitroquinoline 1-oxide, a non-hepatocarcinogen, and generation of DNA adducts in rats. *Cancer Lett* 1995; **89**: 89–94.
4. Lijinsky W. A view of the relation between carcinogenesis and mutagenesis. *Environ Mol Mutagen* 1989; **14**: 78–84.
5. Tsuda H, Lee G, and Farber E. Induction of resistant hepatocytes as a new principle for a possible short-term in vivo test for carcinogens. *Cancer Res* 1980; **40**: 1157–1164.
6. Yasuda M, Okabe T, Itoh J, Takekoshi S, Hasegawa H, Nagata H, Osamura RY, and Watanabe K. Differentiation of necrotic cell death with or without lysosomal activation: application of acute liver injury models induced by carbon tetrachloride (CCl_4) and dimethylnitrosamine (DMN). *J Histochem Cytochem* 2000; **48**: 1331–1339.
7. Wang PY, Kaneko T, Tsukada H, Nakano M, Nakajima T, and Sato A. Time courses of hepatic injuries induced by

- chloroform and by carbon tetrachloride: Comparison of biochemical and histopathological changes. *Arch Toxicol* 1997; **71**: 638–645.
8. Ito N, Imaida K, de Camargo JL, Takahashi S, Asamoto M, and Tsuda H. A new medium-term bioassay system for detection of environmental carcinogens using diethylnitrosamine-initiated rat liver followed by D-galactosamine treatment and partial hepatectomy. *Jpn J Cancer Res* 1988; **79**: 573–575.
 9. Tsuda H, Masui T, Ikawa E, Imaida K, and Ito N. Compared promoting potential of D-galactosamine, carbon tetrachloride and partial hepatectomy in rapid induction of preneoplastic liver lesions in the rat. *Cancer Lett* 1987; **37**: 163–171.
 10. Sohn OS, Ishizaki H, Yang CS, and Fiala ES. Metabolism of azoxymethane, methylazoxymethanol and N-nitrosodimethylamine by cytochrome P450IIE1. *Carcinogenesis* 1991; **12**: 127–131.
 11. Burke MD and Mayer RT. Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab Dispos* 1974; **2**: 583–588.
 12. Trautwein C, Rakemann T, Obermayer-Straub P, Niehof M, and Manns MP. Differences in the regulation of cytochrome P450 family members during liver regeneration. *J Hepatol* 1997; **26**: 48–54.
 13. Lowry OH, Rosebrought NJ, Farr AL, and Randall FJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; **193**: 265–275.
 14. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
 15. Hsu SM, Raine L, and Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 1981; **29**: 577–580.
 16. Abdul-Hussain SK and Mehendale HM. Ongoing hepatocellular regeneration and resiliency toward galactosamine hepatotoxicity. *Arch Toxicol* 1992; **66**: 729–742.
 17. Decker K and Keppler D. Galactosamine induced liver injury. In: *Progress in Liver Diseases*, H Popper and S Fenton (eds), New York: Grune & Stratton, 183–199, 1972.
 18. Murase T, Masuda R, Aoi K, Sawamura T, Shiozaki Y, and Sameshima Y. The effect of galactosamine on rat liver cytochrome P-450 activities. *Jpn J Pharmacol* 1985; **37**: 151–158.