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Oral Exposure of Dimethylarsinic Acid, a Main Metabolite of Inorganic Arsenics, in Mice Leads to an Increase in 8-Oxo-2′-deoxyguanosine Level, Specifically in the Target Organs for Arsenic Carcinogenesis

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We have proposed that oral administration of dimethylarsinic acid (DMA), a metabolite of inorganic arsenics in mammals, rather than inorganic arsenics themselves, promotes lung and skin tumors by way of the metabolic production of free radicals such as dimethylarsenic peroxy radical [$(CH_3)_2AsOO•$]. The purpose of the present study was to examine if dimethylarsenic has the ability to induce oxidative damage. 8-oxo-2′-deoxyguanosine (8-oxodG) was used as a biomarker of DNA oxidation. The oral administration of DMA enhanced significantly the amounts of 8-oxodG specifically in the target organs (skin, lung, liver, and urinary bladder) of arsenic carcinogenesis and also in urine, whereas arsenite did not. The dimethylarsenics thus may play an important role in arsenic carcinogenesis through the induction of oxidative damage, particularly of base oxidation. © 2001 Academic Press

Key Words: **dimethylarsinic acid; free radical; 8-oxo-2′-deoxyguanosine (8-oxodG); arsenic; carcinogenesis.**

Inorganic arsenics such as arsenite and arsenate have been shown epidemiologically to be carcinogenic; nevertheless, experimental animal studies have not conclusively proven their carcinogenicity (1). Recent studies have reported the ability of dimethylarsinic acid (DMA), a main metabolite of inorganic arsenics, to promote tumorigenesis in an experimental model for two stage carcinogenesis using rodents: The oral administration of DMA to mice induced the promotion of tumorigenesis in lung and skin (2–5) and even in liver, kidney, thyroid, and urinary bladder (6–8).

In the course of elucidating the tumorigenic mechanism of dimethylarsenics, we have indicated that DMA in mice is further metabolized to volatile dimethylarsine ($(CH_3)_2AsH$) which is excreted into their expired air (9). Moreover, dimethylarsine promptly reacts with molecular oxygen to be converted into the dimethylarsine radical ($(CH_3)_2As•$) and then the dimethylarsenic peroxy radical ($(CH_3)_2AsOO•$) (10). These radical species may be responsible for promoting multi-organ tumorigenesis in rodents.

Oxidative stress is known to participate in the carcinogenic process and aging (11). The formation of 8-oxo-2′-deoxyguanosine (8-oxodG), a biomarker for oxidative nucleic-base damage, causes mutation based on base-pair substitution during DNA synthesis (12). Furthermore, 8-oxodG formation may take part in any of the tumorigenic processes of initiation (11, 12), promotion and progression (13–15). Recent studies have reported that oral administration of DMA to rats induced the formation of 8-oxodG in liver (16) and in kidney (17). Another study observed an increase in 8-oxodG levels in arsenic-related Bowen's carcinoma but not in an arsenic-unrelated one (18). There is also an interesting report showing *in vitro* toxicity due to dimethylated trivalent arsenics via the production of activated oxygen (19). These findings support the hypothesis that dimethylarsenics play a role in arsenic carcinogenesis via the production of free radicals.

To determine if oral administration of DMA induces oxidative damage in mice, in the present study we examined the formation of 8-oxodG in various tissues after oral administration of DMA to mice. Marked for-

Abbreviations used: DMA, dimethylarsinic acid; 8-oxodG, 8-oxo-2′-deoxyguanosine; ROS, reactive oxygen species; TBA-RS, 2-thiobarbituric acid-reactive substances; DMA(III), dimethylated trivalent arsenic; ODC, ornithine decarboxylase.

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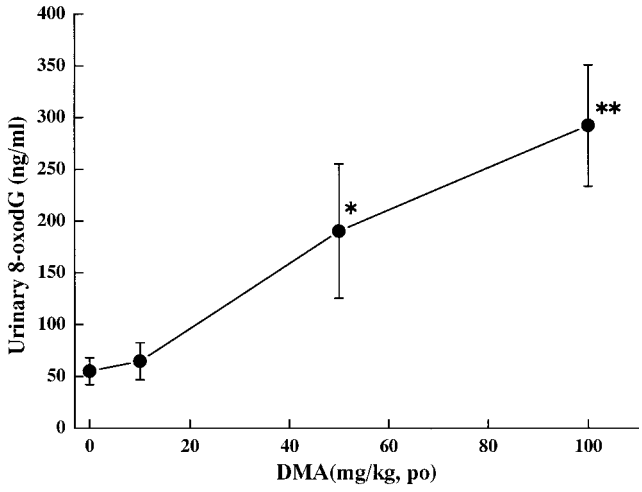

FIG. 1. Urinary 8-oxodG levels in mice 9 h after oral administration of DMA. The male ddY-strain mice were orally administered DMA. Each point represents the mean ± SE (n = 3–5). Significant difference (*P < 0.05, **P < 0.001) from the control (0 h). The statistical analysis was performed using Fisher's PLSD-test.

mation of 8-oxodG was observed in skin, lung, liver, and urinary bladder, i.e., the target organs for arsenic carcinogenesis. A mechanism for 8-oxodG formation by dimethylarsenics is also discussed.

MATERIALS AND METHODS

Animals and chemicals. Six-week-old male ddY and female Hos:HR-1 hairless mice were purchased from Sankyo Laboservice Co., Inc. (Tokyo, Japan). Five mice were housed in a polycarbonate cage and maintained under specific pathogen-free conditions with a 12-h light/dark cycle at 23°C ± 1°C and 55% relative humidity. DMA and arsenic trioxide were obtained from Nacalai Tesque (Kyoto, Japan) and Merck (Darmastadt, Germany), respectively. DMA was purified twice by recrystallization from methanol before use. DMA and arsenic trioxide (arsenite) solutions were prepared by dissolving in sterilized water and in 0.04 M NaOH, respectively. The mice were administered DMA (10–720 mg/kg) or arsenite (15.2 mg/kg) by intragastric gavage or 400 ppm DMA as drinking water *ad libitum*.

Sample preparation from organs. The mice were humanely killed by nembutal anesthesia and then each organ was isolated. Dorsal skin epidermal samples were prepared by removing the dorsal skin by heating at 55°C for 20 s according to a method described previously (20). The tissues and organs (ca. 500 mg) were homogenized with 0.3 M sucrose (5 ml) and centrifuged at 3,000 rpm for 20 min. The crude nuclear pellets were suspended in 1% SDS/1 mM EDTA (pH 8.0) and 2.5 mg proteinase K (Merck, Darmstadt, Germany) added. The mixture was incubated under anaerobic conditions for 1 h at 37°C. The DNA was withdrawn by two sodium iodide/ethanol-extractions. After washing with 70% ethanol, the DNA was suspended in 0.01 × SSC buffer and then treated with RNase T1 (50 units, Sigma-Aldrich, St. Louis, MO) and RNase A (100 μg, Sigma-Aldrich) for 30 min at 37°C. The DNA was extracted and washed with 70% ethanol once again. The DNA was dissolved in 0.5 ml of Chelex 100 Resin (Bio-Rad, Hercules, CA)-treated water. Five milliliters of 200 mM sodium acetate buffer (pH 4.8) and 5 μl of nuclease P1 solution (1 mg/ml of 20 mM sodium acetate buffer) were added to the DNA (50/45 μl water) and then the DNA was digested at 37°C for 1 h under anaerobic conditions. The digested mixture was further digested by adding 0.65 units of alkaline phosphatase type III (Sigma-Aldrich) and 5 μl of 1 M Tris–HCl (pH 7.4). Urine was withdrawn by a syringe through the urinary bladder, put into microtubes (1.5 ml), and then centrifuged at 15,000 rpm for 5 min. The clear supernatant was used as the sample for measuring 8-oxodG by ELISA.

HPLC analysis of 8-oxodG. The analysis was performed in accordance with a method described in a previous paper (21). The HPLC conditions were as follows: column, Pegasil ODS (particle size, 5 μm; 4.6 × 150 mm, Senshu Scientific Co., Tokyo); ECD detector, ESA Coulechem II 5200 (Bedford, MA); HPLC system, Shimadzu LC-10 with UV detector (Tokyo); elutant, 0.6% methanol (pH 5.1) containing 12.5 mM citrate-30 mM sodium hydroxide-25 mM sodium acetate-10 mM acetic acid; flow rate, 1.4 ml/min; ECD-accelerated voltage, 350 mV.

ELISA analysis of 8-oxodG. The amounts of 8-oxodG in the urine samples were determined using a competitive ELISA method with an "8-oxodG Check" (Japan Institute for the Control of Aging, Shizuoka) ELISA kit.

RESULTS AND DISCUSSION

In a previous study we reported an increase in urinary 8-oxodG levels in mice orally administered DMA at a dose of 720 mg/kg body weight (5). Therefore, in the present study, we determined the amount of urinary 8-oxodG at oral doses less than 720 mg/kg. Figure 1 shows that the level of urinary 8-oxodG had increased in a dose-dependent manner 9 h after DMA administration, at least for the dose range of 10–100 mg/kg. On the other hand, one of our earlier reports found that dimethylarsenics but not inorganic arsenics caused DNA damage (22). In the present study, we attempted to clarify whether inorganic arsenics or dimethylarsenics had a greater ability to form 8-oxodG. When the same dose of As (11.5 mg/kg) as arsenite or DMA was orally administered to ddY-strain mice, the amount of urinary 8-oxodG of DMA-treated mice was significantly higher than that of arsenite-treated mice (Table 1). This result supports our previous finding (22) that dimethylarsenics are more genotoxic than inorganic arsenics, and also suggests that dimethylarsen-

TABLE 1

Urinary 8-oxodG Levels in Mice after Oral Exposure to Arsenite or DMA

8-oxodG ng/ml of urine		
Control	Arsenite (15.2 mg (11.5 mg As)/kg)	DMA (21.2 mg (11.5 mg As)/kg)
41.6 ± 7.44	52.3 ± 13.6	69.9 ± 6.11*

Note. The male ddY-strain mice were orally administered arsenite or DMA at a dose of 11.5 mg/kg as arsenic. The urine from each mouse was withdrawn by a syringe through the urinary bladder at 9 h after administration of the arsenics. Each value indicates the mean ± SE (n = 4). Significant difference (*P < 0.05) from the control. The statistical analysis was performed using Student's t-test.

TABLE 2

8-oxodG Levels in Tissues of Mice after Oral Administration of DMA

	8-oxodG/10^5dG	
	Control	400 ppm DMA
(a)		
Lung	1.23 ± 0.26	1.79 ± 0.47*
Liver	1.17 ± 0.25	2.22 ± 0.95*
Spleen	1.21 ± 0.21	1.43 ± 0.46
Kidney	1.56 ± 0.60	1.66 ± 0.49
Urinary bladder	1.24 ± 0.43	2.30 ± 1.32
(b)		
Epidermis of dorsal skin	1.20 ± 0.16	1.55 ± 0.24

(a) The ddY-strain male mice were given drinking water containing 400 ppm DMA *ad libitum* for 4 weeks. Each value indicates the mean ± SD ($n = 5$). Significant difference ($^*P < 0.05$) from control. The statistical analysis was performed using Student's *t*-test.

(b) The female hairless mice were given drinking water containing 400 ppm DMA for 2 weeks. The amounts of 8-oxodG in epidermis of mouse-dorsal skin were determined. Each value indicates the mean ± SD ($n = 5$). Significant difference ($^*P < 0.05$) from the control. The statistical analysis was performed using Student's *t*-test.

ics rather than inorganic arsenics cause the induction of oxidative stress. This also suggests that methylation of inorganic arsenic may be a toxification, rather than a detoxification, pathway from the standpoint of animal carcinogenic systems, as proposed in recent advances in arsenic metabolic research (23, 24).

We examined, using a model of mouse-lung (2) or -skin (5) tumorigenesis developed in our laboratory, whether oral administration of DMA increased 8-oxodG levels in tissues in the tumorigenic process, particularly in the promoting process. Male ddY-strain mice were given drinking water containing 400 ppm DMA, a dose which potently promotes lung tumorigenesis (2), for 4 weeks. As shown in Table 2a, the amount of 8-oxodG was significantly increased not only in lung and liver, but also, though not significantly, in urinary bladder. No increase in the 8-oxodG level was observed in spleen or kidney. On the other hand, a model experiment of mouse-skin tumorigenesis (5), using hairless mice orally administered DMA for 2 weeks, demonstrated a significant increase in the 8-oxodG level in dorsal epidermis (Table 2b). These findings that the tissue-specific increase in the 8-oxodG level was observed only in the target organs, i.e., skin, lung, liver, and urinary bladder, of arsenic carcinogenesis would indicate the participation of oxidative stress even in human arsenic carcinogenesis.

DMA exposure was reported to cause an increase in ornithine decarboxylase (ODC) activity, a biomarker for cell proliferation, in rat liver (16) and kidney (6). Reactive oxygen species (ROS), on the other hand, are also thought to promote the progression of cancer in an animal experimental model of multi-step carcinogenesis (25). Actually, benzoyl peroxide, a well-characterized promoter of skin tumorigenesis, is known to increase ODC activity and to form ROS such as benzoyloxyl radical (26). Based on these facts, the promotion activity of DMA exposure may be caused by way of the production of free radical species.

Next, we attempted to measure the amount of 8-oxodG after DMA administration in ddY-strain mice. When DMA at an acute dose of 720 mg/kg was orally administered, significant increases in 8-oxodG levels were observed in lung, liver, and urinary bladder (Fig. 2), similar to the results shown in Table 2. On the other hand, the data concerning 2-thiobarbituric acid-reactive substance (TBA-RS) values were not completely consistent with that of 8-oxodG; increases in TBA-RS values were observed not only in lung, liver, and urinary bladder, but also in kidney (data not shown). These results suggest that, with regards to oxidative stress induced by arsenics, it may be better to use "8-oxodG formation" as a marker of oxidative stress rather than TBA-RS values.

Recent studies have indicated that oral administration of DMA to rats led to a higher incidence of 8-oxodG formation in liver (16) and kidneys (17). The 8-oxodG level in the kidneys did not increase in a dose-dependent manner. We thought it was important to establish an animal model in order to understand the mechanism of human carcinogenesis by arsenics, and therefore focused our attention on the induction of oxidative damage in skin, lung, liver, and urinary bladder which are target tissues for human arsenic carcinogenesis. The present findings of tissue-specific for-

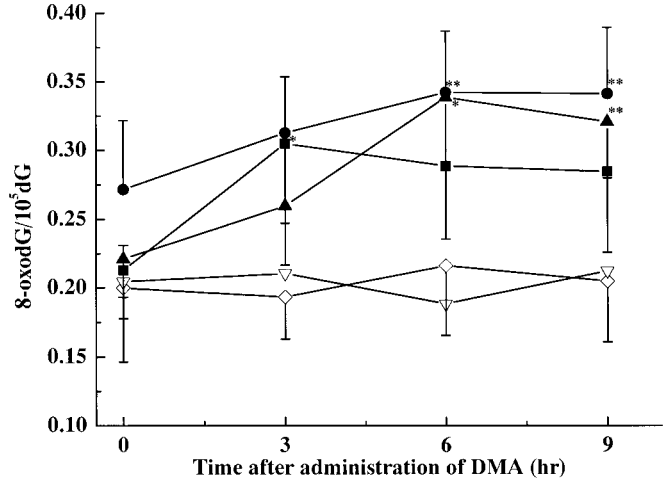

FIG. 2. 8-oxodG levels in various tissues of mice after oral administration of DMA. The male ddY-strain mice were orally administered DMA (720 mg/kg). Each point represents the mean ± SD ($n = 6$–8). Significant difference ($^*P < 0.05$, $^{**}P < 0.01$) from each control (0 h). The statistical analysis was performed using Fisher's PLSD-test. The symbols are: lung (●); liver (▲); urinary bladder (■); kidney (▽); spleen (◇).

mation of 8-oxodG in our model of mouse lung- and skin-tumorigenesis by DMA are useful for understanding the arsenic carcinogenesis mechanism in relation to the formation of free radicals.

It is likely that DMA itself does not induce the oxidative damage but rather its metabolites do. One of our earlier studies has demonstrated that dimethylarsine was produced during the metabolic processing of DMA and excreted into the expired air in mice (9). The arsines then reacted with molecular oxygen to form free radical species such as the dimethylarsenic radical and dimethylarsenic peroxy radical (10). We further assumed that these free radicals led to the induction of lung-specific DNA damage. Among these free radicals, even reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radical might also be produced. We believe that the dimethylarsenic peroxy radical may be responsible for the formation of 8-oxodG.

Some recent reports on arsenic metabolic research suggest that dimethylated trivalent arsenics (DMA (III) dimethylarsinous acid, $(CH_3)_2AsOH$), an extremely unstable metabolite produced in the metabolic methylation of arsenics, have potent biological activity, in particular carcinogenic activity. Among these reports, Ahmad *et al.* (19) reported that DMA (III) was by far the strongest releaser of iron from ferritin, and synergistic iron release by both DMA (III) and ascorbic acid (a well-known iron releaser) from ferritin led to the formation of reactive oxygen species (ROS). These findings suggest that 8-oxodG formation by dimethylarsenics is ascribed to not only the production of dimethylarsenic peroxy radical but also ROS formation by way of release of iron by DMA (III). However, the formation of 8-oxodG by dimethylarsenics may be assigned to the dimethylarsenic peroxy radical, but not the hydroxyl radical, because the 8-oxodG formation was observed in a cell-free *in vitro* experiment in which no ROS such as hydroxyl radical is produced but dimethylarsenic peroxy radical is (data not shown). The formation of 8-oxodG by dimethylarsenic peroxy radical will be reported elsewhere in the near future.

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