Diphenylarsinic acid exerts promotion effects on hepatobiliary carcinogenesis in a rat medium-term multiorgan carcinogenicity bioassay

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Abstract: We have previously demonstrated that diphenylarsinic acid (DPAA) promotes liver carcinogenesis in rats in a medium-term liver carcinogenicity bioassay. However, the effects of DPAA on other organs have not been determined. In the present study, the effects of DPAA on carcinogenesis were investigated using a rat multiorgan carcinogenicity bioassay. A total of 60 six-week-old male F344 rats were treated with the carcinogens diethylnitrosamine, N-butyl-N-(4-hydroxybutyl) nitrosamine, N-methyl-N-nitrosourea, N-bis(2-hydroxypropyl) nitrosamine, and 1,2-dimethylhydrazine dihydrochloride to initiate carcinogenesis in multiple organs. After initiation, DPAA was given at a dose of 0, 5, or 20 ppm in drinking water for 27 weeks. The incidences of moderate and severe bile duct hyperplasia were significantly increased in the 20 ppm DPAA group (29.4%, 70.6%, respectively) compared with the 0 ppm DPAA group (0%, 0%, respectively), and the incidence and multiplicity of cholangioma were significantly increased in the 20 ppm DPAA group (29.4%, 0.4 ± 0.8/rat) compared with the 0 ppm DPAA group (0%, 0/rat). The total number and average area of glutathione S-transferase placenta form-positive foci, preneoplastic lesions in rat livers, were significantly increased in the 20 ppm DPAA group (10.5 ± 2.2/cm², 5.3 ± 1.7 mm²/cm²) compared with the 0 ppm DPAA group (6.2 ± 2.9/cm², 2.4 ± 1.4 mm²/cm²). In conclusion, our results demonstrate that DPAA promotes hepatobiliary carcinogenesis in a rat medium-term multiorgan carcinogenicity bioassay; no promotion effects were observed in other organs. (DOI: 10.1293/tox.2016-0049; J Toxicol Pathol 2017; 30: 39–45)

Key words: diphenylarsinic acid, liver carcinogenesis, cholangioma, bile duct hyperplasia, DMBDD
tap water for 28 days at room temperature was confirmed using an ion chromatography inductively coupled plasma mass spectrometry (IC-ICP-MS) system (IC7000 ion chromatograph, Yokogawa Analytic System Inc., Tokyo, Japan; HP 4500 inductively coupled plasma mass spectrometer, Wilmington, DE, USA) at Osaka City University Graduate School of Medicine. DEN, DMH, and BBN were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). MNU was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and DHPN was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Pentobarbital sodium (Somnopentyl®) was purchased from Kyoritsu Seiyaku Corporation (Tokyo, Japan).

Animals
Five-week-old male F344 rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). They were housed in plastic cages (three or two rats/cage) in an environmentally controlled room maintained at a temperature of 22 ± 2°C and a relative humidity of 50 ± 10%, with a 12-h light/dark cycle. Food (MF pellet diet, Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water were available ad libitum throughout the study. Experiments were initiated after a 1 week acclimation period. Body weights and food and water intake were measured weekly during the experimental period.

Experimental design
The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Osaka City University Graduate School of Medicine. The experimental protocol for the rat multiorgan carcinogenicity bioassay used in this study is shown in Fig. 1. A total of 60 male F344 rats at 6 weeks of age were divided randomly into three groups. All rats were treated with the five carcinogens, DEN, MNU, BBN, DMH, and DHPN (DMBDD), as follows: A single intraperitoneal (i.p.) injection of DEN (100 mg/kg body weight [b.w.]) was given at the beginning of the experiment, followed by four i.p. injections of MNU (20 mg/kg b.w.) during weeks 1 and 2 and then four subcutaneous (s.c.) injections of DMH (40 mg/kg b.w.) during weeks 3 and 4. BBN was administered as 0.05% BBN in drinking water during weeks 1 and 2, and DHPN was administered as 0.1% DHPN in drinking water during weeks 3 and 4. One week after the initiation treatments, the animals were administered DPAA in drinking water for 27 weeks: groups 1, 2, and 3 received 0 (as the control), 5, and 20 ppm, respectively. At the end of week 32, all surviving rats were sacrificed by administration of an overdose (50 mg/kg b.w., i.p.) of pentobarbital sodium. Target organs, including the liver, colon, lung, thyroid gland, urinary bladder, and kidneys, were removed and fixed in 10% phosphate-buffered formalin and embedded in paraffin for histopathological examination. Bile duct hyperplasia was assigned a severity grade (mild, moderate, or severe). If bile duct proliferation was present in a limited area and the bile duct proliferation was slight, lesions were designated as mild hyperplasia. If the portal area was extended geographically by bile duct proliferation, the lesions were designated as severe. Intermediates between mild and severe lesions were designated as moderate bile duct hyperplasia. Fig. 2 shows representative pictures of each type of lesion. Liver tissues embedded in paraffin were processed for immunohistochemical analyses of glutathione S-transferase placenta form (GST-P)-positive foci, which are well-established preneoplastic liver lesions in rats8, and the cell proliferation marker Ki-67. Colons were excised and intraluminally injected with 0.9% saline, cut longitudinally, washed with saline, extended between two filter papers, and then fixed in 10% phosphate-buffered formalin.

Immunohistochemical analysis
Liver tissues embedded in paraffin were examined for GST-P-positive foci and Ki-67 by immunohistochemical staining using the avidin-biotin-peroxidase complex (ABC)
Ki-67 immunostaining was performed in the livers of 10 rats randomly chosen from each group, including livers with cholangiomas. Briefly, paraffin sections were deparaffinized in xylene and then hydrated in graded concentrations of ethanol for 5 min each. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase in distilled water for 5 min. After blocking nonspecific binding with serum for 15 min, sections were incubated overnight at 4°C with rabbit anti-rat GST-P polyclonal antibody (Medical & Biological Laboratories Co., Ltd., Aichi, Japan) diluted 1:1,000 or rabbit anti-Ki-67 polyclonal antibody (Epitomics, Inc., Burlingame, CA, USA) diluted 1:500. Immunoreactivity was detected using a VECSTAIN Elite ABC Kit (Rabbit IgG; Vector Laboratories, Inc., Burlingame, CA, USA).

Quantitative analysis of GST-P-positive foci in the liver

Numbers and areas of GST-P-positive foci larger than 0.2 mm in diameter and the total areas of liver sections examined were measured blindly using a color image processor (IPAP-WIN, Sumika Technoservice Corporation, Hyogo, Japan), and the number and area of GST-P-positive foci per square centimeter of liver tissue were calculated.

Statistical analysis

All mean values are reported as mean ± SD values. Statistical analyses were performed using the StatLight program (Yukms Co., Ltd., Tokyo, Japan) and IBM SPSS Sta-
DPAA Promotes Rat Hepatobiliary Carcinogenesis

Table 2. Histopathological Findings

| Site and type of lesion | Incidence (%) | Multiplicity (No./rat) |
|------------------------|---------------|------------------------|
|                        | 0  | 5  | 20 | 0  | 5  | 20 | 0  | 5  | 20 |
| Liver                  |    |    |    |    |    |    |    |    |    |
| Hepatocellular adenoma | 5 (26.3) | 3 (15.0) | 2 (11.8) | 0.3 ± 0.5 | 0.2 ± 0.4 | 0.1 ± 0.3 |
| Hepatocellular carcinoma | 1 (5.3) | 0 (0) | 2 (11.8) | 0.1 ± 0.2 | 0 | 0.1 ± 0.3 |
| Total hepatocellular tumors | 5 (26.3) | 3 (15.0) | 4 (23.5) | 0.3 ± 0.6 | 0.2 ± 0.4 | 0.2 ± 0.4 |
| Bile duct hyperplasia mild | 19 (100) | 19 (95.0) | 0 (0)* | 0 (0) | 0 (0) | 12 (70.6)* |
| moderate               | 0 (0) | 1 (5.0) | 5 (29.4)* | 0 | 0 | 0 |
| severe                 | 0 (0) | 0 (0) | 12 (70.6)* | 0 | 0 | 0.4 ± 0.8* |
| Cholangioma            | 0 (0) | 0 (0) | 5 (29.4)* | 0 | 0 | 0.4 ± 0.8* |
| Hemangiosarcoma        | 0 (0) | 0 (0) | 1 (5.9) | 0 | 0 | 0.1 ± 0.2 |
| Colon                  |    |    |    |    |    |    |    |    |    |
| Adenoma                | 7 (36.8) | 11 (55.0) | 8 (47.1) | 0.7 ± 1.1 | 0.9 ± 1.1 | 1.0 ± 1.4 |
| Adenocarcinoma         | 5 (26.3) | 7 (35.0) | 1 (5.9) | 0.4 ± 0.7 | 0.6 ± 0.9 | 0.1 ± 0.5 |
| Total tumors           | 10 (52.6) | 13 (65.0) | 8 (47.1) | 1.1 ± 1.4 | 1.5 ± 1.4 | 1.1 ± 1.6 |
| Lung                   |    |    |    |    |    |    |    |    |    |
| Adenoma                | 19 (100.0) | 19 (95.0) | 17 (100) | 3.7 ± 2.7 | 4.5 ± 2.3 | 3.8 ± 2.4 |
| Adenocarcinoma         | 8 (42.1) | 4 (20.0) | 9 (52.9) | 1.5 ± 0.6 | 0.3 ± 0.7 | 1.0 ± 1.1 |
| Total tumors           | 19 (100.0) | 19 (95.0) | 17 (100) | 4.2 ± 2.8 | 4.5 ± 2.8 | 4.8 ± 2.9 |
| Thyroid gland b        |    |    |    |    |    |    |    |    |    |
| Follicular cell hyperplasia | 15 (78.9) | 12 (63.2) | 8 (47.1) | 1.6 ± 1.5 | 1.4 ± 1.3 | 0.5 ± 0.6* |
| Follicular cell adenoma | 5 (26.3) | 5 (26.3) | 2 (11.8) | 0.3 ± 0.6 | 0.3 ± 0.6 | 0.2 ± 0.5 |
| Follicular cell carcinoma | 6 (31.6) | 5 (26.3) | 6 (35.3) | 0.4 ± 0.6 | 0.4 ± 0.7 | 0.4 ± 0.5 |
| Total tumors c         | 10 (52.6) | 10 (52.6) | 8 (47.1) | 0.7 ± 0.7 | 0.7 ± 0.7 | 0.5 ± 0.6 |
| Urinary bladder        |    |    |    |    |    |    |    |    |    |
| PN hyperplasia         | 5 (26.3) | 9 (45.0) | 5 (29.4) | 0.4 ± 0.7 | 0.7 ± 0.9 | 0.4 ± 0.6 |
| Papilloma              | 5 (26.3) | 5 (25.0) | 3 (17.6) | 0.4 ± 0.7 | 0.4 ± 0.8 | 0.2 ± 0.4 |
| Urothelial carcinoma   | 2 (10.5) | 1 (5.0) | 2 (11.8) | 0.1 ± 0.3 | 0.1 ± 0.2 | 0.2 ± 0.5 |
| Total tumors d         | 6 (31.6) | 6 (30.0) | 5 (29.4) | 0.5 ± 0.8 | 0.5 ± 0.8 | 0.4 ± 0.6 |
| Kidney                 |    |    |    |    |    |    |    |    |    |
| Renal cell carcinoma   | 4 (21.1) | 4 (20.0) | 1 (5.9) | 0.2 ± 0.4 | 0.3 ± 0.6 | 0.1 ± 0.2 |
| Nephroblastoma         | 14 (73.7) | 17 (85.0) | 17 (100)* | 1.7 ± 1.4 | 1.5 ± 0.9 | 2.3 ± 1.5 |
| Renal pelvic carcinoma (urothelial carcinoma) | 1 (5.3) | 3 (15.0) | 1 (5.9) | 0.1 ± 0.2 | 0.2 ± 0.4 | 0.1 ± 0.2 |
| Hemangiosarcoma        | 0 (0) | 1 (5.0) | 0 (0) | 0 | 0 | 0.1 ± 0.2 |

* p<0.05, vs. the 0 ppm DPAA group (Group 1), PN, papillary or nodular; † number of rats surviving to week 9 and used for tissue analysis, other than the thyroid gland; ‡ effective number of rats for thyroid gland analysis are 19, 19 and 17 in the 0, 5 and 20 ppm DPAA groups, respectively (see text for explanation); § follicular cell adenoma + follicular cell carcinoma; ¶ papilloma + urothelial carcinoma.

Results

General observations

The number of rats surviving to the end of the study, final body weights, and absolute and relative liver and kidney weights are summarized in Table 1. Four animals died during weeks 7 and 8, possibly due to DMBDD treatment-related reasons, and were therefore not included in the analyses. Therefore, the numbers of rats surviving to week 9 (i.e., the effective number of rats) were 19, 20, and 17 in the 0, 5, and 20 ppm DPAA groups, respectively. However, the effective numbers of rats for examination of the thyroid gland were 19, 19, and 17 in the 0, 5, and 20 ppm DPAA groups, respectively, due to sampling error. The numbers of rats that survived until scheduled sacrifice were 16, 17, and 16 in the 0, 5, and 20 ppm DPAA groups, respectively, and there were no significant differences in survival rates among the groups. Final body weights did not significantly differ among the groups. Absolute and relative liver weights and relative kidney weights of the 20 ppm DPAA group were significantly higher than those of the 0 ppm DPAA group. These factors may be associated with the severity of bile duct hyperplasia and the high incidence of cholangioma and nephroblastoma, as described below.

Histopathological findings

Table 2 summarizes the data regarding the incidence and multiplicity of preneoplastic and neoplastic lesions in the liver, colon, lung, thyroid gland, urinary bladder, and kidney. Bile duct hyperplasia (mild, moderate, severe) was seen in all of the examined rats. The incidences of moderate and severe bile duct hyperplasia were significantly increased in the 20 ppm DPAA group (29.4%, 70.6%, respectively) compared with the 0 ppm DPAA group (0%, 0%, respectively).
There was no cholangiofibrosis, cholangiofibroma, or oval cell hyperplasia. The incidence and multiplicity of cholangioma (Fig. 3) were significantly increased in the 20 ppm DPAA group (29.4%, 0.4 ± 0.8/rat) compared with the 0 ppm DPAA group (0%, 0/rat). There were no significant differences in the incidences or multiplicities of hepatocellular adenoma, hepatocellular carcinoma, or total hepatocellular tumors (adenoma + carcinoma) among the groups.

The multiplicity of follicular cell hyperplasia of the thyroid gland was significantly decreased in the 20 ppm DPAA group (0.5 ± 0.6/rat) compared with the 0 ppm DPAA group (1.6 ± 1.5/rat). There were no significant differences in the incidences of follicular cell hyperplasia, adenoma, carcinoma, or total tumors of the thyroid gland.

The incidence of nephroblastoma was significantly increased in the 20 ppm DPAA group (100%) compared with the 0 ppm DPAA group (73.7%). There were no significant differences in the multiplicities of nephroblastoma, renal cell carcinoma, renal pelvic carcinoma (urothelial carcinoma), or hemangiosarcoma.

**GST-P-positive foci formation in livers**

The numbers and areas of GST-P-positive foci, preneoplastic lesions in rat livers, in DMBDD-treated rats are shown in Fig. 4. Both total number and average area of GST-P-positive foci were significantly increased in the 20 ppm DPAA group compared with the 0 ppm DPAA group. * Significantly different from the 0 ppm DPAA group at p<0.0001.
Quantification of cell proliferation by immunohistochemistry of Ki-67

Fig. 5 shows the Ki-67 data obtained from mild, moderate, and severe bile duct hyperplasia and cholangioma in the livers. The Ki-67 index in moderate and severe bile duct hyperplasias and cholangiomias in the 20 ppm DPAA group was significantly increased compared with that in mild bile duct hyperplasia in livers of rats from the 0 ppm DPAA group. * Significantly different from mild bile duct hyperplasia at p<0.05.

Discussion

The present study demonstrated that DPAA exerts promotion effects on hepatocellular and biliary carcinogenesis in rats, as evidenced by the findings showing that 20 ppm DPAA significantly enhanced the incidence and multiplicity of cholangioma and the development of DMDBD-induced GST-P-positive foci, a well-established preneoplastic liver lesions in rats.

The increase in the number and area of GST-P-positive foci in the DMDBD-treated rat liver agree with our previous findings showing that DPAA promotes liver carcinogenesis in a medium-term rat liver carcinogenicity assay4. GST-P-positive foci are a surrogate end-point marker for predicting liver carcinogenicity in rats and serve as a sensitive maker for liver carcinogenesis in the rat medium-term multiorgan carcinogenicity model9-11. While our findings indicate that DPAA promotes hepatocarcinogenesis in rats, there were no significant changes in the incidences and multiplicities of hepatocellular adenoma or hepatocellular carcinoma in the present study. However, given the increase in preneoplastic lesions in the liver in the 20 ppm DPAA group, it is likely that the incidence and/or multiplicity of hepatocellular tumours would have been different among the 3 groups if the period of the experiment was extended. We are not able to exclude the possibility that factors other than the length of the experiment were associated with the results of the present study.

DPAA activates the aryl hydrocarbon receptor signaling pathway, which promotes oxidative DNA damage and inhibits apoptosis, thereby promoting liver carcinogenesis in rats4. There is little information about biliary carcinogenesis caused by arsenic compounds. The mechanisms of toxicity in the bile duct caused by chemicals remains poorly understood12. In our previous medium-term rat liver carcinogenesis assay, 6 weeks of treatment with 20 ppm DPAA induced mild bile duct hyperplasia regardless of DEN initiation4, and in a more recent study, administration of 20 ppm DPAA alone induced severe bile duct hyperplasia in a 52-week chronic toxicity study in rats, although no bile duct tumors were observed (manuscript in preparation). In the present study, DPAA increased the cell proliferative activity in bile duct hyperplasias and promoted the development of cholangioma. There are some reports indicating that the cytotoxic effects of DPAA are enhanced by the interaction with sulfhydryl compounds, such as glutathione, dimecapropane sulfonate, and dithiothreitol13,14. Taken together, these results suggest that DPAA might be toxic to the bile duct and that increased cholangiocyte proliferation might contribute to the promoting effects of DPAA on biliary carcinogenesis. Further studies are needed to elucidate the mechanism underlying the promoting effects of DPAA on biliary carcinogenesis in rats.

In conclusion, our results demonstrate that DPAA promotes hepatocellular and biliary carcinogenesis in the liver, but not in other organs, in the rat. Our ongoing 2-year rat carcinogenicity study will clarify whether DPAA is a complete carcinogen in the liver.

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