Difference in the Types of Uterine Tumors between Heterozygous p53-deficient and Wild Type CBA Mice Treated with Ethinylestradiol after N-ethyl-N-nitrosoourea Initiation

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Abstract: To cast light on the mechanisms of rapid induction of endometrial stromal sarcomas in p53-deficient CBA mice [p53 (+/–) mice] treated with ethinylestradiol (EE) after a single injection of N-ethyl-N-nitrosourea (ENU), p53 (+/–) and CBA/JNCrj mice [CBA mice] were given diet containing 2.5 ppm EE for 26 and 50 weeks, respectively, after ENU-initiation, and the types of uterine tumors induced were compared. Endometrial adenocarcinomas and stromal sarcomas were induced in 7% and 73%, respectively, of the p53 (+/–) mice given ENU followed by EE. In contrast, the CBA mice demonstrated incidences of adenocarcinomas and sarcomas of 100% and 25%, respectively. These results suggest that the uterine stromal cell is a major target cell of ENU-initiation in p53 (+/–) mice with a role for the tumor suppressor in governing its response to genetic damage due to ENU. The uterine epithelial cell is another target cell but subsequent long-term EE treatment appears prerequisite for the development of adenocarcinomas. While endometrial stromal sarcomas in p53 (+/–) mice were negative for p21 immunohistochemistry, their counterparts in CBA mice were focally positive at a high incidence, suggesting differences in the processes leading to the tumor development in the two cases. (J Toxicol Pathol 2002; 15: 203–207)

Key words: ENU, ethinylestradiol, uterine tumor, p53-deficient mice, p21

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dependent on p53 function\textsuperscript{12–14}, was immunohistochemically evaluated.

**Materials and Methods**

**Animals**

The data for p53-deficient mice given in the present study and the paraffin sections of p53-deficient mice used for the immunohistochemistry were obtained from the experiments described in our previous reports\textsuperscript{5,6}. Briefly, the p53-deficient animals were the F\textsubscript{1} offspring of heterozygous p53-deficient C57BL/6J male mice, in which exon 2 of the lateral p53 allele was inactivated\textsuperscript{15}, backcrossed with CBA female mice [p53 (+/–) mice]. In the present study, for the experiment on wild type CBA mice, a total of fifty-seven 5-week old female CBA/JNCrj mice [CBA mice] were purchased from Charles River Japan (Atsugi, Japan), housed four to a polycarbonated cage with white wood chips for bedding under standard conditions (room temperature, 23 ± 2°C; relative humidity, 55 ± 5%; 12 hr light and dark cycle) and allowed ad libitum access to tap water and basal diet (CRF-1; Oriental Yeast, Tokyo, Japan).

**Experimental design**

The experimental design, including that for our previous study on p53 (+/–) mice is outlined in Fig. 1. Both ENU and EE were obtained from Nacalai Tesque (Kyoto, Japan). The p53 (+/–) mice and CBA mice were divided into two and three groups, respectively. Groups 1 and 2 of the p53 (+/–) mice and 3 and 4 of the CBA mice were initiated with a single intraperitoneal ENU injection at a dose of 120 mg/kg, while the CBA mice of group 5 received the vehicle saline alone. One week after the initiation, basal diet containing 2.5 ppm EE was fed to group 1 for 26 weeks and groups 3 and 5 for 50 weeks. Basal diet was given to groups 2 and 4 for 26 and 50 weeks, respectively. Groups 3–5 consisted of 21, 21, and 15 CBA animals, respectively. The experiments were carried out in accordance with the Guide for Animal Experimentation in the National Institute of Health Sciences of Japan.

**Histopathology and immunohistochemistry**

CBA mice that died during the experimental period were subjected to a complete necropsy as soon as they were found, and all survivors were euthanized at week 50 under ether anesthesia. The uterus and ovaries were excised, fixed in 10% neutral buffered formalin, and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE) for microscopic examination. Other organs and tissues showing macroscopic abnormalities were also processed and examined.

Twelve uterine lesions diagnosed as endometrial stromal sarcomas in p53 (+/–) mice and 3 lesions diagnosed as stromal sarcomas and 11 as adenocarcinomas in CBA mice treated with EE after ENU initiation were immunohistochemically stained for p21 protein using paraffin-embedded sections. A mouse monoclonal antibody to p21 (clone SXM30) was purchased from PharMingen (San Diego, CA) and used at a dilution of 1:100. Antigen retrieval was achieved by heating for 15 min in an autoclave in citrate buffer at pH 6.0. Immunodetection was performed using a secondary biotinylated rabbit antibody (DAKO, Denmark) and the Streptavidin Biotin Complex (DAKO) according to the manufacturer’s recommendations. Light counterstaining with hematoxylin was applied to facilitate microscopic examination. Some sections without the primary antibody were included as negative controls.

**Results**

**Survival**

Four ENU-treated CBA mice (group 4) died in week 3 and were excluded from the evaluation. A further eleven from groups 3 and 4 died from week 38. The cause of death was mainly development of malignant lymphomas. CBA mice found dead from week 45 were included in the evaluation of uterine tumors except for cases where samples were lost by cannibalism. No dead mice were found in CBA mice of the EE alone group (group 5).

**Histopathological observations**

Uterine proliferative lesions, diagnosed as endometrial glandular hyperplasias, atypical hyperplasias of endometrial glands, endometrial adenocarcinomas, endometrial stromal polyps, endometrial stromal sarcomas or hemangiomas, developed in the animals, the incidences being summarized in Table 1. Diagnostic criteria based on the morphological characteristics of each lesion in CBA mice were the same as those previously described\textsuperscript{5,6}. Endometrial stromal sarcomas were induced by the ENU initiation in p53 (+/–) mice (groups 1 and 2, Fig. 2A). In contrast, in CBA mice, stromal sarcomas were induced with the treatment of ENU-initiation followed by EE treatment for 50 weeks (group 3) at only an incidence of 25%. Endometrial stromal polyps were observed in 20–42% of both p53 (+/–) and CBA mice initiated with ENU (groups 1–4). No endometrial stromal...
Table 1. Incidences of Uterine Proliferative Lesions in p53 (+/-) CBA Mice and Wild Type CBA Mice Treated with EE after ENU Initiation

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENU</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>EE</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Effective No. of mice</td>
<td>15</td>
<td>24</td>
<td>12</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Uterus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperplasia, endometrial glands</td>
<td>0* (0)</td>
<td>6 (25)</td>
<td>0** (0)</td>
<td>7 (47)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Atypical hyperplasia, endometrial glands</td>
<td>9 (60)</td>
<td>7 (29)</td>
<td>0*** (0)</td>
<td>4 (27)</td>
<td>9 (60)</td>
</tr>
<tr>
<td>Adenocarcinoma, endometrial glands</td>
<td>1 (7)</td>
<td>0 (0)</td>
<td>12*** (100)</td>
<td>0 (0)</td>
<td>6 (40)</td>
</tr>
<tr>
<td>Polyp, endometrial stroma</td>
<td>3 (20)</td>
<td>10 (42)</td>
<td>4’ (33)</td>
<td>3 (20)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Sarcoma, endometrial stroma</td>
<td>11 (73)</td>
<td>14 (58)</td>
<td>3 (25)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hemangioma</td>
<td>0 (0)</td>
<td>5 (21)</td>
<td>2 (17)</td>
<td>1 (7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ovaries</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesothelioma, ovarian bursa</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>7*** (58)</td>
<td>0 (0)</td>
<td>10 (67)</td>
</tr>
<tr>
<td>Granulosa cell tumor, benign</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (27)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*: p<0.05, **: p<0.01, ***: p<0.001 compared with the ENU alone group.
#: p<0.05, ##: p<0.01 compared with the EE alone group (Fisher’s exact test).

Fig. 2. Uterine endometrial tumor sections from mice fed diet containing 2.5 ppm EE following an i.p. injection of ENU. (A) Stromal sarcoma from a p53 (+/-) mouse killed at week 26. Note marked proliferation of spindle-shaped or pleomorphic cells with atypical nuclei. HE staining (× 120) (B) Adenocarcinoma from a CBA mouse killed at week 50. Note diffusely glandular proliferation of large, atypical epithelial cells. HE staining (× 120) (C) Nuclear p21 expression in a stromal sarcoma from a CBA mouse killed at week 50. Note epithelial cells are negative. Immunohistochemical staining for p21 with nuclear localization in a CBA mouse killed at week 50. Immunohistochemical staining for p21 (× 120).
proliferative lesions were found in CBA mice treated with EE alone (group 5). Endometrial adenocarcinomas were induced at an incidence of 100% in CBA mice initiated with ENU followed by EE treatment for 50 weeks (group 3) and interestingly, also developed in 40% of the EE alone group (group 5) but none of group 4 given ENU alone. All adenocarcinomas, except for one case forming papillary structures, had a predominantly glandular growth pattern (Fig. 2B). In p53 (+/-) mice, atypical hyperplasias of endometrial glands were observed at the incidence of 60% after ENU and EE treatment (group 1), while endometrial adenocarcinomas were induced at very low incidence (7%).

Immunohistochemistry for p21 revealed all examined stromal sarcomas in p53 (+/-) mice (12/12) to be negative, whereas nuclei of those in CBA mice (3/3) were focally positive (Fig. 2C). All adenocarcinomas, with the exception of the one papillary case, were negative for p21 (Fig. 2D). In some uterine sections from CBA mice examined, nuclei of non-proliferative endometrial epithelial cells were slightly positive for p21.

In the ovaries, mesotheliomas of the bursa were induced in EE-treated CBA mice (groups 3 and 5) and benign granulosa cell tumors were observed in ENU-initiated CBA mice (group 4, Table 1). Ovarian atrophy consisted of an absence of developing ova, graafian follicles and corpora lutea was observed and macroscopic lung nodules diagnosed as adenomas were frequently found in CBA mice initiated with ENU (groups 3 and 4). Other macroscopic abnormalities in CBA mice groups included hepatocellular adenomas in group 3 (2/12) and a hepatocellular carcinoma in group 5 (1/15), and forestomach squamous cell carcinomas in groups 3 (1/12) and 4 (2/15).

Discussion

A single intraperitoneal injection of ENU was earlier found to induce endometrial stromal sarcomas in p53 (+/-) mice with additional EE treatment increasing their incidence within 26 weeks8. However, endometrial stromal sarcomas in CBA mice in the present study were rarely induced by ENU followed by EE feeding and no induction was observed in the ENU or EE alone groups after 50 weeks. These results suggest that whereas the uterine stromal cell is a major target cell of ENU-initiation in p53 (+/-) mice, the presence of sufficient p53 probably rescues the stromal cells from genetic damage due to ENU in CBA mice. The finding of GCG to GTG point mutations in codon 135 of exon 5 of the p53 allele in uterine endometrial stromal sarcomas in p53 (+/-) mice induced by ENU3 is of interest in this context. In the present study, immunohistochemistry for p21, widely known to be up-regulated in relation to cell cycle arrest by p5313,16, revealed negative results in all endometrial stromal sarcomas examined in p53 (+/-) mice, while stromal sarcomas in CBA mice were focally positive at a high incidence. In addition, some stromal polyps in CBA mice were also focally positive (data not shown), suggesting that p21 functions as a regulator of the stromal cell cycle in these animals. Its lack in the p53 (+/-) lesions, given their number and rapid development, points to a protective role.

Regarding proliferative lesions originating from epithelial cells, endometrial adenocarcinomas were significantly induced in CBA mice initiated with a single intraperitoneal ENU injection followed by 50-week EE feeding, but not in p53 (+/+) and p53 (+/-) mice given the ENU and only 26-weeks of EE exposure9. In addition, endometrial adenocarcinomas were induced in CBA mice after EE-treatment alone as previously reported for ICR mice8 and newborn CD-1 mice17,18, MNU- or ENU-initiation treatment alone causes only rare uterine adenocarcinomas in mice8,9, as also found here for ENU-initiated CBA mice. These results indicate that epithelial cells are targets of ENU but subsequent long-term EE treatment is a prerequisite for carcinogenesis. The underlying mechanisms might involve hormone imbalance, such as a high level of serum estrogen and a low level of progesterone, which has been demonstrated to be important for uterine carcinogenesis in ICR19 and CD-10 mice.

In the literature, chemicals such as raloxifene19, nortriptiloxone20, nitrofurantoin21, and 1,3-butadiene22 has been reported to induce ovarian tumors, granulosa cell tumor, benign mixed tumors, and/or tubular cell types, along with alteration in endocrine homeostasis and increased incidences of ovarian atrophy and/or tubular cell hyperplasias. In the present study, mesotheliomas of the ovarian bursa and benign granulosa cell tumors developed in EE-treated and ENU-initiated CBA mice, respectively. EE has been reported to be carcinogenic in the mouse uterus and pituitary18,23 and the hamster liver and kidney24 but not the ovary. As far as we know, this is the first report of induction of mesotheliomas originating from the ovarian bursa in mice. Among chemical carcinogens, 7,12-dimethylbenz(a)anthracene is well known to induce ovarian tumors of granulosa cell type25 but this has not been reported for ENU. While granulosa cell tumor induction by ENU initiation could be speculated to associate with ovarian atrophy, the mechanisms of induction of these tumors in the present study remain to be clarified.

In conclusion, p53 (+/-) mice showed high susceptibility to ENU induction of endometrial stromal tumors, possibly associated with lack of p21 expression in the stromal cells. However, p53 appears to rescue stromal cells from the genetic damage due to ENU in CBA mice. Endometrial adenocarcinomas are also induced by ENU in p53 (+/-) and wild-type mice, but subsequent long-term estrogen treatment is a prerequisite for their development.

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References


