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Enhancement of aflatoxin B_1-induced enzyme altered hepatic foci in rats by treatment with carbon tetrachloride

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Abbreviations: AFB_1, aflatoxin B_1; PH, partial hepatectomy; CCl_4, carbon tetrachloride; GST-P, glutathione *S*-transferase placental form; DMSO, dimethyl sulfoxide; BrdU, bromodeoxyuridine; GGT, γ-glutamyltrans-peptidase; MDA, malondialdehyde

Abstract

The effect of carbon tetrachloride ($CC1_4$) on aflatoxin B_1 (AFB_1)-induced enzyme altered hepatic foci has been examined in young male Fischer rats given AIN-76A diet. A single i.p. dose of AFB_1 (0.2 mg/kg body wt) was given to rats 24 h after partial hepatectomy. Two weeks later, CCl_4 (0.8 ml/kg body wt) was injected i.p. once a week for 9 weeks. Animals were sacrificed 24 h after the last dose of $CC1_4$ and glutathione S-transferase placental form (GST-P) and γ-glutamyl transpeptidase (GGT) positive hepatic foci were analyzed by immunohistochemical and histochemical methods, respectively. Ten weeks after AFB_1 dosing, treatment with CCl_4 increased the number of AFB_1-induced enzyme altered foci several fold and produced a ten to twenty-fold increase in area and volume. GST-P was more sensitive than GGT in detecting AFB_1-induced enzyme altered foci. Treatment with AFB_1 or CCl_4 produced mild hepatic fibrosis in zones 1 and 3 respectively, whereas both treatments produced severe fibrosis in zones 1 to 3 areas. Treat-ment with CCl_4 after AFB_1 dosing lowered hepatic GSH levels by 20% and increased lipid peroxidation by 40%. It appears that CCl_4, by being an effective enhancer of AFB_1-induced enzyme altered hepatic foci in the rat, may mimic cirrhosis observed in human hepatocellular carcinoma.

Keywords: Hepatocarcinogenesis, Aflatoxin B_1, Carbon tetrachloride, Enzyme altered foci

Introduction

On the basis of epidemiological data, it has been established that one of the factors responsible for human liver cancer is aflatoxin B_1 (AFB_1), a mycotoxin produced by Aspergillus flavus (IARC Monographs, 1993). Development of human hepatocellular carcinoma is closely associated with liver cirrhosis produced by hepatitis virus infection and heavy consumption of alcohol (Doll and Peto, 1981; Tribelli *et al*., 1989). Even though a rat is the most susceptible laboratory animal species to AFB_1 hepatocarcinogenesis (Newberne and Butler, 1969; Eaton and Gallagher, 1994), cirrhosis is not observed in this species even in the presence of various promoters including phenobarbital (Newberne and Butler, 1969; Kraupp-Grasl *et al.*, 1990; Hiruma *et al.*, 1997).

Compensatory cell proliferation induced by either partial hepatectomy (PH) or carbon tetrachloride (CCl_4) plays an important role in the initiation of rat liver carcinogenesis by various chemicals including AFB_1 as examined by enzyme altered foci (Warwick, 1971; Cayama *et al*., 1978; Pitot *et al.*, 1978; Columbano *et al.*, 1987; Hiruma *et al.*, 1996). Previous studies with diethylnitrosamine and AFB_1 have indicated that early glutathione *S*-transferase placental form (GST-P) positive hepatocytes induced by these carcinogens are the precursors for preneoplastic foci and nodules (Moore *et al.*, 1987; Satoh *et al.*, 1989; Tsuji *et al*., 1992; Dragan *et al*., 1994; Hiruma *et al.*, 1996, 1997). Repeated administration of CCl_4 to rats, has enhanced nitrosamine-induced formation of enzyme altered hepatic foci and nodules (Columbano *et al.*, 1990; Zalatnai *et al.*, 1991; Cho and Jang, 1993). It is also known that repeated administration of CCl_4 to rats results in cirrhosis of the liver (Perez-Tamayo, 1983; Gasso *et al.*, 1996).

In the present study, we have evaluated the effect of CCl_4 on AFB_1-induced enzyme altered hepatic foci. The results of this study indicate that CCl_4 is an effective enhancer of AFB_1-induced enzyme altered hepatic foci in the rat. In addition, CCl_4 treatment enhances hepatic fibrosis and lipid peroxidation.

Materials and Methods

Chemicals

Glycylglycine, diaminobenzidine, AFB_1, dimethylsulfoxide (DMSO), HPLC grade CCl_4, bromodeoxyuridine (BrdU), mouse anti-BrdU antibody, glutathione (GSH) and 5,5'-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma Chemical Co., St. Louis, MO. γ-Glutamyl-4-methoxy-2-naphthylamide was bought from United States Biochemical Corporation, Cleveland, OH. Inhalation anesthetic, methoxyflurane (2,2-dichloro-1-1-difluoroethyl methyl ether) was obtained from Pittman Moore Inc., Washington Crossing, NJ. Vectastain ABC kit for immunohistochemical staining was purchased from Vector Laboratories Inc., Burlingame, CA. AIN-76A diet (Second Report, 1980) was bought from Dyets Inc., Bethlehem, PA. All other chemicals were of reagent grade.

Animals and treatment

Thirty four male Fischer rats, 6-7 weeks old (90-100 g body wt) purchased from Charles River Breeding Laboratories, Wilmington, MA, were housed in plastic cages with hard wood chips for bedding in an air-conditioned room at 23 ± 2°C with a 12 h light/dark cycle. They were maintained on AIN-76A diet and given tap water for 10 days before use.

A two-thirds partial hepatectomy (PH) was performed on all animals under methoxyflurane anesthesia according to the published procedure (Higgins and Anderson, 1931). Earlier studies indicated that the maximum initiation occurs when various carcinogens including AFB_1 are administered at the peak of DNA synthesis. In the rat liver, maximal DNA synthesis occurs 20-24 h after PH (Warwick, 1971; Scherer and Emmelot, 1976; Cayama *et al.*, 1978; Pitot *et al.*, 1978; Columbano *et al.*, 1987; Hiruma *et al.*, 1996;). In the present study therefore, sixteen animals were injected intraperitoneally (i.p.) with DMSO and the remaining 18 animals were injected i.p. with AFB_1 (0.2 mg/kg body wt) dissolved in DMSO 24 h after PH to induce maximum initiation of carcinogenesis. All animals were maintained on AIN-76A diet throughout the experimental period. Two weeks after either DMSO or AFB_1 dosing, sixteen animals were injected i.p. with corn oil and 18 animals were injected i.p. with CCl_4 (0.8 ml/kg body wt) dissolved in corn oil once a week for 9 weeks. Twenty four h after the last dose of either corn oil or CCl_4 treatment, all animals were sacrificed.

To examine liver cell proliferation, all animals were injected i.p. with BrdU (100 mg/kg body wt) dissolved in saline 1 h before sacrifice.

Histopathology

After the animals were sacrificed by exsanguination under CO_2 euthanasia, livers were excised and weighed. Some liver sections cut into 2-3 mm thick sections with a razor blade were fixed in 10% phosphate buffered formalin for routine staining with hematoxylin and eosin (H&E) as well as for immunohistochemical detection of BrdU and GST-P, whereas other sections were fixed in ice-cold acetone for histochemical determination of γ-glutamyl-transpeptidase (GGT).

GST-P staining

The immunohistochemical demonstration of GST-P antibody binding was by the avidin-biotin peroxidase method of Hsu *et al.* (1981). The sites of peroxidase binding were detected by the diaminobenzidine method (Graham and Karnofsky, 1966).

GGT staining

The histochemical staining for GGT was performed according to the published method (Rutenberg *et al.*, 1969) using γ-glutamyl-4-methoxy-2-naphthylamide as a substrate.

Quantitation of GST-P and GGT positive hepatic foci

By taking one section from each lobe, about 3-4 cm^2 of

Table 1. Enhancement of AFB_1-induced GST-P positive hepatic foci in rats by treatment with carbon tetrachloride[a].

Group	GST-P positive hepatic foci (>100 μm in diameter)			
	No./cm^2	Area of Foci (mm^2/cm^2)	No./cm^3	Volume of foci (mm^3/liver)
DMSO + oil	0	0	0	0
DMSO + CCl_4	0	0	0	0
AFB_1 + oil	3.2 ± 1.4[b]	0.3 ± 0.2	274 ± 109	2.9 ± 1.7
AFB_1 + CCl_4	8.6 ± 2.9[c]	3.4 ± 1.8[c]	511 ± 134[c]	31.8 ± 16.1[d]

[a] Each group consisted of 8-10 animals. Other experimental details are as described in 'Materials and Methods'.
[b] Mean ± SD
[c,d] Data highly significant with values of [c]P<0.01 and [d]P<0.001 when compared with respective data of animals without CCl_4 treatment.

liver from each animal was examined for GST-P and GGT. The enzyme altered hepatic foci were counted with the use of a light microscope. Diameters of these foci were determined with an eyepiece micrometer. Areas of these foci were calculated from diameters of these foci. The number of foci per cm^2 was converted to the number of foci per cm^3, with the conditional stereologic estimater of Enzmann *et al.* (1987) as modified recently with the 'Spheres' software program developed by Bayer A.G. in Germany.

BrdU positive cells

BrdU positive cells were detected by an immunohistochemical staining method (Hsu *et al.*, 1981) as described by Sugihara *et al.* (1986). The sites of peroxidase binding were detected by the diaminobenzidine method (Graham and Karnofsky, 1966). At least 1000 hepatocytes were counted for each section to determine BrdU positive cells.

Fibrosis

Fibrosis observed in H&E stained sections was graded as follows: Zone 1 represents a periportal area of the liver lobule only; zone 3 represents a pericentral area of the lobule only; zone 1 to zone 3 represents periportal to pericentral areas including zone 2 area.

Other assays

Hepatic GSH levels were assayed according to Ellman's procedure (1959) in the presence of 1M HEPES buffer, pH 8.3. Concentrations of malondialdehyde (MDA) in liver homogenates were estimated by using a thiobarbituric acid method (Buege and Aust, 1978). The MDA concent-ration was determined using a molar extinction coefficient of 156,000. Results are expressed as nmol of MDA formed per g liver tissue. The significance of the data was statistically evaluated using Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

Ten weeks after AFB_1 dosing, appreciable numbers of GST-P positive hepatic foci could be detected even though the area of these foci was small (Table 1). In the absence of AFB_1 dosing or multiple doses of weekly CCl_4 treatment alone did not yield any GST-P positive hepatic foci. However, weekly treatment of CCl_4 to AFB_1-treated rats produced a 2-3 fold higher number of GST-P positive hepatic foci with a ten fold increase in area and volume compared to animals administered with AFB_1 only.

Treatment with CCl_4 increased the number of AFB_1-induced GGT positive hepatic foci several fold with a twenty fold increase in area and volume (Table 2).

Data on the effects of CCl_4 treatment on hepatic fibrosis, cellular proliferation, GSH levels and lipid peroxidation are summarized in Table 3. Dosing of AFB_1 alone produced hepatic fibrosis in the zone 1 area only, whereas CCl_4 treatment alone produced fibrosis in the zone 3 area only. Treatment of CCl_4 subsequent to AFB_1 dosing produced severe fibrosis (cirrhosis) in zones 1 to 3. Cellular proliferation as measured by BrdU incorporation was very low in DMSO or AFB_1 dosed animals. However, cellular proliferation was several fold higher in CCl_4 treated animals injected with or without AFB_1. Hepatic GSH levels were lowered by 20% in animals treated with $CC1_4$ after either DMSO or AFB_1 dosing. Treatment with CCl_4 increased lipid peroxidation by 25% and 40% in control and AFB_1 injected animals, respectively.

Discussion

In the present study, a single i.p. dose of AFB_1 (0.2 mg/kg body wt) given to rats 24 h after PH without $CC1_4$ treatment did yield some enzyme altered hepatic foci when

Table 2. Carbon tetrachloride enhancement of AFB_1-induced GGT-positive hepatic foci in rats[a].

Group	GGT-positive hepatic foci (>100μm in diameter)			
	No./cm^2	Area of Foci (mm^2/cm^2)	No./cm^3	Volume of foci (mm^3/liver)
DMSO + oil	0	0	0	0
DMSO + $CC1_4$	0	0	0	0
AFB_1 + oil	0.9 ± 0.4[b]	0.06 ± 0.09	74 ± 40	0.6 ± 0.8
AFB_1 + CCl_4	6.1 ± 2.0[d]	1.30 ± 1.19[c]	270 ± 73[d]	12.2 ± 10.7[c]

[a] Same animals used as described in experimental protocols in Table 1. Other experimental details are as described in 'Materials and Methods'.

[b] Mean ± SD of ten analyses on individual livers from ten animals

[c,d] Data highly significant with values of [c]$P < 0.05$ and [d]$P < 0.001$ when compared with data of animals without CCl_4 treatment.

Table 3. Effect of CCl_4 on hepatic cellular proliferation, fibrosis, GSH levels and lipid peroxidation during AFB_1-induced enzyme altered hepatic foci in rats[a].

Group	BrdU Incorporation (%)	GSH levels μmol/g liver	Lipid Peroxidation malondialdehye formed nmol/g liver	Fibrosis
DMSO + oil	0.3 ± 0.1[b]	6.0 ± 0.7	35.4 ± 7.5	-
DMSO + CCl_4	0.8 ± 0.2[d]	4.9 ± 0.6[c]	44.0 ± 7.1	±[e]
AFB_1 + oil	0.3 ± 0.1	6.2 ± 0.8	40.6 ± 10.9	±[f]
AFB_1 + CCl_4	1.9 ± 0.5[d]	4.8 ± 0.9[c]	57.8 ± 10.5[c]	+++[g]

[a] Same animals used as described in experimental protocols in Table 1. Other experimental details are as described in 'Materials and Methods'.

[b] Mean ± SD (n=8-10)

[c,d] Data highly significant with values of [c]P<0.02 and [d]P<0.001 when compared with respective data of animals without CCl_4 treatments.

[e] Fibrosis in zone 3 area.

[f] Fibrosis in zone 1 area.

[g] Fibrosis in zones 1 to 3 areas.

animals were sacrificed at 10 weeks after the carcinogen administration (Tables 1 and 2). However, weekly treatment of CCl_4 enhanced AFB_1-induced enzyme altered foci several fold both in area and volume. The present results are thus in agreement with previous data of other investigators who demonstrated that repeated CCl_4 treatment of various rodent species including rats enhanced nitrosamine-initiated hepatocytes to form enzyme altered foci and nodules (Pound and McGuire, 1978; Dragani *et al*., 1986; Tanaka *et al*., 1987; Columbano *et al.*, 1990; Zalatnai *et al.*, 1991; Cho and Jang, 1993).

In earlier studies, GGT was widely used as an enzyme marker for detecting the production of enzyme altered foci in rodent hepatocarcinogenesis (Kalengayi *et al.*, 1975; Cayama *et al.*, 1978; Pitot *et al.*, 1978; Columbano *et al.*, 1987). In the present study, it is apparent that GST-P is more sensitive than GGT in detecting production of AFB_1-induced and CCl_4-enhanced enzyme altered foci. These results are in agreement with other data on enzyme altered foci obtained with nitrosamine hepatocarcinogenesis from several laboratories (Satoh *et al.*, 1985; Xu *et al.*, 1990; Ledda-Columbano *et al.*, 1992).

It has been shown that CCl_4-induced compensatory cell proliferation and not mitogen-induced cell proliferation is responsible for enhancement of nitrosamine initiated hepatocarcinogenesis (Columbano *et al.*, 1987; Ledda-Columbano *et al.*, 1992). Our previous studies on cellular proliferation examined by BrdU incorporation in the rat indicated optimum hepatic proliferation at 24 h after a single i.p. dose of CCl_4 (0.4 ml/kg body wt) where about 30% of the cells were labeled (Hiruma *et al.*, 1997). In the present study however, cell proliferation examined in the whole liver 24 h after the last CCl_4 treatment was much lower than that observed in our previous study. There is no satisfactory explanation to account for this discrepancy.

Lowering of hepatic GSH levels and enhanced lipid peroxidation after repeated administration of CCl_4 to rats reported previously (Aguilar-Delfin *et al.*, 1996; Corrales *et al.*, 1992; Gasso *et al.*, 1996) are in agreement with our present data. However, in the present study, lipid peroxidation was enhanced significantly in animals dosed with AFB_1 before CCl_4 treatment. It may be useful to employ hepatic lipid peroxidation as a biochemical marker during CCl_4 enhancement of AFB_1-initiated hepatocarcinogenesis.

In experimental CCl_4-induced cirrhosis in the rat, initial necrotic lesions are observed in the centrilobular zone 3 area where fibrosis also occurs (Perez-Tamayo, 1983), whereas during AFB_1 hepatotoxicity, the carcinogen produces periportal zone 1 necrosis (Newberne and Butler, 1969; Moore *et al.*, 1987). Our present data on hepatic fibrosis with AFB_1 or CCl_4 alone occurring in zones 1 and 3 respectively are in agreement with data reported previously by other investigators (Newberne and Butler, 1969; Perez-Tamayo, 1969; Moore *et al.*, 1987). However, CCl_4 treatment subsequent to AFB_1 administration producing severe fibrosis from zones 1 to 3 in the present study suggests that CCl_4 in the rat may mimic cirrhosis observed in human hepatocellular carcinoma (Doll and Peto, 1981; Tribelli *et al.*, 1989).

In summary, repeated treatment of CCl_4 subsequent to AFB_1 administration to male Fischer rats enhanced AFB_1-induced GST-P- and GGT-positive hepatic foci, fibrosis and lipid peroxidation.

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