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Short Communication

Evaluation of the Modifying Effect of Inhalation of Mainstream Cigarette Smoke on Mouse Bladder Carcinogenesis

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Abstract: Cigarette smoking is one of the major risk factors of bladder cancer in humans. To date, however, there is no experimental evidence for the effects of inhalation exposure to mainstream cigarette smoke on bladder carcinogenesis. The purpose of the present study was to evaluate the effect of inhalation of mainstream cigarette smoke on mouse bladder carcinogenesis using a cigarette smoke inhalation exposure system. Six-week-old male C57BL mice were administered 0.025% N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) in their drinking water for 8 weeks and then divided into 2 groups and exposed to 0 or 300 mg/m^3 wet total particulate matter mainstream cigarette smoke for 2 h per day, five times per week, for 22 weeks. The incidences of bladder tumors (papilloma and urothelial carcinoma) tended to increase in the cigarette smoke-exposed group (25.0%) compared with the controls (15.8%), albeit without a statistically significant difference. We also evaluated mRNA expression levels of cytochrome P450 (cyp) enzymes and proliferating cell nuclear antigen (PCNA) in the bladder epithelium. Expression of cyp1a1 was significantly increased in the cigarette smoke-exposed group. Cigarette smoke exposure did not have a significant effect on the expression of cyp1a2, cyp 1b1, cyp 2a4, cyp 2b10, cyp 2e1, or PCNA. In conclusion, limited exposure to mainstream cigarette smoke for 22 weeks, caused a significant increase in cyp1a1 expression. This increase coupled with the nonsignificant increase in bladder tumors suggests that a longer period of exposure is required to clarify the effects of cigarette smoke on bladder carcinogenesis. (DOI: 10.1293/tox.2013-0039; J Toxicol Pathol 2013; 26: 447–451)

Key words: cigarette smoke, bladder carcinogenesis, inhalation exposure, cytochrome P450

Epidemiological studies have demonstrated that cigarette smoking increases the risk of developing several types of cancer including lung, oral, and urinary bladder cancer[1–4]. Cigarette smoke includes more than 7,000 chemicals, and at least 250 are known to be harmful. Among the 250 known harmful chemicals in tobacco smoke, at least 69, including arsenic, benzene, cadmium, and benzopyrene, can cause cancer[4]. One of the major causative agents of bladder cancer in cigarette smoke is aromatic amine, although the underlying mechanism of aromatic amine-induced bladder carcinogenesis is not fully understood[5]. While epidemiological studies have shown that cigarette smoking causes a 2–4-fold increase in the risk of developing bladder cancer[6], there have been no experimental studies on the effect of inhalation exposure to mainstream cigarette smoke on bladder carcinogenesis. The purpose of the present study was to evaluate the effect of inhalation of cigarette mainstream smoke on bladder carcinogenesis using a cigarette smoke inhalation exposure system.

A total of 96 male C57BL mice, 5-weeks old, were obtained from Charles River Laboratories Japan (Yokohama, Japan). They were housed five per plastic cage with paper chips bedding in an experimental animal room with a 12-h light/dark cycle at a temperature of 23 ± 2°C and a relative humidity of 50 ± 20%. Basal diet and drinking water (CE2, Clea Japan Inc., Tokyo, Japan) were available *ad libitum* except for the time when the mice were exposed to clean air (control) or cigarette smoke. Experiments were initiated after a 1-week acclimatization period. The animals were observed daily, and body weights were measured weekly throughout the duration of the experiment. The animal experimentation protocols were approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School.

The experimental protocol is shown in Fig. 1. Ninety-six mice were divided into 2 groups (control and cigarette smoke-exposed groups). They were given 0.025% N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) in their drinking water for 8 weeks, and after a 1-week washout period without BBN, they were exposed to clean air (control) or mainstream cigarette smoke for 2 h/day (9 to 11 AM), 5 days/week, for 22 weeks. During exposure, each mouse in both groups was placed in an acrylic holder that was attached to the inhalation exposure chamber (SIS, Sibata Scientific

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Technology Ltd., Saitama, Japan) and nose-only exposed to cigarette mainstream smoke or clear air. Cigarette mainstream smoke of 3R4F reference cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA) flowed from a cigarette smoke generator (SG-300, Sibata Scientific Technology Ltd., Saitama, Japan) to the inhalation exposure chamber. Humidity in the chamber was 50%. Cigarette smoke was diluted by air to a final concentration of 300 mg/m^3 wet total particulate matter (WTPM), and the air flow was 24.0 L/h. The concentration of CO was also monitored during study, and the CO/WTPM ratio was set between 1.1 and 1.3 using a Glass Fiber Filter (Advantec, Tokyo, Japan). Six mice were found dead in the cigarette smoke-exposed group during weeks 8 to 9: these mice were not included in this study. Ten mice in each group were euthanized at week 22, and all other surviving mice were scheduled to be euthanized at week 31 (control group, 38 mice; exposed group, 32 mice).

All mice surviving for at least 25 weeks were autopsied for macroscopic and histological examination, including mice that died (3) or were killed under anesthesia when becoming moribund (1) during the study. At autopsy, urinary bladders were inflated with 10% buffered formalin, fixed in 10% formalin solution, and processed for histopathological examination. All other major organs (liver, kidney and spleen) were examined grossly and fixed in formalin. After fixation, they were processed for paraffin embedding and stained with hematoxylin and eosin for histological examination. We randomly chose five bladder tissue samples without bladder tumor or papillary/nodular hyperplasia from each group to determine the Ki-67 labeling index. For immunohistochemistry, sections were autoclaved in 10 mM sodium citrate buffer (pH 6.0) at 98°C for 20 min, and then treated with anti-Ki-67 antibody (Epitomics, Burlingame, CA, USA) at a dilution of 1:500. The number of Ki-67-labeled cells was counted and quantified per 1000 total urothelial cells to determine a labeling index.

Total RNA was extracted from formalin-fixed paraffin-embedded tissue using an miRNeasy FFPE Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Briefly, sections (10 μm thickness) were cut and deparaffinized in xylene and rehydrated in alcohol and distilled water. Needle dissection was performed using formalin-fixed paraffin-embedded bladder tissue without tumor lesions (whole layer including muscle), and the sample was dissolved in 240 μl of protein kinase digestion buffer. RT-PCR followed by real-time PCR was performed as previously described[7]. The assay IDs used for real-time PCR were CYP1a1, Mm00487218_m1; cyp1a2, Mm00487224_m1; CYP1b1, Mm00487229_m1; CYP2a4, Mm00487248_g1; CYP2b10, Mm00456591_m1; CYP2e1, Mm00491127_m1; PCNA, Mm00481100_m1; and β-actin, Mm00607939_s1 (Applied Biosystems). The thermal cycle program was 20 seconds at 95°C followed by 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C. Gene expression data were normalized to β-actin levels. Statistical analysis between the two groups was performed using the Mann-Whitney test.

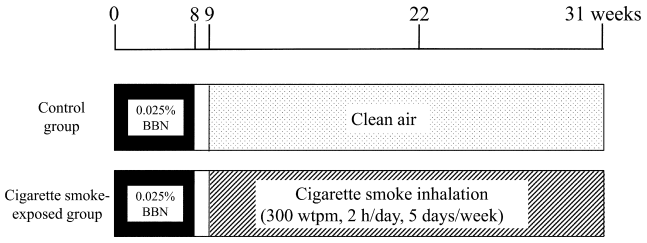

Fig. 1. Experimental design. Mice were divided into 2 groups. All mice were administered 0.025% BBN in drinking water for 8 weeks followed by a 1-week washout period with BBN-free drinking water. Mice in the exposed group were then exposed to mainstream cigarette smoke for 2 h/day, five days per week. Mice in the control group were also restricted and exposed to clean air for the same period. Mice were sacrificed at week 22 (10 mice each) and week 31 (control group, 35 mice; exposed group, 31 mice).

At the week 22 necropsy, no bladder tumors were found except for one papilloma in the exposed group. This result led us to continue the study with the surviving mice for an additional 9 weeks. Table 1 summarizes body and organ weights and the tumor incidence at week 31. Two mice were found dead and 1 mouse became moribund in the control group during weeks 29 to 30; 1 mouse was found dead in the cigarette smoke-exposed group at week 30. These 4 mice were autopsied for histopathological examination and included in this study. There were no cigarette smoke-related mortalities.

In both groups, food consumption was reduced compared with historical controls toward the end of the BBN administration period and through the beginning of the exposure period, and a slight decrease in body weight followed decreased food consumption. At approximately weeks 11–12, after 1 to 2 weeks of being restricted in the acrylic holders attached to the inhalation exposure chamber for 2 h/day, food consumption and body weight began to increase again. Exposure to cigarette smoke resulted in significant decreases in food consumption (Fig. 2C) and body weight (Table 1, Fig. 2A) in the exposed group compared with the control. The body weight loss in the cigarette smoke-exposed group was toxicologically relevant. There was also a significant decrease in relative liver weight in the cigarette smoke-exposed group, but no microscopic changes were observed. Therefore, the decrease in relative liver weight was not a direct effect of cigarette smoke inhalation. There were no significant differences in organ weight or histopathological changes in the kidney or spleen between the groups.

The incidences of bladder tumor (papilloma and urothelial carcinoma) were slightly higher in the cigarette smoke-exposed group, but the increase was not statistically significant (25.0% vs .15.8%, P = 0.3810). Invasive bladder cancers were seen in 5 and 6 mice in the control and exposed groups, respectively. The average tumor sizes were 6.0 and 3.8 mm, and the average tumor numbers were 1.2 and 1.7 per mouse in the control and exposed groups, re-

Table 1. Final Body and Organ Weights and Tumor Incidences in the 31-week Experiment

	Cigarette smoke inhalation	Final number of mice[a]	Body weight (g)	Liver weight		Kidney weight		No. of mice examined[c]	Tumor incidence			
				Absolute (g)	Relative (%)	Absolute (g)	Relative (%)		PN	Papilloma	UC	Total tumor
Control group	-	35	29.9 ± 1.8	1.4 ± 0.1	4.6 ± 0.3	0.4 ± 0.0	1.2 ± 0.07	38	9 (23.7%)	0 (0%)	6 (15.8%)	6 (15.8%)
Exposed group	+	31	27.6 ± 1.1[b]	1.2 ± 0.1[b]	4.3 ± 0.2[b]	0.3 ± 0.0[b]	1.2 ± 0.06	32	8 (25.0%)	1 (3.1%)	7 (21.9%)	8 (25.0%)

[a] Number of mice that survived at week 31. [b] P<0.05 vs. control. [c] Number of mice that survived for at least 25 weeks. PN, papillary or nodular hyperplasia; UC, urothelial carcinoma. PN, papillary or nodular hyperplasia; UC, urothelial carcinoma.

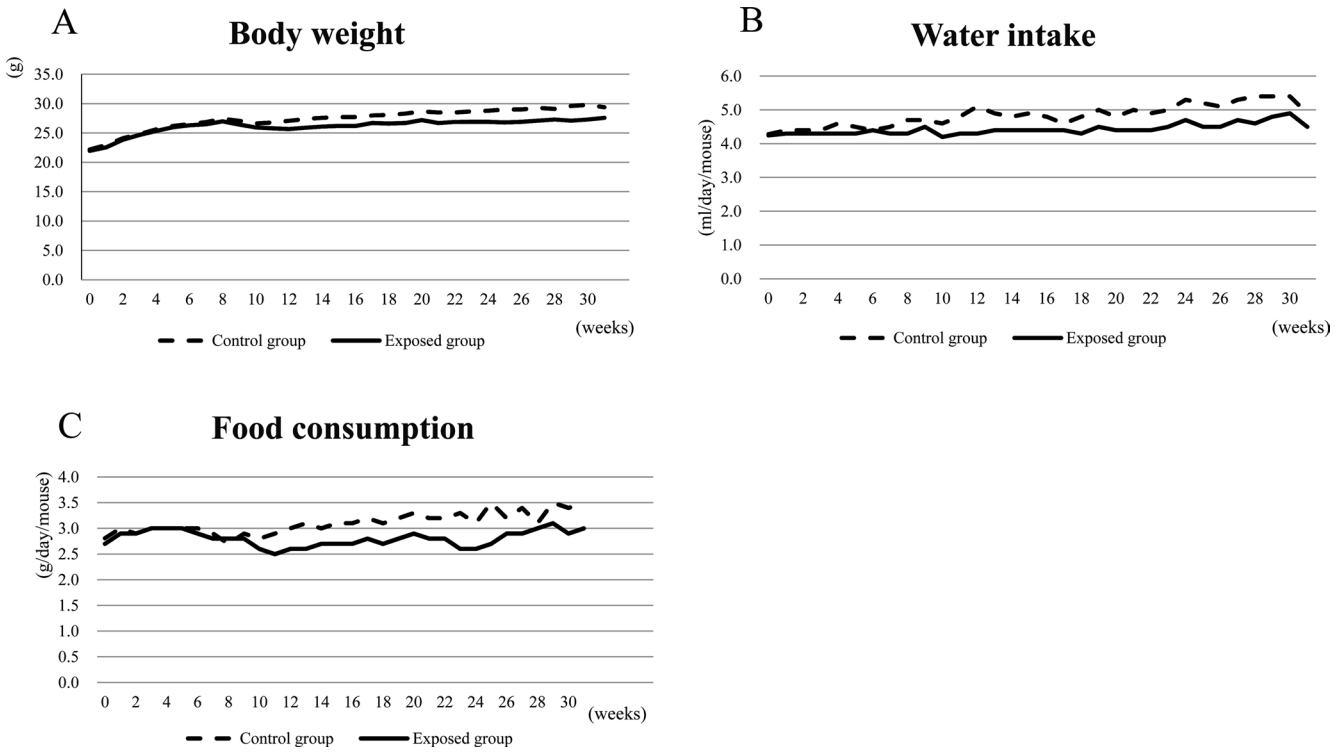

Fig. 2. Body weight, food consumption and water intake in the 31-week experiment. Body weight, food consumption, and water intake of the mice are shown. Mice were administered BBN in their drinking water for 8 weeks, followed by a 1-week washout period and then followed by exposure to clean air or cigarette smoke. Food consumption was reduced toward the end of the BBN administration period and through the beginning of the exposure period. There was a slight decrease in body weight following decreased food consumption. At approximately weeks 11–12, food consumption and body weight began to increase again. Body weight and food consumption of the mice in the exposed group were significantly lower compared with those in the control group (P<0.0001 and P = 0.0002, respectively). Water intake was also lower in the exposed group, but the decrease did not reach statistical significance (P = 0.0703). Statistical analysis was performed using the Mann-Whitney test.

spectively. The Ki-67 labeling indexes were 2.00 ± 0.53 and 2.13 ± 1.05 in the control and exposed groups, respectively. No statistical significance was found in invasive cancer incidences, tumor size, tumor number, or Ki-67 labeling index between the groups.

To evaluate alterations in the mRNA expression levels of cytochrome P450 (CYP) enzymes and PCNA in mouse bladder tissue after exposure to cigarette smoke, RT-PCR followed by real-time PCR was performed using 5 bladder tissue samples without tumor lesions from each group. As shown in Fig. 3, the cyp1a1 expression level was significantly higher in the cigarette smoke-exposed group (P = 0.0286, Mann-Whitney test); no significant differences were found in the expression of the other genes examined.

Ohnishi *et al*. exposed female mice to mainstream and sidestream cigarette smoke for up to 12 months[8]. They reported that cigarette smoke exposure did not result in development of neoplastic or preneoplastic bladder lesions. They did observe an increase in the Ki-67 labeling index of the urothelium at 3 months, but attributed the increased urothelial proliferation to cell cytotoxicity and consequent regenerative proliferation. The increased Ki-67 labeling index

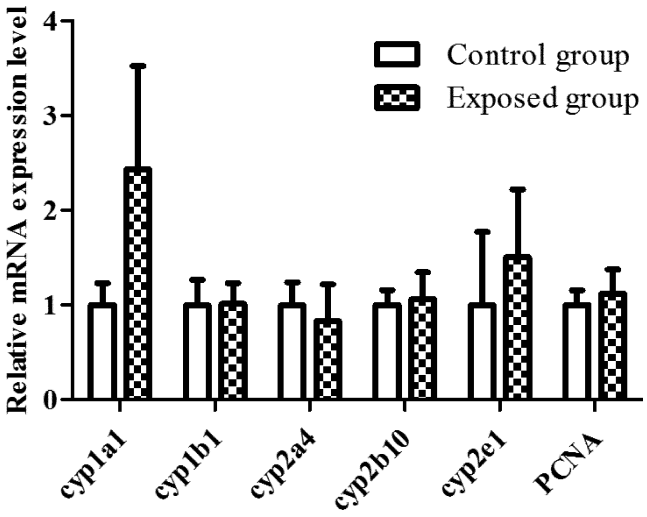

Fig. 3. Gene expression of cytochrome P450 enzymes and PCNA in bladder tissue. mRNA was extracted from bladder tissues without tumor lesions in each group (n = 5) and mRNA expression was evaluated by RT-PCR followed by real-time PCR. Expression of cyp1a2 was not detected in either group. The expression level of cyp1a1 was significantly higher in the exposed group (P = 0.0286). Data are presented as means ± SD, and statistical analysis was performed using the Mann-Whitney test.

was not present at 6, 9, or 12 months. Accordingly, we used an initiation-promotion model in the present study. After initiation of carcinogenesis with BBN followed by 22 weeks of exposure to cigarette smoke, there were no significant increases in bladder tumors compared with the BBN-initiated clean air controls. There was, however, a nonsignificant increase in bladder tumors in the cigarette smoke-exposed group, suggesting that longer exposure to cigarette smoke may enhance bladder carcinogenesis in this model.

The cytochrome P450 family of enzymes plays important roles in the biotransformation of drugs, carcinogens, and environmental toxicants[9–12]. Oxygenation of chemicals by CYP1A1 serves as an initial step in the conversion of the substrates to more polar metabolites, resulting in maintaining chemical homeostasis in the body. CYP1A1 also oxidizes a number of polycyclic aromatic hydrocarbons, some of which are found in cigarette smoke, to carcinogenic intermediates. A notable feature of cyp1a1 is that it is highly inducible at both the mRNA and protein levels by a range of chemicals[13,14]. Because of the critical role of cyp1a1 in chemical carcinogenesis and toxicity, cyp1a1 is a central focus of interest in cancer research, toxicology, and environmental health. Dorrenhaus *et al.* reported that CYP1A1 expression in urothelial cells was higher in smokers than in nonsmokers (44% vs. 6%)[15]. Although the underlying mechanism of CYP1A1 induction by cigarette smoking remains unknown, this study does suggest that human urothelial cells may respond to cigarette smoking by increasing expression of CYP1A1. It is also reported that CYP1A1 levels in liver microsomes were increased by cigarette smoke in NMRI mice and Wistar rats, but not in hamsters[16,17]. In the present study, we found that exposure to cigarette smoke resulted in upregulation of cyp1a1, indicating that exposure did influence this metabolic pathway in the bladder epithelium, and this supports the possibility that longer exposure to cigarette smoke may enhance bladder carcinogenesis in this initiation-promotion model.

We evaluated the effect of cigarette smoke inhalation on bladder carcinogenesis using a novel smoke inhalation experiment system in this study. Only a nonsignificant increase in bladder tumors was seen in the cigarette smoke-exposed group, but exposure resulted in a significant upregulation of cyp1a1. In conclusion, inhalation exposure to mainstream cigarette smoke for 22 weeks caused a significant increase in cyp1a1 expression; however, a longer period of smoke exposure is needed to clarify the carcinogenic effects of cigarette smoke on the mouse bladder.

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