Organ-specific, Carcinogen-induced Increases in Cell Proliferation in p53-deficient Mice

Tokuo Sukata, Keisuke Ozaki, Satoshi Uwagawa, Takaki Seki, Hideki Wanibuchi, Shinji Yamamoto, Yasuyoshi Okuno, and Shoji Fukushima

Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd., [T. S., K. O., S. U., T. S., Y. O.], and First Department of Pathology, Osaka City University Medical School, Osaka 545-8558, Japan [T. S., K. O., S. U., H. W., S. Y., S. F.]

ABSTRACT

Transgenic mice with germ-line p53 alleles disrupted by gene targeting are sensitive to the development of some spontaneous tumors and have provided researchers with much information with respect to cancer. In the present study, to cast light on the organ specificity of chemically induced carcinogenesis, we evaluated carcinogen-induced cell proliferation in target organs in heterozygote p53 knockout mice (p53-deficient mice). Groups of 9- or 10-week-old wild-type (+/+ ) and p53-deficient mice were respectively treated with one of the following carcinogens for 4 weeks: N-butyl-N-(4-hydroxybutyly)nitrosamine (0.0075% in drinking water); dimethylnitrosamine (0.001% in drinking water); dihydroxy-di-N-propynitratosamine (0.1% in drinking water); 1,2-dimethylhydrazine (10 mg/kg body weight s.c. injection once a week); 4-nitroquinoline 1-oxide (4-NQO, 10 mg/kg b.w. s.c. injection once a week); or 7,12-dimethylbenz[a]anthracene (25 μg/kg body weight dermal application once a week). Cell proliferation was evaluated by measuring the 5-bromo-2′-deoxyuridine labeling indices in each target organ. The p53 and p21 statuses were evaluated by comparing the expressions of p53 protein, p21<sup>waf1/cip1</sup> mRNA, and p21<sup>waf1/cip1</sup> protein between the mice. 5-Bromo-2′-deoxyuridine labeling indices were detected with levels obviously lower in the p53-deficient animals. The data suggest that p53-deficient mice have an organ-specific increased sensitivity to the induction of cell proliferation in the urinary bladder and the skin. These are the same organs for which sensitivity to carcinogenesis has been reported. Because a decrease of p21<sup>waf1/cip1</sup> protein overexpression was also observed in the organs in which cell proliferation did not appreciably differ from the level in wild-type (+/+ ) mice, this decrease might have no effect on sensitivity to cell proliferation and carcinogenesis. Alternatively, it might play an important role in the cell cycle regulation of only the sensitive organs.

INTRODUCTION

Many studies have revealed that the p53 tumor suppressor gene demonstrates genetic alteration in various human tumors, including cancers of the lung, the colon, the breast, and the urinary bladder (1–3). Germ-line mutations are associated with an inherited cancer predisposition, the Li-Fraumeni syndrome (4–6), and studies with animal models have provided a number of mechanistic insights. In the present study, we used p21<sup>waf1/cip1</sup> mRNA as an indicator of functional p53 protein by comparing the expression level between p53-deficient and the wild-type (+/+ ) mice. Transcription of the p21<sup>waf1/cip1</sup> gene is activated by p53-dependent and p53-independent mechanisms (20). However, if the overexpression level induced by DNA damage is higher in the wild-type (+/+ ) than in the p53-deficient mice, it can be considered that the overexpression is due to the p53-dependent mechanism, indicating that functional p53 protein might be induced. Furthermore, we examined p21<sup>waf1/cip1</sup> protein to confirm whether it is related to the regulation of cell proliferation at an early stage of carcinogenesis.

MATERIALS AND METHODS

Chemicals. BBN, DMN, and DMH were purchased from Tokyo Kasei Kogyo Inc., Tokyo, Japan; DMBMA and 4-NQO were purchased from Sigma Chemical Co., St. Louis, and DHPN was purchased from Nacalai Tesque Inc., Kyoto, Japan.

Animals and Treatments. Seventy-two male p53-deficient mice were purchased from Taconic, Germantown, NY. The animals were maintained four in a plastic cage with wood chips in a room at 24 ± 2°C temperature and 40–70% humidity with a 12-h light-dark cycle. At 9–10 weeks of age, they were divided into eight groups and were subjected to treatment with chemicals as follows (Fig. 1). All groups except group 8 (16 mice) were composed of 8 animals. These in groups 1–3 were exposed to BBN (0.0075%), DMN

Received 5/20/99; accepted 10/28/99.

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1 Supported by funds from the Project of Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation.

2 To whom requests for reprints should be addressed, at the Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd., 1-98, 3-chome, Kasugade-Naka, Konohana-ku, Osaka 554-8538, Japan. Phone: 81-6-6466-5346; Fax: 81-6-6466-5446; E-mail: sukata@sc.sumitomo-chem.co.jp.

3 The abbreviations used are: CDK, cyclin-dependent protein kinase; BBN, N-butyl-N-(4-hydroxybutyly)nitrosamine; DMN, dimethylnitrosamine; DHPN, dihydroxy-di-N-propynitratosamine; DMBMA, 7,12-dimethylbenz[a]anthracene; DMH, 1,2-dimethylhydrazine; 4-NQO, 4-nitroquinoline 1-oxide; BrdUrd, 5-bromo-2′-deoxyuridine; RT-PCR, reverse transcription-PCR; b.w., body weight.
cells and its control, group 7, were respectively painted with DMBA (25 pM) and acetic acid (painting). Formalin-fixed tissues were embedded in paraffin and sectioned at 4–5 μm thickness, and stained with H&E. Twenty-seven two male C57BL/6J mice, wild-type littermates of p53-deficient mice, were assigned to eight equivalent treatment groups (9–16) receiving the same treatments. After the administration, all animals were killed under ether anesthesia and examined as follows.

Histopathological Examination. The carcinogen-target organs, that is, urinary bladder, liver, lung, and kidney of mice continuously exposed to carcinogens, that is, in groups 1–3, 6–11, and 14–16.

Cell Proliferation Analysis by BrdUrd Labeling Indices. To evaluate early cell proliferation in the carcinogen-targeted organs of p53-deficient and wild-type (+/+ ) mice, five mice in groups 2, 6–8, 10, and 14–16 were infused with BrdUrd 120 mg/ml in 200 μl of saline, a thymidine analogue, using osmotic minipumps for the last 3 days. Five mice in groups 1, 3–5, 8, 9, 11–13, and 16 received BrdUrd intraperitoneally 1 h before sacrifice.

After deparaffinized and rehydration to distilled water, slide sections in sodium citrate buffer (pH 6.0) were boiling in an autoclave for 1 min. Then anti-p53 antibody (clone DO-7, Santa Cruz Biotechnology, CA) was incubated at a 1:50 dilution (except 1:5000 in the urinary bladder case) for 1 h at 37°C with a 1:3000 dilution (except 1:5000 in the urinary bladder case) of 100 μl of RIPA lysis buffer (PBS [pH 7.4]) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml of phenylmethylsulfonyl fluoride, 5 μg/ml of apotinin, 10 μg/ml of leupeptin, 10 μg/ml of pepstatin, 1 mM sodium ortho-vanadate, 1 mM DTT). After incubation for 30 min on ice, the homogenates were clarified by centrifugation at maximum speed (15,000 rpm) in a microcentrifuge for 30 min at 4°C. The protein concentrations of supernatants were measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Richmond, CA). Aliquots of 100 μg of whole-cell lysates were electrophoresed on 12.5% SDS-polyacrylamide gels (GAGEL PC-700, ASTEC Co., Ltd., Fukuoka, Japan). The cycling program was: 94°C/1 min and 22 cycles at 60°C/30 s, 72°C/1 min, and 94°C/30 s. The reaction was stopped by mixing with 2.5 μl of 5% stop solution (25% glycerol, 50 mM EDTA, 0.5% SDS, 0.2% bromphenol blue, 0.2% xylene cyanol), and the mixtures were loaded (2 μl/lane) onto 5% polyacrylamide gels, run at 10 W for 100 min at room temperature, and dried. Signals were measured using a BAS 2000 (BAStation version 1.31 Fuji Photo Film Co., Ltd., Tokyo, Japan).

Immunoblot Analysis of p21 waf1/cip1 Protein. Frozen tissues were homogenized on ice in 100 μl of RIPA lysis buffer (PBS [pH 7.4]) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml of phenylmethylsulfonyl fluoride, 5 μg/ml of apotinin, 10 μg/ml of leupeptin, 10 μg/ml of pepstatin, 1 mM sodium ortho-vanadate, 1 mM DTT). After incubation for 30 min on ice, the homogenates were clarified by centrifugation at maximum speed (15,000 rpm) in a microcentrifuge for 30 min at 4°C. The protein concentrations of supernatants were measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Richmond, CA). Aliquots of 100 μg of whole-cell lysates were electrophoresed on 12.5% SDS-polyacrylamide gels (GAGEL PC-700, ASTEC Co., Ltd., Fukuoka, Japan). The cycling program was: 94°C/1 min and 22 cycles at 60°C/30 s, 72°C/1 min, and 94°C/30 s. The reaction was stopped by mixing with 2.5 μl of 5% stop solution (25% glycerol, 50 mM EDTA, 0.5% SDS, 0.2% bromphenol blue, 0.2% xylene cyanol), and the mixtures were loaded (2 μl/lane) onto 5% polyacrylamide gels, run at 10 W for 100 min at room temperature, and dried. Signals were measured using a BAS 2000 (BAStation version 1.31 Fuji Photo Film Co., Ltd., Tokyo, Japan).

Immunohistochemical Staining of p53 Protein. Immunohistochemical staining of p53 protein was performed on sections of carcinogen-target organs from p53-deficient and wild-type (+/+ ) mice that did not receive BrdUrd. Formalin-fixed tissues were embedded in paraffin and sectioned at 4–5 μm. After deparaffinized and dehydration to distilled water, slide sections in sodium citrate buffer (pH 6.0) were boiled in an autoclave for 1 min. Then anti-p53 staining was carried out with the rabbit polyclonal antiserum CM-5 (Novo-
RESULTS

Histopathological Examination. The body weights of p53-deficient mice in groups 1–8 showed a similar increase to the wild-type(+/+) mice in groups 9–16. Abnormal clinical signs were not observed in any mice. Histologically, simple hyperplasia was noted in the urinary bladder and the skin of mice treated with BBN and DMBA, respectively [p53-deficient mice: 6/6 in the urinary bladder of group 9 and 2/6 in group 14 (Fig. 2; Ref. 42)]. No lesions were noted in the other organs.

BrdUrd Labeling Indices for p53-deficient and Wild-type(+/+) Mice. Table 2 summarizes BrdUrd labeling indices for carcinogen-target organs of p53-deficient and wild-type(+/+) mice. Values for the treated groups were significantly elevated compared with the labeling indices in the control groups. In the urinary bladder and skin of p53-deficient mice, they were significantly higher than in the wild-type(+/+) mice treated with BBN and DMBA, whereas the labeling indices of the other target organs, the liver, the lung, and the kidney were almost the same in both treated groups. Furthermore, the labeling indices were also the same as the control levels in the urinary bladder and the skin of both groups treated with the other chemicals: DMN, 4-NQO, DHPN, and DMH, which have carcinogenicity in other organs (data not shown).

Immunohistochemical Analysis of p53 Protein Expression. p53-specific immunoreactivity was detected in the basal layer of the transitional epithelium of the urinary bladder, the epidermal basal layer of the skin, and the alveolar epithelium and bronchial epithelium of the lung in both the p53-deficient and the wild-type(+/+) mice treated with BBN, DMBA, and DHPN, respectively (Fig. 3). Differences in intensity were not obvious between the two groups of mice. No immunoreactivity was detected in the lungs after treatment with 4-NQO, which is possibly due to the short half-life of p53 protein and the sampling occurring 1 week after a s.c. injection of the carcinogen. p53-specific immunoreactivity was not detected in the other target organs.

Semiquantitative Comparison of p21waf1/cip1 mRNA Expression Between p53-deficient and the Wild-type(+/+) Mice. To further investigate the functional p53 status, we examined p21waf1/cip1 mRNA expression in the relevant target organs. Table 3 summarizes the mRNA expression levels in the treated and control groups. The expression levels were significantly higher in the treated groups compared with the control groups. The expression levels were also higher in the p53-deficient mice compared with the wild-type(+/+) mice treated with the same carcinogen (Student’s t test).
expression by RT-PCR. Fig. 4 shows results of a semiquantitative comparison of p21waf1/cip1 mRNA expression in the urinary bladder, liver, lung, and kidney of mice continuously exposed to the carcinogens. Overexpression was found in all organs examined, but was less pronounced in p53-deficient mice than in wild-type(+/+) mice.

**Immunoblot Analysis of p21waf1/cip1 Protein Expression.**
As shown in Fig. 5, immunoblot analysis revealed an increase of p21waf1/cip1 protein expression in the target organs of all mice exposed to BBN, DMN, or DHPN. However, compared with the wild-type(+/+) mice, this expression was obviously decreased in p53-deficient mice, irrespective of the sensitivity to carcinogens of the individual target organs examined.

**DISCUSSION**
We recently reported that cell proliferation in the urinary bladder of p53-deficient mice was already more elevated as compared to wild-type(+/+) mice after 2 or 4 weeks of the treatment with BBN (33). In the present study, to increase our understanding of the sensitivity to chemical carcinogenesis and its relation to cell proliferation at the early stage, we examined BrdUrd labeling indices in several organs of p53-deficient mice treated with individual carcinogens for 4 weeks.

The present assessment of early stage response to carcinogenic insult demonstrated that p53-deficient mice show organ-specific sensitivity to the induction of cell proliferation from the early stage,
in the organs sensitive to a hemizygous p21 knockout (urinary bladder, liver, lung, and kidney). According to many reports, p53 mutant protein becomes detectable by immunohistochemical staining because of a prolonged half-life, rising from 20 min for the wild-type protein to several hours for the mutant protein (34, 35, 43). Recent results suggest that p53 protein is also stabilized by phosphorylation in response to DNA damage (44). Our studies on chemically induced urinary bladder carcinogenesis in BBN-treated rats revealed no overexpression of p21 waf1/cip1 mRNA or protein (45), whereas p21 waf1/cip1 protein expression was induced by the treatment carcinogen (BBN) in drinking water for 20 weeks in p53-deficient mice treated with BBN, DMBA, and DHPN, respectively. Furthermore, RT-PCR and immunoblot analysis of p21 waf1/cip1 indicated functional up-regulation in all the organs examined (urinary bladder, liver, lung, and kidney). According to many reports, p53 mutant protein becomes detectable by immunohistochemical staining because of a prolonged half-life, rising from 20 min for the wild-type gene product to >2 h (43). Recent results suggest that p53 protein is also stabilized by phosphorylation in response to DNA damage (44).

Our studies on chemically induced urinary bladder carcinogenesis in BBN-treated rats revealed no overexpression of p21 waf1/cip1 mRNA or protein (45), whereas p21 waf1/cip1 protein expression was induced by the treatment carcinogen (BBN) in drinking water for 20 weeks in p53-deficient mice treated with BBN, DMBA, and DHPN, respectively. Furthermore, RT-PCR and immunoblot analysis of p21 waf1/cip1 indicated functional up-regulation in all the organs examined (urinary bladder, liver, lung, and kidney). According to many reports, p53 mutant protein becomes detectable by immunohistochemical staining because of a prolonged half-life, rising from 20 min for the wild-type gene product to >2 h (43). Recent results suggest that p53 protein is also stabilized by phosphorylation in response to DNA damage (44).

On the other hand, RT-PCR and immunoblot analysis showed that p21 waf1/cip1 mRNA overexpression was relatively decreased not only in the organs sensitive to a hemizygous p53 knockout (urinary bladder), but also in the nonsensitive organs (liver, lung, and kidney). This implies that instead of p53-dependent p21 waf1/cip1 overexpression, p53-dependent apoptosis has a fatal effect on the regulation of cell proliferation in all organs examined, or at least the nonsensitive organs like liver, lung, and kidney. Homozygous deletion of p21 waf1/cip1 in the human colon carcinoma cell line HCT116 completely abrogated the G1 checkpoint following γ-irradiation, whereas cell proliferation was suppressed to similar extents in crypts derived from both p21−/− and p21+/+ cells following irradiation of chimeric mice (45, 46), suggesting that p21 waf1/cip1 overexpression is not a dispensable factor for regulating a cell cycle. Therefore, we speculate that the sensitivity to cell proliferation may be due to tissue conditions in the role of p53-induced p21 waf1/cip1. Several CDK inhibitors, especially p27kip1 and p57kip2 proteins, which belong to the same group as p21 waf1/cip1 protein, can also inhibit CDK activity. In the nonsensitive target organs, these may compensate for the decrease of p53-dependent p21 waf1/cip1 overexpression; nevertheless, in a preliminary study, we did not find overexpression of mRNA for p27kip1 or the inducer transforming growth factor β1.

It is also possible that additional p53-dependent genes regulate cell growth arrest. According to a recent report, a temperature-sensitive mutant of human p53 (Val-138) is capable of arresting the growth of rat embryo fibroblasts at the permissive temperature without induction of p21 waf1/cip1 expression (47). Furthermore, p21−/− cells have a phenotype intermediate between p53−/− and wild-type cells (48), suggesting that p53 impacts on an additional gene that participates in cell growth arrest. In conclusion, p53-deficient mice presumably are more sensitive to carcinogenesis in organs in which p53 plays a key role in cell growth such as G1 arrest, but not in organs in which loss of functional p53 has no effect. This appears to be reflected in the sensitivity to induction of cell proliferation. In addition, the present short-term bioassay may be useful to estimate sensitivity to carcinogenesis in p53-deficient mice. It is important that the timing for the examination of cell proliferation be at the end of the latency period for tumor induction to measure the effect of carcinogen. Previous data indicate that exposure to DMN results in an increase in liver hemangiosarcomas, but not until ~12 weeks after the onset (29). In our bioassay, it is possible to evaluate overall sensitivity to chemical carcinogens by assessing the proliferation for parenchymal cells, for example, hepatocytes for hepatocarcinogenesis, but optimal treatment period for different organs may need further consideration.

ACKNOWLEDGMENTS

We thank our fellows in the laboratory for their generous support.

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