Original

Lack of Enhanced Epithelial Cell Proliferation in the Urinary Bladder of Heterozygous p53 Knockout Mice Given Sodium *Ortho*-phenylphenate or Uracil

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Abstract: In the present study, we investigated epithelial cell proliferation in heterozygous p53 knockout (p53^{+/-}) mice after administration of two urinary bladder non-genotoxic carcinogens, in comparison with that in wild-type littermates (p53^{+/+}). Mice at 10-weeks of age were given 2% sodium *ortho*-phenylphenate (Na-OPP) or 2.5% uracil in the basal diet for 4 weeks. Uracil evoked a marked elevation of epithelial cell proliferation and development of papillary or diffuse epithelial hyperplasia associated with calculus formation in p53^{+/-} as well as p53^{+/+} mice. Administration of Na-OPP caused alkalization of the urine in both. Neither Na-OPP nor uracil induced a higher epithelial cell proliferative response in p53^{+/-} mice as compared with p53^{+/+} mice. While we previously reported p53^{+/-} mice to be highly sensitive to a genotoxic urinary bladder carcinogen, N-butyl-N-(4-hydroxybutyl)nitrosamine, the present results suggest that p53^{+/-} mice may not have a high susceptibility to induction of urinary bladder tumors by non-genotoxic carcinogens. (J Toxicol Pathol 2003; 16: 147–152)

Key words: cell proliferation, sodium *ortho*-phenylphenate, uracil, urinary bladder, heterozygous p53 knockout mice

Introduction

Many experimental systems have been developed for the detection of carcinogenic potential of environmental chemicals, the long-term bioassay using both sexes of two rodent species still being most commonly used¹. The protocol for this long-term bioassay has been standardized and the data generated are widely accepted for evaluation of carcinogenic potential of chemicals. However, the length of time required and magnitude of the cost are major disadvantages. Recently, the International Conference on Harmonization on Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) recommended a change in the approach to assessing carcinogenic potential for pharmaceuticals, with the stress on one long-term bioassay (usually in rats) and additional *in vivo* short or medium-term bioassays^{2–4}.

The p53 tumor suppressor gene has received much

attention because of its propensity for genetic alteration in a wide variety of human neoplasms^{5,6}. Wild-type p53 is a negative regulator of cell proliferation, causing cell cycle arrest in late G1 phase to allow repair when DNA is damaged. Cells lacking wild-type p53 function may be unable to arrest their progression through the cell cycle following DNA damage, resulting in fixation of genetic lesions. For this reason, p53 knockout mice are considered to be attractive for *in vivo* short or medium-term bioassay models⁷. Indeed the available p53 knockout mice are well established to be highly susceptible to spontaneous development of tumors^{8–10}. One focus of attention is whether they also feature elevated sensitivity to chemical carcinogens and reports have already appeared that heterozygous p53 knockout (p53^{+/-}) mice demonstrate enhanced development and/or malignant progression of various tumors in targeted organs, including the skin, mesothelium, brain, urinary bladder, and blood vessels^{11–16}.

Previously, we reported that p53^{+/-} mice are highly sensitive to induction of urinary bladder cell proliferation and tumors by a genotoxic carcinogen¹⁵, in line with previous reports^{17–20}, but the question of whether p53^{+/-} mice are similarly sensitive to non-genotoxic urinary bladder carcinogens remained unclear. In the present study, we

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therefore investigated changes in urinary composition and levels of DNA synthesis and degree of morphological alteration in the urinary bladder epithelium of p53^{+/-} mice following oral administration of two non-genotoxic urinary bladder carcinogens, sodium *ortho*-phenylphenate (Na-OPP) or uracil, and compared the results with the findings for wild-type (p53^{+/+}) mice.

Materials and Methods

Animals and chemicals

Male p53^{+/-} and p53^{+/+} mice were purchased from Taconic (Germantown, NY, USA) and housed in plastic cages (5 mice to a cage) with wood chips for bedding in a room kept at 24 ± 2°C temperature and 40–70% humidity with a 12-hr light-dark cycle (light on, 8:00–20:00). After a one-week acclimatization period, 10-week-old mice were used for the following experiment. Na-OPP was purchased from Tokyo Chemical Industry Co. (Tokyo) and uracil was obtained from Yamasa Shoyu Co. (Chiba).

Experimental design

Totals of 30 p53^{+/-} and p53^{+/+} mice were randomly divided into 3 groups, as shown in Table 1. They were given powdered basal diet (Oriental MF, Oriental Yeast Co., Ltd., Tokyo) containing no supplement (control) or 2% Na-OPP or 2.5% uracil, for 4 weeks. The dose of uracil chosen is known to induce urinary bladder tumors in mice²¹, while 2% Na-OPP has been reported to target the urinary bladder in male rats but not mice^{22,23}. Food and water were available *ad libitum*. During the experiment, body weights were measured weekly, and food consumption and water intake were assessed at week 4, when fresh urine was collected from mice in each group by forced micturition at 8 a.m. for measurement of urinary pH using a pH meter (Twin pH, Horiba, Kyoto).

DNA synthesis and pathological examination

All mice received a single i.p. injection of 5-bromo-2'-deoxyuridine (BrdU, Sigma Chemical Co., St Louis, MO, USA) at a dose of 100 mg/kg body wt, one hour prior to being killed¹⁵. Each urinary bladder was inflated by intraluminal injection of 10% phosphate-buffered formalin solution, and placed in the fixative. After two-hours fixation, they were bisected and one half was cut longitudinally into 2 strips, preserved again in the fixative overnight, then embedded in paraffin. Tissue sections cut at approximately 5 µm were stained for immunohistochemistry using anti-BrdU antibody (DAKO A/S, Copenhagen, Denmark) and a Vectastain ABC kit (Vector laboratories Inc., Burlingame, CA, USA) with 3,3'-diaminobenzidine (Sigma Chemical Co., St Louis, MO, USA). For scoring BrdU labeling indices, 1000 epithelial cells were examined for each group under a light microscope (LM) and the labeling indices expressed as percentage values. Sections were also stained with hematoxylin and eosin for LM examination.

Statistical analysis

Statistical analysis was conducted using the Yukms statistical library I (Yukms Corporation, Tokyo). Body weights and BrdU labeling indices were analyzed using analysis of variance. With regard to parameters for which a significant difference was detected at the 5% level, the least significant difference (LSD) method was used as a test for significant differences from the control values. Urine pH data were analyzed using the Kruskal-Wallis and Mann-Whitney tests.

Results

No deaths occurred during the treatment period. Clinical observation revealed soft feces for both p53^{+/-} and p53^{+/+} mice treated with Na-OPP. Data on final body weights as well as food consumption and water intake are summarized in Table 1. The final body weights of the Na-OPP and uracil treated groups were significantly reduced in both p53^{+/-} and p53^{+/+} mice as compared with those of the matched control values. Food consumption was slightly decreased in both p53^{+/-} and p53^{+/+} mice treated with uracil while water intake was moderately increased. In the case of Na-OPP, food consumption of both p53^{+/-} and p53^{+/+} mice was slightly lower than that of non-treated mice, with no difference between the treated groups.

Urinalysis

Data for urinary pH are shown in Table 2. In both p53^{+/-} and p53^{+/+} mice treatment with Na-OPP resulted in significant elevation as compared to the matched control values. In the uracil-treated case, urinary acidity was significantly increased.

DNA synthesis

Data for BrdU labeling indices are shown in Table 2. Values for both p53^{+/-} and p53^{+/+} mice given Na-OPP were not increased as compared with those of matched controls. While p53^{+/-} and p53^{+/+} mice given uracil showed marked increase in BrdU labeling indices, there was no significant difference between the p53^{+/-} and p53^{+/+} cases (Fig. 1).

Pathological examination

No calculus formation was evident in the urinary bladders of p53^{+/-} and p53^{+/+} mice treated with Na-OPP and microscopic features were comparable with those for controls. Uracil treatment caused calculus formation and microscopic examination revealed papillary or diffuse epithelial hyperplasia of the bladder epithelium in both p53^{+/-} and p53^{+/+} mice.

Discussion

The results of the present study clearly demonstrated a lack of elevated epithelial cell proliferation in the urinary bladder of p53^{+/-} mice as compared with p53^{+/+} mice after administration of two non-genotoxic urinary bladder

Table 1. Data for Final Body Weights, Food Consumption, and Water Intake

Group	Chemical	No. of mice	Final body weight (g) (mean $\pm$ SD)	Food consumption (g/animal/day)	Water intake (ml/animal/day)
p53 ^{+/+}	Control	5	29.9 $\pm$ 1.5	3.6	5.4
	2% Na-OPP	5	26.5 $\pm$ 1.2**	2.3	5.7
	2.5% Uracil	5	25.7 $\pm$ 1.2**	3.3	7.7
p53 ^{+/-}	Control	5	29.8 $\pm$ 1.4	3.5	5.4
	2% Na-OPP	5	27.6 $\pm$ 0.8**	2.9	4.9
	2.5% Uracil	5	28.4 $\pm$ 0.9*, ###	2.7	7.3

Significantly different from the relevant control value at *, $P < 0.05$, **, $P < 0.01$.

Significantly different from the value for p53^{+/+} mice treated with the same chemical at ###, $P < 0.01$.

Table 2. Data for Urinary pH and Urinary Bladder BrdU Labeling Indices

Group	Chemical	No. of mice	Urinary pH (mean $\pm$ SD)	BrdU labeling index (%) (mean $\pm$ SD)
p53 ^{+/+}	Control	5	7.2 $\pm$ 0.2	0.11 $\pm$ 0.13
	2% Na-OPP	5	8.1 $\pm$ 0.2**	0.13 $\pm$ 0.05
	2.5% Uracil	5	6.5 $\pm$ 0.7*	17.37 $\pm$ 2.48**
p53 ^{+/-}	Control	5	7.4 $\pm$ 0.1	0.07 $\pm$ 0.03
	2% Na-OPP	5	8.1 $\pm$ 0.1**	0.08 $\pm$ 0.04
	2.5% Uracil	5	6.5 $\pm$ 0.4**	17.94 $\pm$ 1.73** a)

Significantly different from the relevant control value at *, $P < 0.05$; **, $P < 0.01$.

a): Effective number of mice was four.

carcinogens, Na-OPP and uracil.

After administration of uracil, formation of urinary calculi, as well as papillary or diffuse hyperplasia and cell proliferation of the epithelium were observed in both p53^{+/-} and p53^{+/+} mice. However, with neither parameter did the extent differ between p53^{+/-} and p53^{+/+} mice so that p53^{+/-} mice do not have greater susceptibility to uracil-induction of epithelial cell proliferation in the urinary bladder. This may indicate that mechanical agents, such as uracil-induced calculi, affect the urinary bladder epithelium by a p53 independent pathway. Uracil has been reported to induce urinary bladder tumors in mice due to formation of urinary calculi that mechanically damage the epithelial cells of the urinary bladder, resulting in cell death and compensatory epithelial cell proliferation and tumor development^{21,24}. The present results would suggest that p53^{+/-} mice might not be expected to develop more tumors than their wild-type counterparts on chronic exposure to such mechanical agents.

Na-OPP is reported to be a urinary bladder carcinogen in male rats but not in females or in either sex of mice^{22,23}. For sodium salt-induced urinary bladder carcinogenesis, changes of urinary components, with elevation of the urinary pH value and Na ion concentration, are considered essential factors for activity in male rats²⁵⁻²⁷. In fact, OPP itself does not show any modifying potential for urinary bladder carcinogenesis in rats^{28,29}. It has been reported that sodium L-ascorbate and Na-OPP treated mice show elevation of urinary pH value and Na ion concentration, but no morphological changes or enhanced urinary bladder

carcinogenesis^{30,31}. Regarding species and sex differences a urinary protein, α 2u-globulin, is considered to be an essential factor²⁷. In fact, sodium L-ascorbate caused no induction of epithelial cell proliferation or any promotion effects on urinary bladder carcinogenesis in NCI-Black Reiter (NBR) male rats that lack the capacity for α 2u-globulin synthesis^{32,33}. It is well known that administration of Na-OPP or other sodium salts is associated with enhanced DNA synthesis in the urinary bladder epithelial cells, resulting in epithelial hyperplasia in rats³⁴. Therefore, the present finding that p53^{+/-} mice showed elevation of urinary pH, reflecting the elevation of Na ion concentrations in urine, but no increase in epithelial cell proliferation, supports the existence of species differences in Na-OPP induced urinary bladder carcinogenesis.

OPP is not genotoxic, although its semiquinone and quinone metabolites, phenylhydroquinone (PHQ) and phenylbenzoquinone (PBQ), might form DNA adducts^{35,36}. Furthermore, it has been reported that after injection of PHQ and PBQ into the urinary bladder of rats directly, DNA strand breaks were only detected at high doses of PBQ, which were very unlikely to be reached with oral uptake²⁹. In the present study, production of these metabolites was not measured, but if they were present in the urine, DNA damage could have occurred in the urinary bladder epithelium, since p53^{+/-} mice have high susceptibility to genotoxic urinary bladder carcinogens. The present data indicating no elevation of epithelial cell proliferation as compared with that in the wild-type may thus point to a lack of high levels

Fig. 1. BrdU immunostaining of urinary bladders of mice treated with 2.5 % Uracil. A: $p53^{+/+}$. B: $p53^{+/-}$. Both A and B show marked increase in BrdU-stained epithelial cells.

of semiquinone and quinone metabolites in urine after oral administration of Na-OPP.

Recently, organ specificity has been reported regarding susceptibility to induction of cell proliferation in $p53^{+/-}$ mice, with the p53 gene playing a key role in regulation of cell growth in the urinary bladder and skin, but not the liver, lung, and kidney³⁷. In fact, $p53^{+/-}$ mice exhibit enhanced malignant progression of skin tumors induced by DMBA¹¹ and ultraviolet radiation¹² and development of urinary bladder tumors after treatment with BBN¹⁵ or *p*-cresidine²⁰, but not liver lesions after treatment with genotoxic carcinogens, 2-amino-3-methylimidazo[4,5-*f*]quinoline

(IQ)³⁸, 2-amino-3,8-dimethylimidazo [4,5-*f*] quinoxaline (MeIQx)³⁹, dimethylnitrosamine⁴⁰, 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP)⁴⁰, 6-nitrochrysene⁴⁰ or N-di-N-butyl nitrosamine⁴¹. These results clearly demonstrate organ specific sensitivity in $p53^{+/-}$ mice, even if they are exposed to genotoxic carcinogens by which DNA damage would occur in the target organs. In the nonsensitive target organs, other cell cycle regulation factors may compensate for decrease of p53 function in $p53^{+/-}$ mice. However, the present demonstration of no enhancement of cell proliferation in $p53^{+/-}$ mice after treatment with two non-genotoxic carcinogens, Na-OPP and uracil, was in the

urinary bladder, where p53 onco-suppressor gene does have an essential regulatory role.

The present data indicate that p53^{+/-} mice may not have high sensitivity to non-genotoxic carcinogens. However, one example of the latter, the immunosuppressive cyclosporin A, did enhance tumorigenesis in p53^{+/-} mice¹⁸. We speculate that this may have been due to its effects on lymphoid tissues, which spontaneously give rise to lymphomas in p53^{+/-} mice. Therefore, the question of whether p53^{+/-} mice have high sensitivity to non-genotoxic carcinogens may depend on the target organ, and this now needs to be confirmed by long-term studies with the emphasis on underlying mechanisms.

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