Lack of Modifying Effects of Bisphenol A and Roasted-Ground Soybean (Kinako) on N-ethyl-N-nitrosourea-Induced Uterine Carcinogenesis in Heterozygous p53 Deficient CBA Mice

Makoto Ueda¹, Kunitoshi Mitsumori¹, Hiroshi Onodera¹, Hisayoshi Takagi¹, Kazuo Yasuhara¹, Tamotsu Takizawa¹, and Masao Hirose¹

¹Division of Pathology, National Institute of Health Sciences, 1–18–1, Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan
²Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3–5–8, Saiwai-cho, Fuchu, Tokyo 183–8509, Japan

Abstract: In a previous study, we established a 2-stage uterine carcinogenesis model that is useful for detecting modifying effects of endocrine disrupting chemicals (EDCs) featuring administration of N-ethyl-N-nitrosourea (ENU) as an initiator to female heterozygous p53 deficient CBA mice [p53 (+/-) mice]. In addition, we demonstrated that ethinylestradiol with strong estrogenic activity, but not methoxychlor with weak estrogenic activity, showed promoting effects on uterine carcinogenesis. In the present study, to clarify the effects of other EDCs with weak estrogenic activity on development of uterine tumors, female p53 (+/-) CBA mice received an intraperitoneal injection of 120 mg/kg body weight of ENU followed by the diet containing 1% bisphenol A (BpA), 20% roasted-ground soybean (Kinako) (SB) or no further treatment for 26 weeks. Animals of the ENU+BpA and ENU+SB groups showed no significant differences in body weight gain compared to the ENU alone group. Lower values in absolute and relative uterine weights were observed in the ENU+BpA and ENU+SB groups compared to the ENU alone group, but no significant differences were observed in the incidence of uterine endometrial stromal sarcomas and their PCNA labeling indices among the groups. The results in the present study indicate that 1% BpA and 20% SB in diet have no modifying effects on uterine carcinogenesis in p53 (+/-) CBA mice initiated with ENU. (J Toxicol Pathol 2001; 14: 129–134)

Key words: N-ethyl-N-nitrosourea, bisphenol A, soybean, uterine carcinogenesis, heterozygous p53 deficient mice

Introduction:

Several environmental xenoestrogens have become an important social problem because of their potential endocrine disrupting effects. Since natural and synthetic estrogens have been shown to express biological influence mainly by binding to estrogen receptors and to be major etiological agents for uterine carcinogenesis in humans¹,², evaluation of the carcinogenic risk or tumor-promoting ability of such endocrine disrupting chemicals (EDCs) regarding the uterus is clearly a high priority. In our previous study, N-ethyl-N-nitrosourea (ENU)-initiated female heterozygous p53 deficient mice of the CBA strain [p53 (+/-) mice], with one allele of the p53 gene inactivated, were found to be useful for detecting uterine tumor modifying effects of EDCs with estrogenic activities³. In addition, we reported that ethinylestradiol (EE) with strong estrogenic activity, but not methoxychlor with weak estrogenic activity, showed promoting effects on uterine tumorigenesis in this study model⁴. Since the potential of other environmental agents to influence neoplasia is unclear, a 6-month study using our p53 (+/-) mouse model was performed with dietary administration of the weak xenoestrogens, BpA and roasted-ground soybean (Kinako) (SB) after initiation with ENU, to clarify modifying effects on uterine carcinogenesis.

Bisphenol A (BpA), which possesses a weak estrogenic activity⁵–⁹, is an important monomer of plastics widely used in the manufacture of polycarbonate and epoxy resins, dental sealants, and stabilizing agents in plastics³⁵. As a result, it is present in many household plastic products and in food- and drink-packaging materials. Since the maximum tolerable dose (MTD) of BpA was reported to be 1% in 104-week feeding carcinogenicity study using B6C3F1 mice¹¹, a 6-month study using our p53 (+/-) mouse model was performed with the
same dose level. Soybean is famous because it contains many isoflavones such as genistein and daidzein\textsuperscript{12,13} which are also known to show weak estrogenic activity\textsuperscript{14,15}. Kimura et al. reported that feeding of 40% defatted soybean with an iodine deficient diet for 6 to 12 months resulted in the induction of thyroid follicular cell tumors in rats\textsuperscript{16}, but it is unknown whether this carcinogenicity can be attributed to soybean itself and would extend to SB. Because of the high amount of fat included in this SB, it proved difficult to perform a 6-month feeding study at a dose of 40% (w/w). Therefore in the present study, we selected the dose of 20% SB as one that would not overly affect the nutritional balance of the mice.

Materials and Methods

Animals and housing

The animals used in the present study were heterozygous female p\textsubscript{53} deficient CBA mice [p\textsubscript{53} (+/-) mice] in which exon 2 of the lateral p\textsubscript{53} allele was inactivated. They were the F1 offspring of heterozygous p\textsubscript{53} deficient C57 BL/6J male mice back-crossed with CBA inactivated. They were the F1 offspring of heterozygous female p\textsubscript{53} deficient CBA mice \textsuperscript{17}. Twenty seven female p\textsubscript{53} (+/-) mice, 6 weeks of age, were purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). Through the acclimatization and experimental periods, animals were housed at a maximum of 5 per plastic cage with absorbent hardwood bedding (White Flakes, Charles River Inc., Tokyo, Japan) in an air-conditioned animal room (room temperature, 24 ± 2°C; relative humidity, 60 ± 10%; lighting cycle, 12 light/12 dark). All animals were transferred to clean cages with fresh bedding twice weekly. The mice were quarantined for 4 weeks in the animal room assigned for the study and only those without any abnormal findings at the end of this acclimatization period were selected for experimentation. CRF-1 pellet diet (Oriental Yeast Co., Ltd.) and tap water via automatic stainless steel nozzles were freely available throughout the study. In addition, CRF-1 powdered diet (Oriental Yeast Co., Ltd.) was used as a basal diet. The analytical data performed by the manufacture showed that this diet contained about 100 ppm of both genistein and daidzein. This study was carried out in accordance with the Guide for Animal Experimentation in National Institute of Health Sciences of Japan.

Test materials

The materials used were obtained from the following manufactures; ENU, Nacalai Tesque Inc. (Kyoto, Japan); BpA, Wako Chemical Co. (purity, 99.9%; Osaka, Japan); and SB, Mitake-Syokuhin Co., Ltd. (Saitama, Japan).

Experimental design

Female p\textsubscript{53} (+/-) mice were divided into 3 groups of 15, 7, and 5 animals for the BpA treatment, SB treatment, and ENU alone groups, respectively. All mice received an intraperitoneal injection of 120 mg/kg body weight of ENU in physiological saline followed by the diet containing 1% BpA (Group 1; ENU+BpA, 15 animals), diet containing 20% SB (Group 2; ENU+SB, 7 animals) or no supplement (Group 3; ENU alone, 5 animals) for 26 weeks. Filtration of the carcinogen through a filter (MILEX-GV, Japan Millipore Ltd., Tokyo, Japan) was performed before the injection. An intraperitoneal dose of 120 mg/kg ENU was considered to be appropriate for uterine tumorigenesis in mice based on the results of our previous study\textsuperscript{3}. BpA and SB were mixed into powdered diets for \textit{ad libitum} consumption. Individual body weights and food consumption in each group were determined every week. Since ENU treatment induces ovarian atrophy and it disrupts estrous cycle to the mice, we did not monitor it in this study.

Histopathology and immunohistochemistry

At the end of the 26-week experimental period, the surviving animals were killed by exsanguination from the posterior \textit{vena cava} under ether anesthesia and subjected to a full autopsy. After measuring the uterine weights, the uterus, vagina, ovaries, liver, spleen, kidneys, heart, lungs, adrenal glands, pituitary gland, thyroid glands, and grossly abnormal lesions were fixed in 10% neutral buffered formalin. These tissues were processed routinely, embedded in paraffin, sectioned at 4–5 μm, and stained with hematoxylin and eosin (H-E) for microscopic examination. Immunohistochemical staining using a monoclonal antibody against proliferating cellular nuclear antigen (PCNA) (DAKO, Glostrup, Denmark) was performed for determining cell proliferation activity on the uterus at a dilution of 1: 100. Avidin-biotin peroxidase complex kits (DAKO) were applied for the performance of immunohistochemistry with 3,3’-diaminobenzidine as the chromogen and hematoxylin for counterstaining. The numbers of PCNA positive cells per 100 cells in each proliferative lesion were counted in ten different areas. The PCNA labeling indices were calculated as the percentage of positive cells in each proliferative lesion.

Statistical analysis

The incidences of proliferative lesions observed were analyzed by the Fisher’s exact probability test for significant differences between the ENU alone group and the ENU+ BpA or ENU+SB groups. Variation in the mean food consumption, final body weight, and PCNA labeling indices for uterine endometrial stromal sarcomas were also analyzed by the Student’s \textit{t} test.

Results

Three of 15 mice of the ENU+BpA group and 2 of 7 mice of the ENU+SB group died during the experimental period. The causes of these deaths were mainly malignant lymphomas. Other animals demonstrated a healthy condition throughout the experimental period. No significant differences in body weight gain, food consumption, and final body weights were observed in ENU+BpA and ENU+SB groups, as compared to those for
On autopsy at the terminal sacrifice, hypertrophy or nodules of the uterine horn suggestive of uterine tumors were observed in 9, 5, and 5 animals treated with ENU+BpA, ENU+SB, and ENU alone groups, respectively. The absolute uterine weights and uterine weight/body weight ratios in the ENU+BpA and ENU+SB groups were lower than those in the ENU alone group but no significant differences were observed (Table 1).

Histopathologically, endometrial stromal sarcomas, endometrial hyperplasias, and atypical hyperplasias of the endometrial gland were observed in the uterus (Figs. 1–3). Incidences of endometrial stromal sarcomas were 83.3%, 100%, and 100% in the ENU+BpA, ENU+SB, and ENU alone groups, respectively. Incidences of endometrial hyperplasias were 25.0%, 20.0%, and 20.0% in the ENU+BpA, ENU+SB, and ENU alone groups, respectively. Incidences of atypical hyperplasias of endometrial glands were 66.7%, 60.0%, and 60.0% in the ENU+BpA, ENU+SB, and ENU alone groups, respectively (Table 2). There were no significant differences in the incidences of these lesions among the groups.

The PCNA labeling indices for the endometrial stromal sarcomas were 25.9%, 22.6%, and 24.6% in the ENU+BpA, ENU+SB, and ENU alone group, respectively, there being no significant differences among the groups (Table 2). In other organs, lung adenomas and malignant lymphomas were observed without any statistically significant intergroup differences. Incidences of lung adenomas were 75.0%, 60.0%, and 60.0% in the ENU+BpA, ENU+SB, and ENU alone groups, respectively. Incidences of malignant lymphomas were 8.3%, 20.0%, and 0% in the ENU+BpA, ENU+SB, and ENU alone groups, respectively. With regards to non-neoplastic lesions, atrophic changes of the ovary, as well as reduced numbers of the corpora lutea and follicles, were observed in all ENU-treated mice. No changes due to BpA or SB treatment were observed in other organs including genital and endocrine organs.

**Discussion**

BpA is a monomer component of polycarbonate plastics which can migrate from food-packing materials to food and also from certain dental sealants. Estrogenic activity of BpA has been demonstrated in a number of in vivo and in vitro assays. In vitro assays, BpA demonstrated an estrogenic effect on MCF-7 human breast cancer cells, like that of 17β-estradiol (E2), but at approximately 5,000-fold lower levels with affinities for estrogenic receptors α.
(ERα) and β (ERβ). 10,000-fold lower than those of E2. In vivo studies, BpA has mimicked effects of E2 in a number of rodents studies, including induction of vaginal cornification, uterotrophic effects in immature or ovariectomized rodents, hypertrophy of the pituitary gland, and increase in c-fos mRNA levels in the uterus. Some of these studies showed estrogenic effects at lower dose levels than the expected dose of genistein in the present study. However, in spite of the fact that we selected relatively higher dose levels for not only SB but also BpA, there were no clear changes suggestive of estrogenic action on genital and endocrine organs attributable to BpA or SB administration. ENU-induced atrophic changes of the ovary and expansive growth of uterine endometrial stromal sarcomas would be the major reasons why it was difficult to detect the estrogenic changes in our study.

In our previous study, EE, but not methoxychlor, exhibited tumor promoting effects on stromal and epithelial proliferative lesions of the uteri in p53 (+/-) mice induced by ENU. In the present study, 1% BpA or 20% SB did not affect the incidences of stromal and epithelial proliferating lesions and PCNA labeling indices of endometrial stromal sarcomas in female p53 (+/-) mice given ENU. Since the uterine weights in the ENU+BpA and ENU+SB groups were about 3 times lower than those in the ENU-alone group, BpA and SB might inhibit the growth of endometrial stromal sarcomas. However, no differences were observed on the PCNA labeling indices of the endometrial stromal sarcomas among the groups. The results in the present study thus suggest that BpA and SB have no modifying effects on ENU-induced uterine tumors in p53 (+/-) mice. The results obtained in the present study and our previous study may suggest that EDCs with weak estrogenic activity exert no modifying effects on uterine carcinogenesis.

There have been many reports on the experimental induction of uterine tumors in rodents using chemical carcinogens, but there seem to be no appropriate uterine carcinogenesis models in which the experimental procedures are not complicated and tumor-modifying effects can be detected within a relatively short treatment period. Recently, we have developed a new uterine carcinogenesis model, in which rasH2 mice (transgenic mice carrying human c-Ha-ras gene) given ENU intraperitoneally

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**Table 2. Incidence of Uterine Proliferative Lesions and PCNA Labeling Index of Uterine Endometrial Stromal Sarcomas in ENU-initiated p53 (+/-) Mice**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>No. of mice examined</th>
<th>Incidence of uterine proliferative lesions (%)</th>
<th>PCNA labeling index of ESS (%) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AHE   EH   ESS</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>ENU+BpA</td>
<td>12</td>
<td>66.7  25.0  83.3</td>
<td>25.9 ± 9.0</td>
</tr>
<tr>
<td>2</td>
<td>ENU+SB</td>
<td>5</td>
<td>60.0  20.0  100</td>
<td>22.6 ± 7.2</td>
</tr>
<tr>
<td>3</td>
<td>ENU alone</td>
<td>5</td>
<td>60.0  20.0  100</td>
<td>24.6 ± 14.3</td>
</tr>
</tbody>
</table>

posses a high incidence of uterine endometrial adenocarcinomas (unpublished data). Such malignancies are common human uterine neoplasms whereas endometrial stromal sarcomas are relatively rare. Therefore, this ENU-initiated rasH2 model would be beneficial for research on uterine carcinogenesis. Additional studies using an ENU-initiated rasH2 model are now in progress to clarify whether EE or EDCs have any tumor-modifying effects on uterine epithelial malignancies.

Acknowledgments: This work was supported in part by a grant-in-aid for research on modifying effects of endocrine disrupting chemicals on tumorigenesis from the Ministry of Health, Labor, and Welfare of Japan.

References


