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Promotion of Rat Hepatocarcinogenesis by Dimethylarsinic Acid: Association with Elevated Ornithine Decarboxylase Activity and Formation of 8-Hydroxydeoxyguanosine in the Liver

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Arsenicals are epidemiologically significant chemicals in relation to induction of liver cancer in man. In the present study, we investigated the dose-dependent promotion potential of dimethylarsinic acid (DMAA), a major metabolite of inorganic arsenicals in mammals, in a rat liver carcinogenesis model. In experiment 1, glutathione-*S*-transferase placental form (GST-P)-positive foci, putative preneoplastic lesions, were employed as endpoints of a liver medium-term bioassay for carcinogens (Ito test). Starting 2 weeks after initiation with diethylnitrosamine, male F344 rats were treated with 0, 25, 50 or 100 ppm of DMAA in the drinking water for 6 weeks. All animals underwent two-thirds partial hepatectomy at week 3 after initiation. Examination of liver sections after termination at 8 weeks revealed dose-dependent increases in the numbers and areas of GST-P-positive foci in DMAA-treated rats as compared with controls. In experiment 2, ornithine decarboxylase activity, which is a biomarker of cell proliferation, was found to be significantly increased in the livers of rats treated with DMAA. In experiment 3, formation of 8-hydroxydeoxyguanosine, which is a marker of oxygen radical-mediated DNA damage, was significantly increased after administration of DMAA. These results indicate that DMAA has the potential to promote rat liver carcinogenesis, possibly via a mechanism involving stimulation of cell proliferation and DNA damage caused by oxygen radicals.

Key words: Dimethylarsinic acid — 8-Hydroxydeoxyguanosine — Ornithine decarboxylase — Rat hepatocarcinogenesis

Epidemiological investigations have revealed that ingested inorganic arsenic is carcinogenic to humans, especially in the skin and lung.[1,2] In a blackfoot disease endemic area of Taiwan, where the arsenical level in the drinking water is relatively high, increased incidence of and mortality from skin, bladder, kidney, lung, and liver cancers in man have been reported with a defined dose-response relationship.[3–7]

However, in experimental animals, arsenicals have not been found to be carcinogenic,[1,8] although we recently revealed promotion potential for dimethylarsinic acid (DMAA), a main metabolite of ingested inorganic arsenicals in most animals, in the urinary bladder, kidney, liver and thyroid gland of rats.[9,10] The tumor incidences were dose-dependently elevated.[9,10] In rat bladder carcinogenesis initiated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), the amount of DMAA ingested was found to correlate with urinary excretion of the compound, as well as tumor development. DMAA also induced increasing cell proliferation of urinary bladder epithelium.[10]

In the present study, we examined the dose-dependence of DMAA promoting activity in rat hepatocarcinogenesis using a liver medium term bioassay (Ito test).[11] To assess possible mechanisms, formation of 8-hydroxydeoxyguanosine (8-OHdG), a DNA adduct generated by activated oxygen radical species, was examined,[12] since a contribution of oxygen radicals to carcinogenic processes has been proposed.[13–16] In addition, ornithine decarboxylase (ODC) and spermidine/spermine N^1-acetyltransferase (SAT), which are rate-limiting enzymes of polyamine biosynthesis reported to be increased concomitantly with promotion in skin, bladder and liver carcinogenesis,[17–19] were measured.

MATERIALS AND METHODS

Animals A total of 110, 6-week-old male F344 rats (Charles River Japan, Inc., Hino) was used. They were housed with wood chips for bedding in an animal room with a 12 h light/dark cycle at 22±2°C and 44±5% relative humidity. Body weights were measured weekly and drinking water consumption was measured throughout each administration period. Diet and water were available *ad libitum* throughout.

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Chemicals Diethylnitrosamine (DEN) was obtained from Tokyo Chemical Industry Co., Tokyo. DMAA with a purity of 99% was purchased from Wako Pure Chemical Industries, Ltd., Osaka.

Experiment 1 Sixty male F344 rats were used for the liver medium-term bioassay (Ito test).[11] In groups 1 to 4, rats were given a single i.p. injection of DEN (200 mg/kg body weight) dissolved in saline to initiate hepatocarcinogenesis, and in groups 5 and 6, rats received the saline vehicle alone. After 2 weeks on basal pellet diet (CE2, Clea Japan, Inc., Tokyo) and water, they were given one of several concentrations of DMAA in the drinking water (groups 1–4; 0, 25, 50 and 100 ppm, group 5 and 6; 0 and 100 ppm) for 6 weeks. They were fed basal pellet diet during this period. Animals were subjected to two-thirds partial hepatectomy (PH) at week 3 to maximize the influence of the compound by enhancing liver cell proliferation. At week 8, the rats were killed under ether anesthesia, and their livers were examined immunohistochemically for glutathione-*S*-transferase placental form (GST-P) expression.

Experiment 2 Thirty male F344 rats were used for examination of ODC and SAT activity after DMAA treatment. All were given a single i.p. injection of DEN (200 mg/kg body weight) dissolved in saline. After 2 weeks on basal diet and water, they were given one of three concentrations of DMAA in the drinking water (groups 1–3; 0, 10, and 50 ppm) for 2 weeks. Animals were subjected to PH at week 3. At week 4, the rats were killed under ether anesthesia, and their livers were examined for ODC and SAT activities.

Experiment 3 Twenty male F344 rats were employed for detection of 8-OHdG formation in liver DNA after DMAA treatment. They were divided into 4 equal groups, of which 1 and 2 were given a single i.p. injection of DEN (200 mg/kg body weight) dissolved in saline and 3 and 4 received saline alone. After 1 week on basal diet and water, rats in groups 1 and 3 were given 100 ppm of DMAA in their drinking water for 3 weeks, and rats in groups 2 and 4 were given water. At week 4, all rats were killed under ether anesthesia and their livers were examined for 8-OHdG formation in DNA.

Tissue processing At autopsy, the livers were excised and sections of 2–3 mm thick were cut with a razor blade. Three slices, one each from the right posterior, anterior and caudate lobes, were fixed in ice-cold acetone for immunohistochemical examination of GST-P in experiment 1. For the measurement of ODC and SAT activities in experiment 2 and 8-OHdG in experiment 3, tissue was frozen in liquid nitrogen and stored at −80°C until processed.

GST-P immunohistochemistry The avidin-biotin-peroxidase complex method was used to visualize GST-P-positive liver foci, which are putative preneoplastic lesions.[11, 20, 21] After deparaffinization, liver sections were treated sequentially with normal goat serum, rabbit anti-GST-P (1 : 8000) (provided by Dr. K. Sato, Hirosaki University), biotin-labeled goat anti-rabbit IgG (1 : 400) and avidin-biotin-peroxidase complex. The sites of peroxidase binding were demonstrated by the diaminobenzidine method. Sections were counter-stained with hematoxylin for microscopic examination. As a negative control for the specificity of anti-GST-P antibody binding, preimmune rabbit serum was used instead of antiserum. The numbers and areas of GST-P-positive foci >0.2 mm in diameter and the total areas of the liver sections examined were measured using a color video image processor (VIP-21CH).

Measurement of ODC and SAT activities and polyamine biosynthesis ODC and SAT activities were measured by the methods of Matsui *et al.*[22] and Otani *et al.*,[23] respectively. Frozen pieces of rat liver were suspended in 0.5 ml of 50 m*M* Tris (pH 7.5) containing 0.25 *M* sucrose and disrupted with a homogenizer for 30 s. The homogenized suspensions were centrifuged at 100,000*g* for 30 min, and the supernatants were assayed for ODC and SAT by measurement of the amount of radioactive putrescine produced from [5-^{14}C]ornithine and the amount of acetyl moiety transferred from [1-^{14}C]acetyl-CoA to spermidine, respectively.

Detection of 8-OHdG formation in DNA Liver samples of approximately 2 g wet weight were taken from each animal and the DNA was isolated and digested into deoxynucleosides by combined treatment with nuclease P1 (Yamasa Shoyu Co., Ltd., Chohshi, Chiba) plus alkaline phosphatase (Sigma, St. Louis, MO), as described by Takagi *et al.*.[24] The level of 8-OHdG in each resultant preparation was determined by high-performance liquid chromatography using an adaptation of the methods of Floyd *et al.*[25, 26] and Kasai *et al.*,[12, 27, 28] as detailed elsewhere.[29] The level of 8-OHdG formation was expressed as the number of 8-OHdG residues/10^5 of total deoxyguanosine.

Statistical analysis Statistical analyses were completed with Stat-View software for the Macintosh microcomputer, and significant differences were determined by the use of Student's *t* test.

RESULTS

Experiment 1 Six of 10 rats in group 4 (DEN→DMAA, 100 ppm), and 5 of 10 rats in group 6 (saline→DMAA, 100 ppm) died because of the toxicity of DMAA between weeks 3 and 4 after the start of DMAA treatment. Effective numbers of rats, final body weights and relative liver weights are shown in Table I. No statistically significant variation in final body weights or relative liver weights was noted. Fig. 1 shows the numbers and areas of

Table I. Average Final Body and Relative Liver Weights of Experiment 1

Treatment		Effective no. of rats	Final body wt (g)	Relative liver wt (g/100 g body wt)
DEN	DMAA (ppm)			
+	0	10	231±11	3.16±0.22
+	25	10	230±8	3.14±0.14
+	50	10	229±14	3.39±0.23
+	100	4	224±12	3.18±0.14
−	0	10	252±8	3.03±0.14
−	100	5	242±9	3.30±0.16

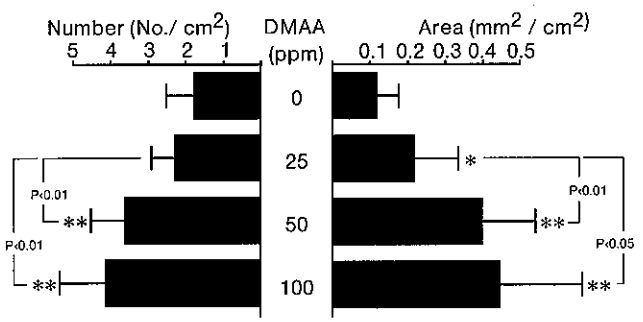

Fig. 1. Numbers and areas of GST-P-positive foci in the livers of rats initiated with DEN followed by treatment with DMAA. Significantly different from the control group (DMAA 0 ppm) at * $P<0.05$, ** $P<0.01$ (Student's t test). Bars indicate the SD.

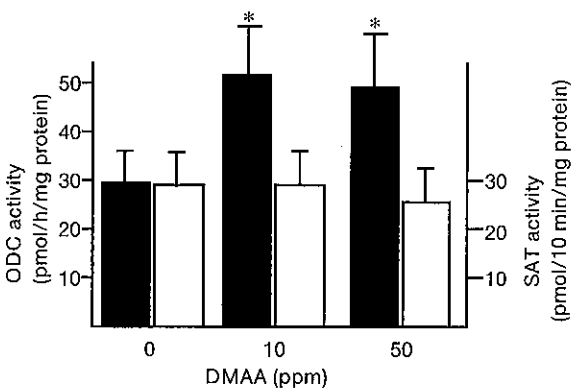

Fig. 2. ODC and SAT activity in the livers of rats treated with DMAA. Significantly different from the control group (DMAA 0 ppm) at * $P<0.05$ (Student's t test). Bars indicate the SD.

GST-P-positive foci per unit area of liver sections in rats initiated with DEN. Values were increased in a DMAA dose-dependent manner. The areas of GST-P-positive foci were significantly increased in the groups treated

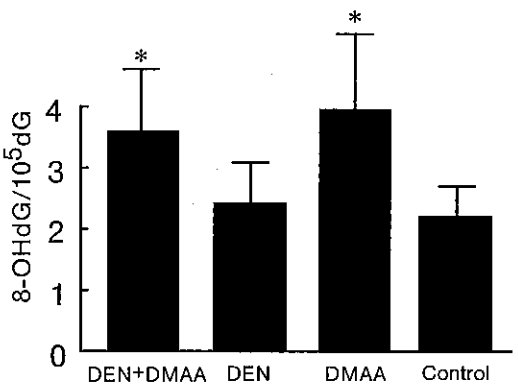

Fig. 3. Formation of 8-OHdG in the livers of rats treated with DMAA. Significantly different from the control group (DMAA 0 ppm) at * $P<0.05$ (Student's t test). Bars indicate the SD.

with more than 25 ppm of DMAA and the numbers were significantly increased with more than 50 ppm. In the groups without DEN initiation, no GST-P-positive foci were seen.

Experiment 2 Fig. 2 shows the ODC and SAT activities in the livers of rats treated with DMAA. ODC activity was significantly increased (10 ppm; 52.0±18.7 pmol/mg, 50 ppm; 49.3±12.6 pmol/mg) as compared to the control level (30.0±6.3 pmol/mg). On the other hand, SAT activity was not altered.

Experiment 3 Fig. 3 shows the results of determination of 8-OHdG in liver DNA of rats treated with DMAA after initiation by DEN or saline injection. Formation of 8-OHdG was significantly increased over the control levels in both cases.

DISCUSSION

In the present study, DMAA exhibited dose-dependent promoting activity for rat liver carcinogenesis in a medium-term bioassay for carcinogens (Ito test). This assay system examines the post-initiation modifying potential of chemicals,[11] but positive results mean that the chemicals tested have promoting or carcinogenic activities for rat liver. The degree of induction of GST-P-positive foci, used as a marker in this bioassay system, has been shown to correspond with the incidence of hepatocellular carcinomas in long-term *in vivo* experiments.[30,31] Consequently, the present study strongly indicates that DMAA can promote rat hepatocarcinogenesis. This finding is in agreement with our previous study using a rat multiorgan carcinogenesis model, in which DMAA at a concentration of 200 ppm enhanced hepatocellular carcinoma development.[9]

In the present study, promoting activity was evident at the relatively low dose of 25 ppm in the drinking water. This is, however, in line with the epidemiological data from an area of Taiwan endemic for blackfoot disease, where the arsenic concentration in drinking water was found to be related to the liver cancer mortality in a dose-response manner.[3-7]

Concerning the toxicity of DMAA, in experiment 1, the rats given 100 ppm demonstrated a 60% mortality rate. The main histological changes were necrosis and fatty accumulation around the central veins in the liver. We recently found that 7 of 10 rats starting 200 ppm DMAA treatment at 6 weeks of age died within 4 weeks (unpublished data). In contrast only one and none of ten rats died in the groups aged 8 weeks and 10 weeks, respectively, at the commencement. Thus DMAA administration is primarily toxic to young rats.

As regards the mechanism of hepatopromoting activity of DMAA, the present results clearly point to an influence on cell proliferation. ODC activity is a biomarker of epithelial cell proliferation and is well known to be elevated in the skin and bladder in response to promoting agent administration.[17,18] Similarly, Takada *et al.*[19] observed an increase of ODC activity in liver cells treated with promoting agents. In this experiment, ODC activity increased in the groups treated with DMAA, albeit without dose-dependence. Although the reason for the lack of dose-dependency of ODC activities is not clear, a similar situation was observed for the BrdU labeling index of rat bladder epithelium after treatment with DMAA employed as a promoter of bladder carcinogenesis.[10] Namely, in the case of DMAA promotion, other agents may influence the dose-dependence. Although levels of SAT activities did not change at all, the reason for this discrepancy between the levels of ODC and SAT activities was not clear. Brown and Kitchin[32] found that sodium arsenite increased both ODC and heme oxygenase activities, but caused no DNA damage in the rat liver, suggesting that it may be a promoter rather than a carcinogen.

The present examination of 8-OHdG formation in the livers of rats treated with DMAA revealed a significant increase. Kasai *et al.*[12] earlier reported the formation of 8-OHdG in cellular DNA by agents producing oxygen radicals. The possible contribution of these radicals to carcinogenic processes including the stages of initiation, promotion and progression, has attracted a great deal of attention.[13-16] Our results thus indicate that DMAA or its active metabolites cause DNA damage via oxygen radicals.

It is therefore not clear whether DMAA should be regarded as a carcinogen or a promoter. Yamanaka *et al.*[33-40] revealed that it can induce DNA damage in the lung, i.e., DNA single-strand breaks and the clumping of heterochromatin, again via the production of free radicals. From these data and our own 8-OHdG findings, we speculate that DMAA might itself be carcinogenic. However, in our previous study,[9] DMAA did not induce liver tumors during 30 weeks' treatment. Further long-term examination is needed to clarify this point.

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