Rapid induction of uterine tumors with p53 point mutations in heterozygous p53-deficient CBA mice given a single intraperitoneal administration of N-ethyl-N-nitrosourea

Kunitoshi Mitsumori2, Hiroshi Onodera, Takeo Shimo, Kazuo Yasuhara, Hisayoshi Takagi, Takatoshi Koujitan, Masao Hirose, Chika Maruyama3 and Shigeharu Wakana1

Division of Pathology, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501 and 1Central Institute for Experimental Animals, 430 Nogawa, Miyamae-ku, Kawasaki 216-0001, Japan

To investigate the sensitivity of heterozygous p53-deficient CBA mice to carcinogens, 20 female mice [p53(+/–)] and 20 wild-type littermates [p53(+/+)] were given an intraperitoneal injection of 120 mg/kg body wt of N-ethyl-N-nitrosourea (ENU) and were maintained without any other treatment for a further 26 weeks. Histopathology showed that uterine tumors (endometrial polyps and stromal sarcomas) and lung adenomas were induced in both p53(+/+) and p53(+/–) mice. The incidence of uterine tumors and lung adenomas (94% and 81%, respectively) in p53(+/+) mice was significantly greater than that in p53(+/–) mice (37% and 42%, respectively). Malignant lymphomas were only induced in p53(+/–) mice, at an incidence of 31%. Concerning uterine tumors and preneoplastic lesions, there were endometrial stromal sarcomas and atypical hyperplasias of the endometrial gland in 90% and 63%, respectively, of p53(+/–) mice, with significantly greater incidences than in p53(+/+) mice. Gene analysis revealed GCG→GTG point mutations in codon 135 of exon 5 of the p53 allele in all of the uterine endometrial stromal sarcomas examined. Our results suggest that female p53(+/–) CBA mice are very susceptible to uterine carcinogenesis, providing a useful model for ENU-induced uterine tumors.

Introduction

Recently, the p53 suppressor gene has attracted the attention of many investigators studying carcinogenesis, since its mutation has been detected in diverse types of human tumors such as urinary bladder carcinomas (1), breast cancers (2,3), esophageal cancers (4), gastric cancers (5), lung cancers (3,6), uterine cancers (7) and prostate cancers (8). The p53 suppressor protein exerts checkpoint functions at the G1–S (9–11) and G2–M (12) transitions of the cell cycle, and thus is involved in the regulation of DNA replication and cell division (13). In addition, it has been postulated that genomic instability, defined as the loss or gain of chromosomes as well as genetic changes at the level of signal genes such as rearrangements, translocations, amplifications, deletions and point mutations, is also caused by loss of function of the p53 tumor suppressor gene (14). The fact that homozygous p53-deficient mice [p53(–/–)], in which both p53 alleles have been inactivated, develop tumors early in their life can be explained by such genomic instability contributing to tumorigenesis (14,15).

Long-term carcinogenicity bioassays using rodents have been used to assess the carcinogenic potential of newly developed chemicals, but they are expensive and time consuming. In this respect, the establishment of new animal models as short-term alternatives for effective detection of carcinogenic potential is of great concern. One alternative under study is the heterozygous p53 knockout mouse [p53(+/–) mouse] model, in which one p53 allele has been inactivated; extensive short-term evaluation studies of such mice have been carried out (16,17). There have been several reports that p53(+/–) mice are highly sensitive to genotoxic carcinogens, thymic lymphomas being induced by phenolplhalin (18), urinary bladder tumors by N-butyl-N-(4-hydroxybutyl)nitrosamine (19) or p-cresideine (16), and skin tumors by 4-vinyl-cyclohexene diepoxide (16). Such findings provide a useful basis for short-term carcinogenicity testing, but further analyses of other types of carcinogens are pivotal for drawing definite conclusions.

N-Ethyl-N-nitrosourea (ENU) is an alkylating carcinogen that is potentially carcinogenic to various organs of several animal species (20). This study was performed as part of the continuing evaluation of the carcinogenic susceptibility of p53(+/–) mice to clarify which organs are targets of intraperitoneally administered ENU in p53(+/–) mice. In addition, the types of p53 point mutations present in induced tumors were investigated.

Materials and methods

Animals and test materials

The mice used in this study were heterozygous p53-deficient CBA mice [p53(+/–)] in which exon 5 of the lateral p53 allele was inactivated (21). They were F1 offspring of heterozygous p53-deficient C57 BL/6J male mice back-crossed with CBA female mice. Twenty female p53(+/–) mice and 20 wild-type littermates [p53(+/+)], 7 weeks of age, were purchased from Oriental Yeast (Tokyo, Japan). Through the acclimatization and experimental periods, animals were group-housed at a maximum of five per cage in plastic cages with absorbent hardwood bedding (Beta Chips, Oriental Yeast) in an air-conditioned animal room (room temperature, 23 ± 2°C; relative humidity, 55 ± 5%; lighting cycle, 12 h light/12 h dark). Mice were transferred to clean cages with fresh bedding twice weekly. The mice were quarantined for 2 weeks in the animal room before intraperitoneal (i.p.) injection.

Experimental design

The p53(+/–) and p53(+/+) mice received a single i.p. injection of 120 mg/kg body wt ENU and were then maintained without any further treatment for 26 weeks. This dose and route of application were selected based on the finding that tumors of the lung, Harderian gland, forestomach and lymphoreticular system could be induced by a single i.p. injection of 120 mg/kg ENU into B6C3F1 mice aged 42 days (22).

Pathological analysis

After the end of the 26 week experimental periods, animals were subjected to a full autopsy and extensive organs and tissues were dissected out, fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin, sectioned at 4–5 μm and stained with hematoxylin and eosin (H-E) for microscopic examination. Uterine endometrial lesions were basically diagnosed according to World Health Organization criteria (23).

Abbreviations: ENU, N-ethyl-N-nitrosourea; MNU, N-methyl-N-nitrosourea.
higher than the latter (Table I). The incidence of lung adenomas
There were uterine tumors in 15 of 16 (94%) p53(/H11001
in diameter, were noted in nine p53(/ and four p53(/ mice.
Ten different uterine tumors were trimmed free of surrounding non-tumor
results because of the onset of malignant lymphomas
Anatomical location p53(/ mice (n = 16) p53(+/+) mice (n = 19)
Uterus (polyp, endometrial stromal sarcoma) 15 (94)* 7 (37)
Lung (adenoma) 13 (81)* 8 (42)
Hematopoietic organs (lymphoma) 5 (31)* 0 (0)
Others (rhabdomyosarcoma/leiomysarcoma, malignant schwannoma, schwannoma NOS) 4 (25) 0 (0)
*Significantly different from the p53(+/+) value at P < 0.05.

Molecular analysis of p53 gene mutations
Ten different uterine tumors were trimmed free of surrounding non-tumor tissue. In addition, three samples of normal uterine tissues were also taken from p53(+/+) mice. Small pieces of these tumors and of normal uterine tissue were digested with proteinase K (Merck, Darmstadt, Germany) and high-molecular-weight genomic DNA was extracted using the phenol-chloroform-isooamyl-alcohol method. PCR was performed for exons 5–8 of the p53 gene as follows. The temperature profile of the cycles was: denaturation at 98°C for 10 s, annealing at 55°C for 2 s and extension at 74°C for 2 s. Then 38 cycles of amplification were performed at the final annealing temperature. The PCR primers were designed by Okamoto et al. (24) and included both exon and intron portions to avoid amplification of pseudogenes (Figure 1). PCR amplification was carried out in a 100 µl mixture that contained 100 ng of DNA, dNTPs, PCR reaction buffer and 1 unit of KOD DNA polymerase (Toyobo, Osaka, Japan). PCR products were separated in 1% low-melting-point agarose gel and purified using the β-agarase reaction. Finally, PCR-direct sequencing was performed with an ALFred automated laser fluorescence DNA sequencer (Pharmacia, Uppsala, Sweden). Exons 5–8 of the p53 gene in all tumors were analyzed by PCR-direct sequencing.

Statistical analysis
The incidences and multiplicities of proliferative lesions observed were analyzed by the Fisher's exact test and Student’s t-test, respectively. Comparison was between p53(+/+) and p53(+/+) groups.

Results
Four of 20 p53(+/+) and one of 20 p53(+/+) mice treated with ENU died or were killed in a moribund condition during the observation period because of the onset of malignant lymphomas and uterine endometrial stromal cell sarcomas. Autopsy revealed multiple nodules (5–10 mm in diameter) of the uterine horn, thymic masses (5–10 mm in diameter) and subcutaneous masses (1–2.5 cm in diameter) limited to 10, five and four p53(+/+) mice, respectively. Lung nodules, 0.5–1 mm in diameter, were noted in nine p53(+/+) mice and seven p53(+/+) mice. Histopathology showed that uterine tumors and lung tumors were induced in both p53(+/+) and p53(+/+) mice. There were uterine tumors in 15 of 16 (94%) p53(+/+) mice and seven of 19 (37%) p53(+/+) mice; the former was significantly higher than the latter (Table I). The incidence of lung adenomas

Discussion
It has been reported that tumors of the liver, kidney, lung, ovary, Harderian gland, stomach and lymphoreticular system are induced in F1 hybrids of C57BL/6J×C3He/F1 and C3Heb/Fe/J×A/J mice given a single i.p. injection of 120 or 60 mg/kg body wt ENU and reared without any treatment until 90 weeks (22). Young adult mice given a single i.p. injection of ENU at 42 days of age proved especially susceptible to induction of tumors in the lung, Harderian gland, forestomach and lymphoreticular system (22), but uterine tumors have not been described. The most striking finding of our study was, therefore, the uterine endometrial stromal sarcomas found within 26 weeks of ENU administration in p53(+/+) CBA mice. Since malignant fibrous

Table I. Anatomical location and incidence (number (%)) of tumors detected histopathologically in p53(+/+) or p53(+/+) mice induced by ENU

<table>
<thead>
<tr>
<th>Anatomical location</th>
<th>p53(+/+) mice (n = 16)</th>
<th>p53(+/+) mice (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterus (polyp, endometrial stromal sarcoma)</td>
<td>15 (94)*</td>
<td>7 (37)</td>
</tr>
<tr>
<td>Lung (adenoma)</td>
<td>13 (81)*</td>
<td>8 (42)</td>
</tr>
<tr>
<td>Hematopoietic organs (lymphoma)</td>
<td>5 (31)*</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Others (rhabdomyosarcoma/leiomyosarcoma, malignant schwannoma, schwannoma NOS)</td>
<td>4 (25)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Significantly different from the p53(+/+) value at P < 0.05.

Table II. Number (%) of uterine proliferative lesions in p53(+/+) or p53(+/+) mice induced by ENU

<table>
<thead>
<tr>
<th>Proliferative lesion</th>
<th>p53(+/+) mice (n = 16)</th>
<th>p53(+/+) mice (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrial stromal sarcoma</td>
<td>12 (75)*</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Endometrial stromal polypl</td>
<td>3 (19)</td>
<td>7 (37)</td>
</tr>
<tr>
<td>Atypical hyperplasia of endometrial glands</td>
<td>10 (63)*</td>
<td>5 (26)</td>
</tr>
<tr>
<td>Endometrial hyperplasia</td>
<td>0 (0)</td>
<td>6 (32)</td>
</tr>
</tbody>
</table>

*Significantly different from the p53(+/+) value at P < 0.05.
Uterine tumorigenesis in p53-deficient mice

Fig. 2. (a) Uterine endometrial stromal polyp from a heterozygous p53-deficient [p53(+/−)] female mouse given an i.p. injection of ENU and killed at week 26. Note histological features characterized by pedunculated masses protruding into the uterine lumen consisting of loosely organized endometrial stromal cells with scattered endometrial glands and well-differentiated cuboidal epithelial cells on the surface. H–E staining (×100). (b) Uterine endometrial stromal sarcoma from a p53(+/−) female mouse given an i.p. injection of ENU and killed at week 26. Note marked proliferation of spindle-shaped or pleomorphic cells with atypical nuclei, associated with mitoses. H–E staining (×200). (c) Uterine atypical hyperplasia of the endometrial gland from a p53(+/−) female mouse given an i.p. injection of ENU and killed at week 26. Note histological features characterized by proliferative foci of atypical endometrial glandular epithelia. H–E staining (×200). (d) Uterine endometrial hyperplasia from a p53(+/−)female mouse given an i.p. injection of ENU and killed at week 26. Note increased well-differentiated endometrial glands with cysts in the endometrium. H–E staining (×200).

Histiocytomas and stromal sarcomas were induced when 1,2-dimethylhydrazine was administered to CBA mice (25), the findings may indicate a particular strain specificity with p53(+/−) CBA mice being especially susceptible to ENU-induced uterine carcinogenesis.

Another strain of p53(+/−) C57BL/6 mice in which exon 2 of the p53 gene is inactivated, called p53(+/−) TSG mice, is maintained by Taconic Farms (Germantown, NY). This strain is highly sensitive to genotoxic carcinogens, urinary bladder carcinogenesis occurring with N-butyl-N-(4-hydroxybutyl) nitrosamine (19) or p-cresidine (16), and skin carcinogenesis with 4-vinyl-1-cyclohexene diepoxide (16). However, experimental studies with ENU have yet to be performed.

Generally, spontaneous or chemically induced neoplasms of the uterus in mice are mainly adenocarcinomas (26). Maekawa et al. reported production of uterine endometrial hyperplasias after intra-uterine application of ENU to CD-1 mice (26). There were significantly greater incidences of endometrial adenocarcinomas and atypical hyperplasias in the uterus in ICR mice whose diet included 5 p.p.m. 17β-estradiol for 20 weeks after the intravaginal instillation of 10 mg/kg body wt of N-methyl-N-nitrosourea (MNU) than in mice given MNU alone (27). The atypical hyperplasia of the endometrial gland observed in our study looks similar, morphologically, to that described in ICR mice induced by the treatment of MNU and 17β-estradiol (27). Thus it can be considered as a precancerous stage, with the potential to become an endometrial adenocarcinoma when the observation period is prolonged or a cancer-promoting treatment such as 17β-estradiol administration is applied. Additional studies in which p53(+/−) mice are fed diet containing ethinyl estradiol during the promotion stage after the ENU initiation are now in progress to test this hypothesis.

It has been postulated that p53(+/−) mice do not produce sufficient p53 protein after DNA damage resulting in less p53-dependent p21, a cyclin-dependent kinase inhibitor and, consequently, that they are more susceptible to carcinogenesis than their p53(+++) counterparts (10). Gene analysis revealed a GCG (Ala) to GTG (Val) transition at codon 135 of exon 5 of p53 in all
tumors examined. In contrast, a G:C to A:T transition mutation at codons 148, 241, 242 and 263 of p53 was found in uterine sarcomas of CBA mice induced by 1,2-dimethylhydrazine (25). Administration of ENU to pregnant p53+/- mice resulted in rapid development of brain tumors, such as glioblastomas, with loss of heterozygosity for exons 2 and 6 of the p53 gene (28). In vitro experiments with alkylating chemicals such as ENU (29) and MNU (30) demonstrated a high frequency of G-G to G-A transitions in p53. These findings indicate that p53 point mutations differ depending on the type of tumor or carcinogen used.

In conclusion, this study demonstrated that endometrial stromal sarcomas with point mutations of the p53 gene and atypical hyperplasia of the endometrial gland can be induced at high incidence within 26 weeks after a single administration of ENU. Although uterine sarcomas are relatively uncommon in humans (their incidences are approximately 1–3% of all uterine malignant neoplasms (31)), female p53+/- CBA mice given ENU are useful models for both mesenchymal and epithelial transitions in p53. These and MNU (30) demonstrated a high frequency of G:G to G:A transitions in p53 wild type allele in heterozygous p53-deficient (+/-) mice. Toxicol. Pathol., 25, 533–540.

References


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