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

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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 carcinoogens for 4 weeks: N-butyl-N-(4-hydroxybutyl)nitrosamine (0.007% in drinking water); dimethylnitrosamine (0.001% in drinking water); dihydroxy-di-N-propylnitrosamine (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 waf1/cip1 mRNA, and p21 waf1/cip1 protein between the mice. 5-Bromo-2-deoxyuridine labeling indices in the urinary bladder and the skin were significantly increased in several groups, and it is also known as WAF1 (wild-type p53 activation factor), CIP1 (CDK 2 interacting protein), SDH1 (senescent cell-derived inhibitor), and MDA-6 (melanoma differentiation-associated; Refs. 16–18, 19, and 20). p21 waf1/cip1 is a member of a family of CDK inhibitors, including p27 Kip1 (21, 22) and p57 Kip2 (23–26), which effectively inhibit several CDKs, such as CDK2, CDK3, CDK4, and CDK6, by preventing their phosphorylation (27).

Using gene targeting techniques, p53 knockout mice have been developed, and it has been demonstrated that homozygotes for null alleles develop normally but show a high sensitivity to early onset of spontaneous tumors, such as malignant lymphomas, soft tissue sarcomas, and osteosarcomas (28, 29). Additionally, mice with a single null p53 allele, heterozygote p53 knockout mice, show an increased sensitivity to radiation (30, 31) or chemically induced carcinogenesis, such as skin carcinogenesis induced with the initiator DMBA followed by continued application of 12-O-tetradecanoyl-phorbol-13-acetate (32) and BBN (1)-induced urinary bladder carcinogenesis (33, but no change in sensitivity regarding liver (34) and breast carcinogenesis (35).

Recently, attention has been concentrated on using transgenic mice as model animals to provide advantages in shortening the time and improving the accuracy of carcinogen identification and characterizing risk (36). To increase our understanding of the p53 knockout model, the present study of carcinogenesis at an earlier stage was conducted.

In the present study, we used p21 waf1/cip1 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 waf1/cip1 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 waf1/cip1 protein to confirm whether it is related to the regulation of cell proliferation at an early stage of carcinogenesis.

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 inherited cancer predisposition, the Li-Fraumeni syndrome (4–6), and studies with animal models have provided a number of mechanistic insights. With regard to primary functions, p53 plays important roles in G1 arrest (7–11) and apoptosis (12–15) induced by DNA damage and other agents. Especially in G1 arrest, p53 functions as a transcription factor for p21 waf1/cip1 (16–18).

Cloning of cDNA for the latter has been achieved independently by several groups, and it is also known as WAF1 (wild-type p53 activated factor), CIP1 (CDK 2 interacting protein), SDH1 (senescent cell-derived inhibitor), and MDA-6 (melanoma differentiation-associated; Refs. 16–18, 19, and 20). p21 waf1/cip1 is a member of a family of CDK inhibitors, including p27 Kip1 (21, 22) and p57 Kip2 (23–26), which effectively inhibit several CDKs, such as CDK2, CDK3, CDK4, and CDK6, by preventing their phosphorylation (27).

MATERIALS AND METHODS

Chemicals. BBN, DMN, and DMH were purchased from Tokyo Kasei Kogyo Inc., Tokyo, Japan, DMBA 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 to 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...
and its control, group 7, were respectively painted with DMBA (25 mg/kg in oil (olive oil:cholesterol 20:1) once a week, respectively. Those in group 6 and its control, group 7, were respectively painted with DMBA (25 mg/kg b.w. acetone) and the acetone vehicle to skin once every week. Animals of group 8 were given deionized drinking water for 4 weeks as control for groups 1–5. Seventy-two male C57BL/6 mice, wild-type littermates of p53-deficient mice, (Taconic, Germantown, NY) were also assigned to eight equivalent groups (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 for BBN (33), liver for DMN (37), lung for DHNP (38) and 4-NQO (39), kidney for DHNP (38) and DMH (40), and skin for DMBA (41) were excised from six animals each in groups 1–16 and preserved partially in 10% phosphate-buffered formalin. The preserved tissues were dehydrated, embedded in paraffin, sectioned at ~5-μm thickness, and stained with H&E, and then examined on the target organs for each chemical under a light microscope.

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.

To evaluate BrdUrd labeling indices, the number of labeled nuclei in the lungs of each animal were counted under a light microscope.

Protein Expression Analysis. Protein expression, levels of p21 waf1/cip1 mRNA were examined in the urinary bladder, liver, lung, and kidney of mice continuously exposed to carcinogens, that is, in groups 1–3, 6–11, and 14–16.

Total RNA was isolated from tissues frozen in liquid nitrogen using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) and digested with DNase at 37°C for 1 h. Primer sequences used for RT-PCR were indicated in Table 1. RT-PCR was performed semiquantitatively according to the protocol for the RNA PCR kit version 2 (Takara Shuzo Co., Ltd., Osaka, Japan) with minor modifications in accordance with our preliminary experiment. Namely, to perform RT-PCR semiquantitatively, we confirmed that PCR products were increased linearly in line with the input cDNA concentration under the following conditions. 32P-end labeling of PCR primers was carried out using a MEGALABEL kit (Takara Shuzo Co., Ltd., Osaka, Japan), and PCR was performed in a 10-μl reaction mixture using a Program Temp Control System 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/plate) onto 5% polyacrylamide gels, run at 10 W for 100 min at room temperature, and dried. Signals were measured using a BAS 2000 (BASStation 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 aprotinin, 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 (PAGEL ISOGEN, Nippon Gene Co., Ltd., Tokyo, Japan) and digested with DNase at 100 min at room temperature, and dried. Signals were measured using a BAS 2000 (BASStation version 1.31 Fuji Photo Film Co., Ltd., Tokyo, Japan). Statistical Analysis. The two-tailed Student’s t test was performed to compare the BrdUrd labeling indices using a Yukms statistical computer package (Yukms Co., Ltd., Kawasaki, Japan). Statistical significance was evaluated with P of 0.05 and 0.01.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21 waf1/cip1</td>
<td>5′AGCAAGAGTGCTGGCTTGCAGC3′ (sense)</td>
</tr>
<tr>
<td>β-actin</td>
<td>5′AGGAGTACAGTACGGTAC3′ (antisense)</td>
</tr>
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Table 1. Sequences of mouse p21 waf1/cip1 and β-actin primers used for RT-PCR analysis to quantitate mRNA expression in organs exposed to carcinogens.
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 1 and 4/6 in the skin of group 6; wild-type(+/+) mice: 2/6 in 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-treated groups. In the urinary bladder and skin of mice 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 \( p21^{\text{waf1/cip1}} \) mRNA Expression Between p53-deficient and the Wild-type(+/+) Mice. To further investigate the functional p53 status, we examined \( p21^{\text{waf1/cip1}} \) mRNA 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).

Table 2. BrdUrd labeling indices in the carcinogen target organs

<table>
<thead>
<tr>
<th>Group</th>
<th>Urinary bladder</th>
<th>Liver</th>
<th>Lung</th>
<th>Kidney</th>
<th>Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (+/+) mice Control</td>
<td>0.10 ± 0.12</td>
<td>2.06 ± 1.09</td>
<td>0.44 ± 0.05</td>
<td>3.86 ± 1.04</td>
<td>10.90 ± 1.96</td>
</tr>
<tr>
<td>Treatment 1</td>
<td>3.58 ± 0.24</td>
<td>6.42 ± 2.98</td>
<td>2.27 ± 0.46</td>
<td>5.50 ± 1.96</td>
<td>68.69 ± 2.86</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>1.51 ± 0.54</td>
<td>7.76 ± 2.54</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>p53-deficient mice Control</td>
<td>0.07 ± 0.02</td>
<td>1.98 ± 1.52</td>
<td>0.40 ± 0.05</td>
<td>2.28 ± 0.36</td>
<td>8.57 ± 3.52</td>
</tr>
<tr>
<td>Treatment 1</td>
<td>6.89 ± 2.97</td>
<td>6.58 ± 1.08</td>
<td>2.50 ± 0.62</td>
<td>5.59 ± 1.94</td>
<td>93.78 ± 3.58</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>1.49 ± 0.29</td>
<td>6.42 ± 2.27</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Treatment 1: 0.0075% BBN for urinary bladder; 0.001% DMN for liver; 0.1% DHPN for lung and kidney; 25 μg/kg DMBA for skin.

\( a \) p < 0.01 versus the relevant control group.

\( b \) p < 0.05 versus the relevant control group.

\( c \) Treatment 2: 10 mg/kg b.w., 4-NQO for lung; 10 mg/kg b.w., DMH for kidney.

\( d \) p < 0.001 versus the wild-type(+/+) mice treated with the same carcinogen (Student’s t test).

\( e \) p < 0.01 versus the wild-type(+/+) mice treated with the same carcinogen (Student’s t test).

\( f \) p < 0.05 versus 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 \( p21^{waf1/cip1} \) 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 \( p21^{waf1/cip1} \) Protein Expression.**

As shown in Fig. 5, immunoblot analysis revealed an increase of \( p21^{waf1/cip1} \) 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 addition, we observed simple hyperplasia of the urinary bladder and the skin in most p53-deficient mice treated with BBN and DMBA, respectively. Thus, in the organs that are more sensitive to chemical carcinogenesis in p53-deficient animals, some abnormal effects due to loss of functional p53, such as acceleration of cell growth, may occur from an early stage. Kemp et al. (32) found that loss of functional p53 has no effect on initiation but greatly enhances malignant progression in the skin carcinogenesis. However, our protocol for DMBA treatment was different from their method.

The present investigation of whether functional p53 protein is induced by a treatment carcinogen demonstrated immunoreactivity in the urinary bladder, skin, and lung of the p53-deficient and wild-type (+/+ ) mice revealed no p53 mutations in early stage lesions such as simple hyperplasia (33). Furthermore, functional p21\(^{\text{wt}}\)/p21\(^{\text{cip1}}\) overexpression is induced by the treatment carcinogen (16). Therefore, in the present study, the immunoreactivity presumably reflected functional p53 stabilized by phosphorylation, and it was clarified that functional p53 protein was induced by the treatment carcinogen in all target organs examined. However, it was not clear why the immunoreactivity was not detectable in the liver and kidney treated with carcinogen targeted to those organs.

On the other hand, RT-PCR and immunoblot analysis showed that p21\(^{\text{wt}}\)/p21\(^{\text{cip1}}\) 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\(^{\text{wt}}\)/p21\(^{\text{cip1}}\) 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\(^{\text{wt}}\)/p21\(^{\text{cip1}}\) in the human colon carcinoma cell line HCT116 completely abrogated the G\(_1\) checkpoint following \(\gamma\)-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\(^{\text{wt}}\)/p21\(^{\text{cip1}}\) overexpression is not indispensable 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\(^{\text{wt}}\)/p21\(^{\text{cip1}}\). Several CDK inhibitors, especially p27\(^{\text{kip1}}\) and p57\(^{\text{kip2}}\) proteins, which belong to the same group as p21\(^{\text{wt}}\)/p21\(^{\text{cip1}}\) protein, can also inhibit CDK activity. In the nonsensitive target organs, these may compensate for the decrease of p53-dependent p21\(^{\text{wt}}\)/p21\(^{\text{cip1}}\) overexpression; nevertheless, in a preliminary study, we did not find overexpression of mRNA for p27\(^{\text{kip1}}\) or the inducer transforming growth factor \(\beta\) (1).

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\(^{\text{wt}}\)/p21\(^{\text{cip1}}\) 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 G\(_1\) 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 hepatocarcinogenicity, but optimal treatment period for different organs may need further consideration.

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**REFERENCES**


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