Carcinogenesis vol.24 no.11 pp.1827–1835, 2003
DOI: 10.1093/carcin/bgg143

Liver tumorigenicity of trimethylarsine oxide in male Fischer 344 rats—association with oxidative DNA damage and enhanced cell proliferation

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Arsenic is a notorious environmental toxicant known to be carcinogenic for the skin, lung and urinary bladder in human beings. The carcinogenicity of trimethylarsine oxide (TMAO), one organic metabolite of inorganic arsenics in humans and experimental animals, was investigated here in male Fischer 344 rats in a 2-year carcinogenicity test. TMAO was administered to a total of 129 male rats *ad libitum* at concentrations of 0 (Control), 50 or 200 p.p.m. in the drinking water. In animals that died or were killed from the 87th week until the end of 104th week, incidences of hepatocellular adenomas were 14.3, 23.8 and 35.6% in the 0, 50 and 200 p.p.m.-treated groups, respectively; the multiplicities were 0.21, 0.33 and 0.53. Both were significantly increased in the 200 p.p.m.-treated group. While a variety of other tumors developed in various organs, they were present in all groups, including the controls, and were histologically diagnosed as those known to occur spontaneously in F344 rats. To test the contribution of reactive oxygen species (ROS) to TMAO tumorigenicity in the liver, 8-hydroxydeoxyguanosine (8-OHdG) formation was assessed by high performance liquid chromatography. The 8-OHdG values for the 200 p.p.m. TMAO group were significantly higher than those for the control group. Furthermore, as assessed by the proliferating cell nuclear antigen index, cell proliferation in the normally appearing parenchyma was elevated by the TMAO treatment. These results indicate that TMAO exerts liver tumorigenicity with possible mechanistic roles for oxidative DNA damage and enhanced cell proliferation.

Introduction

Arsenic, an environmental toxicant known as a carcinogen for human beings (1,2), naturally occurs as a metalloid that exists in inorganic as well as organic forms. Inorganic examples include the trivalent meta arsenite [As(III)], and the pentavalent arsenate [As(V)]. Millions of people in many countries are chronically exposed to various levels of arsenic, mainly through ingestion of contaminated water and food and to some extent due to inhalation of arsenic in the ambient air (3). In areas of high arsenic exposure in India, arsenic is found in drinking water at concentrations that can reach over 200–600 μg/l. According to WHO, the safe level of arsenic in water is below 10 μg/l and the maximum permissible accepted limit is 50 μg/l (3). From epidemiological studies, the US National Research Council reported that there is conclusive evidence that ingestion of arsenic may cause bladder, lung and skin cancers. Increased risks of other cancers, such as liver and kidney, in exposed people have also been reported (2).

Methylation of inorganic arsenics is an important step during the process of their elimination in many mammals. Generation of monomethylarsonic acid [MMA(V)], monomethylarsonous acid [MMA(III)], dimethylarsinic acid [DMA(V)], dimethylarsinous acid [DMA(III)] and trimethylarsine oxide (TMAO) is due to methyltransferase enzymes in reactions that require *S*-adenosyl-methionine and trimethylarsine [TMA(III)] is the product of this chemical reduction *in vivo*, the methylated arsenic metabolites then being excreted via the urine (Figure 1) (4). During this process, arsenic is almost quantitatively reduced from arsenate to arsenite in plasma and subsequently methylated to MMA(V), MMA(III) and DMA(V) in the liver (5). The population group or ethnicity, gender, age, dose level, route and form of exposure, pregnancy, nutrition and genetic polymorphisms, all affect methylation of arsenic (6,7). For instance, rats metabolize DMA(V) to TMAO to a larger extent [~30% of the administered DMA(V)] than do mice, hamsters and humans (~3–6%) (8).

Despite extensive research on the toxicity of arsenic, many questions remain unanswered, making risk assessment difficult. For instance, it is still not known how the carcinogenicity of arsenic is mechanistically determined. Moreover, there is increasing debate on whether the metabolic methylation of arsenic should be considered a toxification or detoxification process (9). Animal studies have shown that the liver is a major target organ for arsenic toxicity, being a vital organ for methylation of inorganic arsenite (10). However, an experiment in mice revealed that exposure to DMA(V) was without profound effect on this organ despite earlier induction of tumors with increased multiplicity (11). In experiments on rats, two studies demonstrated urinary bladder carcinogenicity with long-term exposure to DMA(V), when administered in either the diet or drinking water (12–14). DMA(V) can act as a tumor promoter in different animal organs, such as bladder, liver, kidney, thyroid and skin (15,16) and dose dependence has been confirmed for promoting effects on urinary bladder (17) and liver carcinogenesis (18). Yamanaka *et al.* (19–21) found that DMA(V) induced DNA damage in mouse and rat lung cells and increased 8-hydroxydeoxyguanosine (8-OHdG) levels in target organs of carcinogenicity in the mouse, such as liver, lung, skin and urinary bladder. Recently, another long-term

Abbreviations: AsBe, arsenobetaine; DMA(V), dimethylarsinic acid; DMA(III), dimethylarsinous acid; HCC, hepatocellular carcinoma; MMA(V), monomethylarsonic acid; MMA(III), monomethylarsonous acid; 8-OHdG, 8-hydroxydeoxyguanosine; PCNA, proliferating cell nuclear antigen; RBC, red blood cell; ROS, reactive oxygen species; TeMA, tetramethylarsonium; TMAO, trimethylarsine oxide; WBC, white blood cell.

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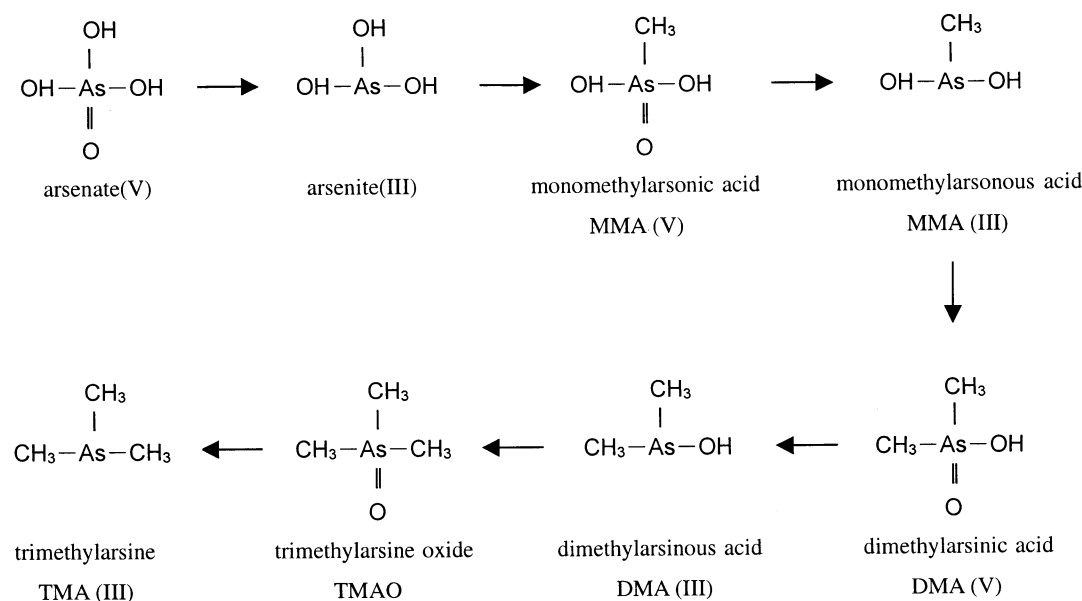

Fig. 1. Metabolic pathway of arsenics in rats. Inorganic arsenate and arsenite are metabolized to organic MMA(V), MMA(III), DMA(V), DMA(III), TMAO and TMA(III).

study showed that MMA(V) has no carcinogenicity, but induced glutathione *S*-transferase placental form (GST-P) foci in the liver and hyperplasia in the urinary bladder in male F344 rats when administered in drinking water (22).

Of particular importance, the findings from long-term studies of DMA(V) and MMA(V) using F344 rats revealed effects at sites where human arsenic-associated cancers developed, such as the urinary bladder and liver. However, a complete bioassay of TMAO, a key metabolite of arsenic in humans, has not yet been performed and there is no evidence for possible carcinogenic effects of TMAO at the experimental level. Toxicological data of TMAO in F344 rats are limited, but we found DMA(V) exerted urinary bladder carcinogenicity (13), and MMA(V) induced pre-neoplastic lesions when administered at 50 and 200 p.p.m. in drinking water (22). Therefore, the present experiment was conducted to examine effects of 50 and 200 p.p.m. of TMAO in F344 rats in a 2-year study. Given the accepted important role of free radicals in chemical carcinogenesis, formation of 8-OHdG, a marker of oxidative damage to DNA commonly generated under conditions of oxidative stress (23), was targeted as an additional oxidative stress parameter. Recently, it has attracted attention as an important biomarker of carcinogenic risk to humans (24). Furthermore, levels of cell proliferation were assessed as another aid to understanding arsenic's mechanisms of actions.

Materials and methods

Test material

TMAO (CAS No. 4964-14-1), was obtained from Tri Chemical Laboratory (Yamanashi, Japan), with a purity of >99.0%, as confirmed by high performance liquid chromatography (HPLC).

Animals and husbandry

Male Fischer 344/DuCrj rats were purchased at 5 weeks of age from Charles River Japan (Hino, Japan), housed three to a plastic cage, and fed standard CE-2 basal pelleted diet (Clea Japan, Tokyo, Japan) and water *ad libitum*. The animals were kept in an air-conditional barrier-system at a temperature of 22 ± 2°C and a humidity of 55 ± 5% with a 12:12 light: dark cycle. Rats were used in this study after a 5-week acclimation period.

Experimental design

129 10-week-old rats were randomly subdivided into three groups, and administered TMAO in the drinking water as follows: group 1, TMAO 0 p.p.m. (control) (42); group 2, TMAO 50 p.p.m. (42); group 3, TMAO 200 p.p.m. (45). Rats were observed daily for any abnormalities. The body weights, water intake and food consumption were measured weekly for the first 12 weeks, then every 4 weeks thereafter to calculate the actual intakes of TMAO.

Urine and blood analysis

Urinary samples were collected by forced urination in the morning at the 30th, 60th and 100th weeks of the experiment. A combined ion chromatography system (IC), as the separation technique (model IC 7000, Yokogawa Analytical Systems, Tokyo, Japan) and ICP-MS (model HP 4500, DE, USA) as an element-selective detector was used to analyze the arsenic species. The levels of TMAO, arsenobetaine (AsBe), tetramethylarsonium (TeMA), MMA(V) and DMA(V) were estimated. Analysis conditions of the ion chromatography with inductively coupled plasma mass spectrometry system were described previously in detail (25).

After 104 weeks at final killing, blood samples were drawn via the abdominal aorta from 11 to 13 rats under ether anesthesia randomly chosen from each group. Small amounts of each sample were utilized for hematology values such as white blood cells (WBC) and red blood cells (RBC). WBC and RBC were determined with an automated hematology analyzer (Sysmex XE-2100, Hyogo, Japan). Serum biochemistry data for total protein (TP), glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), blood urine nitrogen (BUN) were determined by automatic analyzer (Olympus AJ-5200, Tokyo, Japan).

Necropsy and tissue sampling

For all of the surviving rats at 104 weeks, at the final scheduled necropsy, the liver, spleen and kidneys were weighed. After careful gross examination, complete necropsies were performed for all rats after they were killed when moribund or at 104 weeks. The following tissues were collected and kept in 10% phosphate-buffered formalin: brain, cervical spinal cord, pituitary glands, eyes, Harderian glands, ears, Zymbal glands, thyroids and parathyroid glands, heart, aorta, thymus, lymph nodes (cervical, mesenteric and mediastinal), salivary glands (submandibular, parotid and sublingual), sternum (including bone marrow), nasal cavity and oral mucosa, trachea, lungs, esophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, liver, spleen, kidneys, adrenal glands, pancreas, prostate, testes, seminal vesicles, skin and skeletal muscle. During the course of the study, dead animals were also completely autopsied to determine the reason for mortality, and their data were included.

Histopathology

Histopathological examination was performed for all tissues collected at necropsy and autopsy from all groups. After checking the tissues very

carefully, all tumor tissues, as well as the organs mentioned above were routinely processed, sectioned at ~3 μm, and stained with hematoxylin and eosin.

Diagnostic criteria for liver proliferative lesions were based on a review by a working group of Japanese Society of Toxicologic Pathology (26). Histopathological lesions of the liver were classified into: focus of cellular alteration and hepatocellular adenoma.

Measurement of 8-OHdG formation in liver DNA

Liver samples of ~500 mg wet weight were taken at random from eight rats in the 0, 50 and 200 p.p.m. TMAO-treated groups. The DNA was isolated and digested into deoxynucleosides by combined treatment with nuclease P1 (Tamasa Shoyu, Chiba, Japan) and alkaline phosphatase (Sigma, St Louis, MO). The level of 8-OHdG formation in each resulting preparation was determined by HPLC (27,28), expressed as the number of 8-OHdG residues/10^6 total deoxyguanosines.

Immunohistochemical staining of proliferating cell nuclear antigen (PCNA)

PCNA immunohistochemical staining was carried out for the normal appearing liver tissue in 16 rats from each group to evaluate cell proliferation activity using anti-PCNA antibody (PC-10, IgG2a; Dako, USA) with the avidin–biotin complex (ABC) method, as described previously (29). Briefly, liver sections were deparaffinized with xylene, hydrated through a graded ethanol series and incubated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The sections were then incubated with 10% normal horse serum at room temperature for 30 min to block background staining and then with PCNA antibody diluted 1:500 in Tris (hydroxymethyl) aminomethane-buffered saline overnight. The sections were then exposed to biotinylated horse anti-mouse IgG (Vector Labs, Burlingame, CA) for 30 min. Finally, peroxidase activity in the liver cell nuclei was visualized by treatment with 0.02% diaminobenzidine. Nuclei were counterstained with hematoxylin. For each slide, numbers of positive labeled cells were counted under a light microscope and labeling indices were generated as percentages of positive cell numbers (with a minimum of 3000 cells counted per slide).

Statistical analysis

Data for body weights, food and water consumption, organ to body weight ratios, 8-OHdG levels, PCNA indices, urine metabolite levels, hematology and clinical chemistry parameters, were analyzed by one-way analysis of variance (ANOVA), and mean values were compared using the Dunnett's test ($P < 0.05$). Kaplan–Meier analysis was conducted to determine survival curves. Logrank (Mantel–Cox) analysis was used to compare tumor latency. The Fisher's exact probability test was used for comparison between final survival rates, incidences of tumors and foci of alterations in livers in different groups (StatView Software ver.5, Abacus Concepts, CA).

Results

Clinical signs and body weights, consumption of food and TMAO

During the 104-week experimental period, no notable changes were observed as clinical signs resulting from the administration of TMAO. The final body weights of rats did not demonstrate significant inter-group differences (Table I). Daily food consumption and water intake of each group showed no significant differences among the groups, and total TMAO intake was consistent and dose-dependent (Table I).

Organ weights

In 0, 50 and 200 p.p.m. TMAO-treated group, the mean values for relative organ weights (organ weight/100 g of body wt) of liver were 3.07 ± 0.55, 3.13 ± 0.31 and 3.16 ± 0.33, respectively; spleen were 0.38 ± 0.25, 0.57 ± 0.75 and 0.30 ± 0.14, respectively; kidneys were 0.61 ± 0.09, 0.60 ± 0.05 and 0.58 ± 0.05, respectively. No significant differences were found among the groups. In each group, some rats had increased spleen weights or splenomegaly, which mainly resulted from leukemia or malignant lymphomas. However, the incidences did not significantly vary with the treatment.

Survival rates, cause of death and tumor latency

In the 0, 50 and 200 p.p.m.-treated groups, the final survival rates were 69.0, 73.8 and 86.7%, respectively; average survival times were 99, 102 and 103 weeks respectively (Table I). There were no differences in survival curves among the groups. The numbers of unscheduled necropsies and autopsies were 13, 11 and six in the 0, 50 and 200 p.p.m. groups, respectively. Most unscheduled necropsies and autopsies occurred after 80 weeks. In the 0 and 50 p.p.m. TMAO-treated groups, a single rat died from unknown causes at the 92nd and the 77th week, respectively. Generally, the main reason for death was tumor formation in all groups. There was no difference of tumor latency among the 0, 50 and 200 p.p.m. TMAO-treated groups. The lesions were those usually developing in aged male Fischer rats (30). In the 42nd week, the first tumor was found in the 0 p.p.m. TMAO group. From the 42nd week to the 84th week, the incidence increased slowly and thereafter dramatically in all groups, with no significant differences.

Histopathology

The histological types, sites and incidences of primary tumors of each group are summarized in Table II. The first unscheduled necropsy was performed at the 42nd week and a subcutis fibroma was found. All rats that survived beyond the 42-week time point were therefore included in the effective numbers.

Tumors were detected in many organs and tissues in all groups, including the controls. However, the incidence of hepatocellular adenomas in the 200 p.p.m. TMAO-treated group was significantly increased as compared with the control value. In all groups, neoplastic lesions were most frequent in the testis, followed by the hematopoietic organs and thyroid glands. Most testicular tumors were of benign interstitial cell type, the most frequently encountered spontaneous tumors in male F344 rats (30). The total numbers of tumors were 120, 141 and 152 in the 0, 50 and 200 p.p.m.-treated group, respectively. The average tumor multiplicities (number of tumor/rat) were 2.9 ± 1.1, 3.4 ± 1.8 and 3.4 ± 1.2, respectively, with no significant differences in the 0, 50 and 200 p.p.m. groups. Only the incidence and number of liver hepatocellular adenomas in

Table I. Data for average body weights, food and water intakes, total TMAO intakes, final survival rates and average survival times

TMAO treatment (p.p.m.)	Body wt (g)		Food intake (g/rat/day)	Water intake (g/rat/day)	Total intake of TMAO (mg/rat)	Final survival rate (%)	Average survival time (week)
	Initial	Final					
0	230 ± 10[a]	441 ± 45	14.1	17.6	0	69.0	99
50	230 ± 9	437 ± 41	14.3	17.6	638	73.8	102
200	229 ± 8	434 ± 35	13.9	17.1	2,475	86.7	103

[a]Values are mean values ± SD.

Table II. Sites, types, incidences and numbers of primary tumors in F344 rats treated with TMAO

Site	Type of tumors	TMAO treatment (p.p.m.)		
		0	50	200
Effective number of rats		42	42	45
Small intestine	Adenoma	0[a]	0	1 (1)[b]
Colon	Leiomyosarcoma	1 (1)	0	0
Liver	Hepatocellular adenoma	6 (9)	10 (14)	16 (24)*
Lung	Alveolar/bronchiolar adenoma	3 (4)	2 (2)	1 (1)
	Alveolar/bronchiolar carcinoma	0	2 (2)	0
	Adenocarcinoma	0	1 (2)	1 (1)
Pituitary	Adenoma	2 (2)	3 (3)	5 (5)
Thyroid	C-cell adenoma	8 (8)	13 (13)	9 (9)
	Parathyroid adenoma	1 (1)	0	0
	C-cell carcinoma	1 (1)	1 (1)	2 (2)
Adrenal	Cortical adenoma	1 (1)	2 (2)	3 (3)
Spleen	Hemangiosarcoma	0	1 (1)	1 (1)
Kidney	Renal cell carcinoma	1 (1)	1 (1)	0
Haematopoietic organs	Large granular lymphocyte leukaemia	14 (14)	15 (15)	11 (11)
	Malignant lymphoma			
Thymus	Thymoma	1 (1)	0	0
Skin	Squamous cell carcinoma	0	2 (2)	0
	Malignant fibrous histiocytoma	1 (1)	1 (1)	2 (2)
	Basal cell carcinoma	1 (1)	0	0
	Sebaceous adenoma	1 (1)	0	1 (1)
Subcutis	Fibroma	2 (2)	1 (1)	1 (1)
	Hemangioendothelioma	0	1 (1)	0
	Lipoma	3 (3)	0	3 (3)
Testis	Interstitial cell tumor	35 (66)	37 (72)	43 (80)
	Mesothelioma	0	2 (2)	0
Prostate	Adenoma	0	2 (2)	0
Mammary gland	Fibroma	0	0	2 (2)
	Fibroadenoma	3 (3)	2 (2)	1 (1)
Preputial gland	Adenoma	0	1 (1)	3 (3)
Thoracic cavity	Mesothelioma	0	1 (1)	0
Abdominal cavity	Mesothelioma	0	0	1 (1)
Total number of benign tumors		100	112	134
Total number of malignant tumors		20	29	18
Total number of benign and malignant tumors		120	141	152
Total number of rats with benign tumors		40	39	44
Total number of rats with malignant tumors		18	21	14
Total number of rats with tumors		42	40	45
Average tumor/rat		2.9 ± 1.1[c]	3.4 ± 1.8	3.4 ± 1.2

[a]Numbers show the number of rats with tumor.
[b]Parentheses show the number of tumors.
[c]Values are means ± SD.
*$P < 0.05$ compared with the control group.

TMAO 200 p.p.m.-treated group were higher than the control group.

Liver histopathology

Data for liver lesions, including foci of cellular alteration, hepatocellular adenomas and total proliferative lesions are summarized in Table III. The first hepatocellular adenoma was found in a 200 p.p.m.-treated animal at the 87th week. From then on, hepatocellular adenomas were found in each group (Figure 2). The final incidences of adenomas in the 0, 50 and 200 p.p.m. TMAO-treated groups were 14.3, 23.8 and 35.6%, respectively; multiplicity (tumors/rat) were 0.21 ± 0.61, 0.33 ± 0.65 and 0.53 ± 0.84, respectively. The elevated incidence and multiplicity in the 200 p.p.m.-treated group were statistically significant when compared with the control group. No hepatocellular carcinomas (HCC) or hemangiomas were found. Non-neoplastic lesions, such as myocardial fibrosis and bile duct proliferation were observed in each group.

Table III. Incidence and multiplicity of liver tumors, altered foci and total proliferative lesions found in livers of F344 rats treated with TMAO

TMAO treatment (p.p.m.)	0	50	200
Effective number of rats	42	42	45
Adenoma:			
Incidence (%) of tumor-bearing rats	6 (14.3)	10 (23.8)	16 (35.6)*
Multiplicity/tumor-bearing rats	1.50 ± 0.83[a]	1.40 ± 0.52	1.50 ± 0.73
Multiplicity/effective number of rats	0.21 ± 0.61	0.33 ± 0.65	0.53 ± 0.84*
Foci of alteration:			
Incidence (%)	12 (28.6)	14 (33.3)	14 (31.1)
Total proliferative lesions (adenoma + foci)			
Incidence (%)	18 (42.9)	24 (57.1)	30 (66.6)

[a]Values are means ± SD.
*$P < 0.05$ compared with control group.

8-OHdG formation

Data for 8-OHdG levels are shown in Figure 3. Values for the 0, 50 and 200 p.p.m.-treated groups were 0.537 ± 0.043, 0.627 ± 0.173 and 0.654 ± 0.093, respectively, with a statistically significant increase in the 200 p.p.m. group ($P < 0.05$).

PCNA indices

Livers were stained for the analysis of PCNA by an immunohistochemical method (Figure 4). The data for PCNA indices (positive cell numbers/100 cells) are presented in Figure 5, the average values for the TMAO 0, 50 and 200 p.p.m.-treated groups being 3.01 ± 1.16, 5.41 ± 2.35 and 6.11 ± 1.32, respectively. The PCNA index in 200 p.p.m. TMAO-treated group was significantly higher than in the control case.

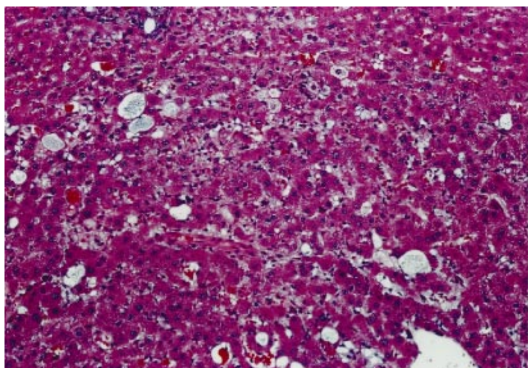

Fig. 2. Representative appearance of hepatocellular adenoma in a 200 p.p.m. TMAO-treated rat (H&E staining, original magnification ×100).

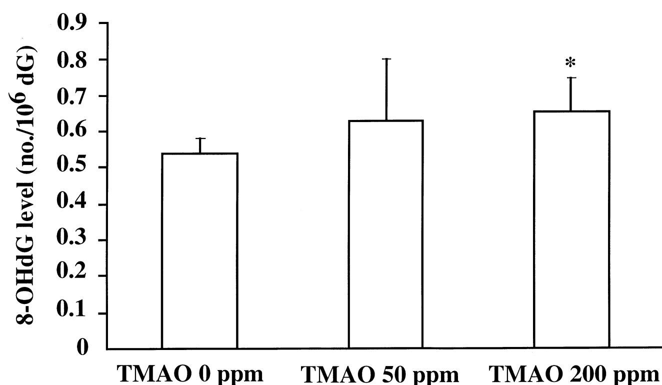

Fig. 3. 8-OHdG levels in the liver of rats treated with TMAO ($^{*}P < 0.05$ compared with 0 p.p.m. TMAO-treated group).

Arsenic metabolites in the urine

As shown in Table IV, the arsenic metabolites found in urine after chronic administration TMAO at the 60th week were TMAO, DMA(V), TeMA and AsBe, as well as one unidentified arsenic peak, M1, which eluted just after MMA(V) (25). The major compound was TMAO itself, accounting for 97.39% and 96.67% of the total arsenic found in the 50 and 200 p.p.m.-treated groups, respectively. Small amounts of TeMA, DMA(V) and AsBe were found, with traces of MMA(V). Metabolite levels of TMAO, TeMA, M1 and total arsenic contents in 200 p.p.m. TMAO-treated group were significantly higher than in the 50 p.p.m. group. Except for AsBe and MMA(V), levels of the metabolites in 50 and 200 p.p.m.-treated groups were significantly higher than in the 0 p.p.m.-treated group.

Hematology and serum biochemistry data

In the 0, 50 and 200 p.p.m. TMAO-treated groups, hematology data for WBC (/ml) were 4358 ± 3982, 5364 ± 3817 and 3596 ± 966, respectively; for RBC ($\times 10^4$/ml) values were 871 ± 96, 778 ± 186 and 899 ± 75, respectively. Serum biochemistry data for TP (g/dl) were 6.7 ± 0.1, 6.6 ± 0.5 and 6.9 ± 0.2, respectively; for GOT (IU/l) were 93 ± 32, 105 ± 29 and 86 ± 24, respectively; GPT (IU/l) were 60 ± 22, 72 ± 26 and 61 ± 18; and for BUN (mg/dl) were 18 ± 4, 19 ± 4 and 18 ± 3. The TMAO-treated groups demonstrated no significant changes in any parameters when compared with the control values.

Discussion

Although a large number of epidemiological studies have provided strong evidence for carcinogenic effects of inorganic arsenic compounds in humans, the mechanisms of carcinogenicity have remained largely unknown. Because arsenics can exert many diverse carcinogenic effects, in both human and animal experiments, more than one mechanism may be involved (8) and defining these mechanisms using animal models is critical to determine the nature and extent of human health hazard presented by environmental arsenic exposure.

The liver is a major target organ of arsenic toxicity and hepatocellular carcinoma, angiosarcoma, cirrhosis and

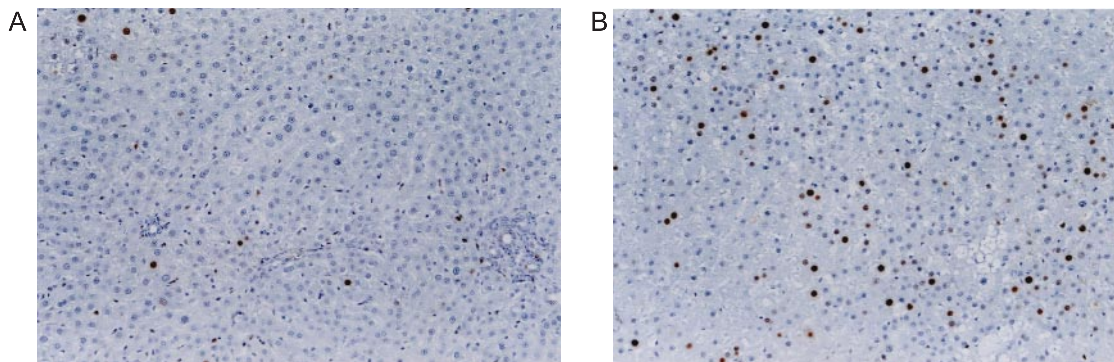

Fig. 4. Immunohistochemical findings. (**A**) A photomicrograph of the liver in a 0 p.p.m. TMAO-treated rat, showing less numbers of PCNA-labeled cells. (**B**) A photomicrograph of the liver in a 200 p.p.m. TMAO-treated rat, showing increased numbers of PCNA-labeled cells (immunohistochemical PCNA staining, original magnificant ×80).

Table IV. Arsenic metabolite levels in urine of F344 rats treated with TMAO at the 60th week

TMAO treatment (p.p.m.)	Urinary metabolites (μg/ml)						
	MMA	DMA	TMAO	TeMA	AsBe	M1	Total arsenic content
0	<0.01	0.16 ± 0.04[a]	<0.01	<0.01	0.17 ± 0.05	0	0.4 ± 0.1
50	0.02 ± 0.02	0.16 ± 0.02	81 ± 17	1.03 ± 0.23	0.13 ± 0.02	0.80 ± 0.16	83 ± 17
200	0.01 ± 0.00	0.18 ± 0.03	326 ± 55	6.36 ± 1.52*	0.11 ± 0.02	4.55 ± 1.88*	338 ± 57*

[a]Values are means ± SD.
*$P < 0.01$ compared with TMAO 50 p.p.m. group.
All of the values (except AsBe and DMA) in TMAO 50 and 200 p.p.m.-treated groups are significantly different to TMAO 0 p.p.m.-treated group at $P < 0.01$. M1 is an unidentified peak.

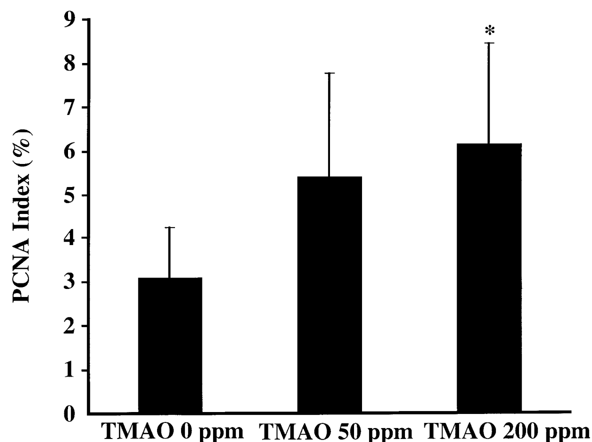

Fig. 5. PCNA index in normal appearing liver tissues of rats treated with TMAO. *$P < 0.05$ compared with 0 p.p.m. TMAO-treated group.

hepatoportal sclerosis have all been associated with arsenic exposure (31). In mice chronically exposed to arsenite or arsenate via drinking water for 48 weeks, liver parenchymal cell degeneration and fatty infiltration are widespread, with inflammation, focal necrosis and early proliferative lesions (32). In another study, Santra *et al.* (33) observed that the liver histology of male BALB/c mice remained normal for the first 9 months after being exposed to 3.2 mg/l arsenic in drinking water, but showed fatty infiltration after 12 months and histologic evidence of fibrosis was observed after 15 months, with increasing oxidative stress in liver, reflected in changes in parameters such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, glutathione, glutathione *S*-transferase, glutathione reductase, glutathione peroxidase and malondialdehyde. Waalkes *et al.* (34) found hepatocellular proliferative lesions (adenoma and foci of cellular alteration) increased by repeated arsenate injections in female Swiss mice in a 96-week study. More recently, they showed that brief exposure of pregnant C3H mice to arsenite in the drinking water results in a marked increase in hepatocellular carcinoma induction in a dose-related fashion in male offspring after they reach adulthood, showing that the liver is a target organ for carcinogenesis (35). Nishikawa *et al.* (36) found 100 p.p.m. TMAO in the drinking water to significantly increase the numbers and areas of the GST-P foci in male F344 rats after *N*-diethylnitrosamine initiation in a medium-term liver bioassay (Ito test). In the latter case this was accompanied by a significant increase of CYP2B1 protein, CYP2B1/2 mRNA and 8-OHdG levels, indicating up-regulation of oxidative promotion pathways in the liver. Santra *et al.* (33) described the fact that arsenic-induced portal fibrosis in mice was consistent with long-term consumption of arsenic-contaminated water patients in West Bengal, India. Lu *et al.* (37) examined liver disease in patients living in arsenic-contaminated areas in Guizhou, China, and observed similarities to the effects of chronic arsenic exposure in mice (34). Our study also found that the liver is a target organ of arsenic tumorigenesis in F344 rats, consistent with Waalkes *et al.*'s recent result that generation of HCC after transplacental arsenic exposure in mice (35).

Among several mechanisms that were recently proposed for arsenic carcinogenecity or promotion such as those well reviewed by Kitchin (8) and Hughes (38), the induction of oxidative stress and cell proliferation recently have been considered as two major parts. Oxidative stress arises when ROS are generated that can react with cellular constituents such as thiols and lipids (38). ROS, damaging almost all classes of subcellular components, are produced in numerous pathophysiological states and have been recognized as participating in the development of carcinogenesis and a wide variety of diseases (39). Oxidative damage is thought to underlie several chronic liver diseases induced by arsenics (33). Oxidative stress, which is based on activated molecular species of oxygen, is a complex process that can result in the peroxidative damage of the major cellular components including amino acids, carbohydrates, lipids, proteins and nucleic acids. Damage to membrane lipids and the associated alterations in bulk properties of membranes frequently are, however, considered to be the primary basis for chemical-induced hepatocellular injury and a loss of cell viability (33).

Chemically induced cancer is a multistage process, which can be divided into at least three stages: initiation, promotion and progression (40). The activation of the carcinogen to an electrophilic moiety capable of causing DNA-damage is a necessary step for the initiation stage. Oxidative stress may play a pivotal role in arsenic carcinogenesis, possible by generation of adducts in DNA. 8-OHdG has long been utilized as a DNA oxidative stress marker (8,41). Oxygen radicals attack DNA bases and deoxyribose residues, producing damaged bases and single strand breaks. In addition, oxygen radicals, oxidize lipid and protein molecules, generating intermediates, which can react with DNA and form adducts which can cause DNA strand breakage (42). Formation of 8-OHdG in DNA can generate G:C to T:A transversions, which frequently occur in the *p53* gene with the development of HCC. The 8-OHdG content in the liver is increased with chronic hepatitis, a condition predisposing to HCC (42).

Arsenic appears to induce oxidative stress both *in vitro* and *in vivo* (38). Wanibuchi *et al.* (18) earlier found hepatic 8-OHdG to be increased in a long term rat DMA(V) carcinogenesis study. Elevation was furthermore demonstrated after

8-week exposure of rats to DMA(V), MMA(V) or TMAO (36). Yamanaka *et al*. (21) showed that oral administration of DMA to mice significantly enhanced the amounts of 8-OHdG levels specifically in the target organs (skin, lung, liver and urinary bladder) and also in urine. Elevated 8-OHdG is typical of arsenic-related Bowen's carcinoma (43). *In vitro* treatment of human-hamster hybrid (A_L) cells with sodium arsenite induced a dose-dependent increase in the formation of 8-OHdG (44). In this study we found that 8-OHdG increased significantly in livers of TMAO 200 p.p.m.-treated group, the group, which demonstrated a significant increase in the incidence of hepatocellular adenomas. This finding is in line with the hypothesis that tissue-specific increase in the 8-OHdG level is observed only in the target organs (21,43). In an acute exposure study, Kitchin and Ahmad (45) found that MMA(V) and DMA(V) damaged rat lung DNA *in vivo*.

There may be two explanations for the finding that 8-OHdG was increased only in the 200 p.p.m. TMAO-treated group and then only slightly. First, this was a 2-year study and since the overall efficiency of the antioxidant defence systems decreases during aging, all of the aged male F344 rats had high spontaneous 8-OHdG levels. Accumulation with age of spontaneous mutations has long been considered to play key roles in senescence. Different factors determine 8-OHdG levels at a particular age (39). One study reported aging-dependent increase of 8-OHdG in the liver of male F344 rats from 1 to 24 months (46). Secondly, there are obvious differences between mutations and 8-OHdG, both spontaneously generated in nuclear DNA in terms of the age-dependency. The simplest reason for such differences is the fact that 8-OHdG is pro-mutagenic but requires DNA replication without being repaired to cause mutation (39).

The development of cancer involves in part the uncontrolled proliferation of cells (47). Two common causes of cell proliferation are mitogenic stimulation and cell toxicity and death followed by compensatory regeneration. Errors of replication resulting from unrepaired DNA damage present at the time of DNA replication can result in mutation of the genetic material (8). Cell turnover can result from cytotoxicity and consequent regeneration and/or direct mitogenesis (47).

Arnold *et al*. (48) have shown that DMA(V) treatment of female F344 rats increased cell proliferation in urinary bladder. In intact mice chronically treated with arsenite or arsenate, proliferative pre-neoplastic lesions in the liver were also observed (32,34), and continued cell cycle disruption may well have resulted in hyperproliferation. In mice, PCNA is highly over-expressed in arsenic-induced pre-neoplastic uterine hyperplasias (34). Similarly, in chronic arsenite-transformed rat liver TRL 1215 cells, dramatic increases in c-*myc*, PCNA and cyclin D1 were accompanied by a marked enhancement of cell proliferation (49). Induction of ornithine decarboxylase, an indicator of cellular proliferation, has been observed in rat liver after administration of arsenite and DMA (18,50).

In the present study, 50 p.p.m. TMAO-treated group was not associated with liver tumor development, or increase in 8-OHdG and cell proliferation. Chemical-induced initiated cells may have a potential to progress to neoplasia, but they do not always develop into tumors without enhanced cell proliferation, inhibition of apoptosis and disturbed intercellular communication (26). In this case, we considered that 50 p.p.m. dose level did not provide sufficient growth advantage to initiated cells in line with existence of a threshold.

The implications of this animal model with respect to human arsenic-induced carcinogenesis remain to be elucidated. To begin with, this is an organic arsenic compound, rather than inorganic arsenic, which appears to be the carcinogenic form in humans. Secondly, the dose exerting a tumorigenic effect in rats is significantly higher than that experienced with human exposure, even in areas where arsenic levels in the drinking water are considered to pose a significant risk to human health (2). Also, high concentrations of TMAO were found in the urine of DMA(V)-treated rats, whereas the concentration was only ~0.5% in human urine following ingestion of an arsenosugar and 4% following ingestion of DMA(V) (51,52). Finally, there are also many differences in the rate of clearance of arsenic between rats and mice and humans. In the rat, because of binding of DMA to erythrocytes, the half-time of arsenic is ~60–90 days, much longer than in mice and humans (5). Furthermore, humans are the only species to excrete MMA in significant amounts, with normal urinary excretion profiles ranging from 10 to 20% arsenic, 10–20% MMA and 60–80% DMA; although rats and mice are more efficient at methylating arsenic than humans, the binding of DMA to rat erythrocytes results in a low urinary excretion of DMA (4%) compared with mice (70%) and humans (60–80%) (5).

To our knowledge, this is the first report of liver tumorigenicity for TMAO in F344 rats after 2 years' exposure. Based on the observations of this study and the results from the literature, we conclude the potential modes of action for TMAO with regard to rat liver tumorigenesis to be as follows: first, in the initiation stage, TMAO exerts multiple effects by direct attack of ROS leading to oxidative DNA damage and subsequent mutations. Then in the promotion stage, TMAO acts via increased cell proliferation in the liver. The arsenic compound could thus act as both tumor initiator and promoter, both effects being essential for the process of liver tumorigenesis.

Acknowledgements

The authors deeply appreciate the technical assistance of Kaori Touma and Masayo Imanaka, and the help of Mari Dokoh and Yoko Ohnishi during preparation of this manuscript. The research was supported by grants from the Ministry of Health, Labor and Welfare and the Japan Science and Technology Corporation, including the Project of Core Research for Evolutional Science and Technology (CREST) in Japan.

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Received April 28, 2003; revised July 17, 2003; accepted August 4, 2003