Original

Promoting Effects of Ethinylestradiol but not Atrazine on N-ethyl-N-nitrosourea-induced Uterine Carcinogenesis in ICR Mice

Takao Watanabe¹, Yoko Kashida¹, Makoto Ueda², Hiroshi Onodera², Masao Hirose², and Kunitoshi Mitsumori¹

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

Abstract: In order to assess the modifying effects of atrazine (ATR) on uterine endometrial carcinogenesis, female ICR mice received an intra-uterine injection via the vagina of 50 mg/kg body weight of N-ethyl-N-nitrosourea (ENU), followed by no further treatment, by a diet containing 5, 50 or 500 ppm ATR or by a diet containing 2.5 ppm ethinylestradiol (EE) for 26 weeks. In the ENU + EE (positive control) group, depression of body weight gain was seen throughout the treatment period, and the incidence of uterine endometrial proliferative lesions such as adenocarcinomas and atypical hyperplasias, and PCNA positive indices were significantly increased as compared to the ENU alone group. On the other hand, although only slight depression of body weight was seen throughout the study in the ENU + 500 ppm ATR group, but no significant differences in uterine weights, incidence of uterine proliferative lesions with their PCNA positive indices and the immunohistochemical expression of ERα were found in all of the ENU + ATR groups as compared with those of the ENU alone group. The results in the present study indicate that ATR up to 500 ppm in diet has no modifying effects on uterine carcinogenesis in ICR mice initiated with ENU. (J Toxicol Pathol 2003; 16: 139–145)

Key words: N-ethyl-N-nitrosourea, atrazine, uterine carcinogenesis, ICR mice

Introduction

Possible adverse effects to various organs in human beings resulting from the release of man-made endocrine disrupting chemicals (EDCs) with estrogenic activities into the environment have been recently pointed out as an important social problem. Especially, it has been reported that such EDCs may play an important role in developing uterine adenocarcinoma¹⁻³ that is one of the most common malignant tumors in women⁴. Natural and synthetic estrogens have been shown to express their biological effects mainly by binding to estrogen receptors, and have been found to be major etiological agents for uterine carcinogenesis in humans⁵⁻⁶. Therefore, evaluation of the carcinogenic risk of these EDCs on the uterus is clearly a high priority.

Atrazine (ATR), a chloro-s-triazine-derived compound commonly used as a herbicide for the control of broad leaf weeds and some types of grasses⁷, is one of the EDCs. ATR has been classified as 2B class (possibly carcinogenic to humans) according to the classification of IARC⁸. Although ATR did not induce chromosomal aberrations and sister chromatid exchanges in Chinese hamster ovary (CHO) cells, it was reported to induce chromosomal aberrations in the mouse bone marrow and dominant lethal mutation in mice⁹. The 50% lethal dose (LD₅₀) value of ATR is 3000 mg/kg in rats administered orally¹⁰ and ATR is slightly irritating to the rabbit skin¹¹. With regard to the uterine carcinogenesis, in long-term carcinogenicity studies, the incidences of mammary and uterine endometrial carcinomas were increased in F344 rats given ATR at a dose of 750 ppm for over 24 months¹². On the contrary, ATR possess anti-estrogenic action by preventing the binding of endogenous 17β-estradiol from estrogen receptor (ER)¹³⁻¹⁶, the link between the ATR administrations and uterine carcinogenesis having not be proven.

Uterine carcinogenesis is frequently studied in mouse or rat models that employ specific chemical carcinogens, with or without promoters, for the tumor induction¹⁷,¹⁸.
Maekawa et al. reported high production of uterine endometrial adenocarcinomas in CD-1 mice given subcutaneous implantation of 17β-estradiol for 15 weeks after a single intra-uterine application of N-ethyl-N-nitrosourea (ENU)19. In addition, the incidences of endometrial adenocarcinomas and atypical hyperplasias in the uterus were significantly increased in ICR mice fed diet containing 5 ppm 17β-estradiol for 20 weeks after the intra-vaginal instillation of 10 mg/kg body weight of N-methyl-N-nitrosourea (MNU), as compared with those in mice given MNU treatment alone20.

In the present study, to clarify whether ATR has any tumor-modifying effect on uterine carcinogenesis, we performed a short-term carcinogenicity study using the ENU-initiated uterine carcinogenicity model in ICR mice.

Materials and Methods

Animals and housing
Eighty-five female Slc:ICR mice, 6 weeks of age, were purchased from Japan SLC Inc. (Shizuoka, 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 of 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 1 week 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. In order to avoid the modifying effects of phytoestrogen, the soybean (SB) free powdered diet (Oriental Yeast Co., Ltd., Tokyo, Japan) was used as a basal diet. The analytical data performed by the manufacturer showed that this diet contained less than detectable value of daidzin, daidzein, genistin, and genistein. SB free powdered diet and tap water in bottles with automatic stainless steel nozzles were freely available throughout the experimental period. This study was carried out in accordance with the Guide for Animal Experimentation in the National Institute of Health Sciences of Japan.

Chemicals
ENU and ethinylestradiol (EE) were both purchased from Nacalai tesque, Inc. (Kyoto, Japan) and ATR from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). EE was used as a positive control.

Experimental design
After the acclimatization period, all female mice were divided into 5 groups, consisting of 17 animals in each group. All the ICR mice received a single intra-uterine administration via the vagina of ENU at a dose of 50 mg/kg body weight in polyethylene glycol, followed by no further treatment, a diet containing 5 ppm ATR, 50 ppm ATR, 500 ppm ATR or 2.5 ppm EE for 26 weeks. The maximum tolerated oral dose (MTD) in chronic toxicity studies has been estimated to be 300 ppm in the diet (50 mg/kg body weight) in rats21. Eldridge et al. reported that estrogenic effects such as prolonged diestrus and persistent estrus of estrus cycle were seen in SD rats by the administration of 400 ppm ATR in the diet by 26 weeks22. In our previous study in which female p53(+/–) mice received an intraperitoneal injection of 120 mg/kg body weight of ENU followed by a diet containing 400 ppm ATR, no significant differences on the development of uterine proliferative lesions were observed in the ENU + ATR group as compared to the ENU alone group (unpublished data). In addition, with regard to the mammary tumorigenesis, differences in strain and species were demonstrated in previous reports21,23,24. Therefore, in the present study, to clarify the effect of ATR on uterine carcinogenesis, 500 ppm over MTD was set as a maximum dose. With regard to the dose of EE, in our previous study in which 5 ppm EE in the diet was administered to transgenic mice carrying human prototype c-Ha-ras gene (rasH2) initiated with ENU, severe reduction of body weight gain was seen in the ENU + 5 ppm EE group (unpublished data). In the 2.5 ppm EE group, in contrast, although a depression of body weight gain was seen, the incidences of uterine tumors such as endometrial hyperplasias and atypical hyperplasias were enhanced as compared to the control mice given ENU alone. So that, in the present study, 2.5 ppm EE was administered to animals of the positive control group for 26 weeks after ENU initiation. Filtration of the carcinogen through a Millipore filter (MILEX-GV, Japan Millipore, Ltd., Tokyo, Japan) was performed before the administration. An intra-uterine dose of 50 mg/kg ENU was set in the present study, based on the results of the previous report25. Either ATR or EE was mixed into powdered diets for ad libitum consumption. These test diets were replaced two times a week for animals, and individual body weights and food consumption in each group were measured every week.

Necropsy and light microscopic examination
All surviving animals were checked for estrous cycle by vaginal smears at 2 weeks before the terminal sacrifice. At the termination, animals were exsanguinated from the posterior vena cava under ether anesthesia, and subjected to autopsy. After measuring the uterine weights, a wide variety of organs and tissues consisting of the heart, lung, liver, kidney, spleen, adrenal gland, ovary, vagina, and uterus were fixed in 10% neutral buffered formalin. The tissues including the uterus were processed routinely, embedded in paraffin, sectioned at 4–5 µm, and stained with hematoxylin and eosin (H.E.) for microscopic examinations. In the present study, uterine endometrial proliferative lesions were basically diagnosed according to the World Health Organization criteria26. Briefly, endometrial hyperplasias were classified into three degrees of severity on the basis of atypia and size: slight (+), moderate (++), and marked (+++). Atypical hyperplasias and adenocarcinomas were
characterized by adenomatous or papillary foci and/or nests consisting of irregularly proliferating atypical cells, and the latter having a clear evidence of invasion into the muscularis. Immunohistochemical staining using a monoclonal antibody against proliferating cell nuclear antigen (PCNA, DAKO, Glostrup, Denmark) for determining cell proliferation activity in the uterus at a dilution of 1:200, and that against estrogen receptor α (ERα, Novocastra Laboratories Ltd., UK) for determining the expression of ERα at a dilution of 1:100 were performed on uterine tissues in the ENU alone, ENU + 500 ppm ATR, and ENU + EE groups. An avidin-biotin peroxidase complex kit (Histofine, Nichirei Ltd, Tokyo, Japan) was applied for the performance of immunohistochemistry with 3,3’-diaminobenzidine as the chromogen and hematoxylin for counterstaining. The percentages of PCNA positive cells per 100 cells in each proliferative lesion were counted in ten different areas to give PCNA positive indices. The staining intensity of the ERα positive cells was evaluated by the number of the positive nuclear area per total nuclear area of sections, and categorized as slight (+), moderate (++), or marked (+++) with increasing order of the number of positive cells.

Statistical analysis
Quantitative data are represented as the mean ± standard deviation (SD). Data for incidences of uterine proliferative lesions including adenocarcinomas observed were analyzed by Fisher’s exact probability test for significant different between the ENU alone and ENU + ATR, or ENU + EE groups. Variations in the body weights, food consumptions, and organ weights were analyzed by Dunnett test, those in the PCNA positive indices being analyzed by Student’s t-test.

Result

Dead animal
Six treated mice (two of the ENU alone group, two of the ENU + 50 ppm ATR group, and two of the ENU + 500 ppm ATR group) died during the experimental period. The causes of the deaths of four mice (two of the ENU alone group, one of the ENU + 50 ppm ATR group, and one of the ENU + 500 ppm ATR group) were thymic lymphomas, and those of other two mice were unclear by cannibalism.

Body weight gain, food consumption, estrus cycle, and uterine weight

Body weight gains of the ENU + EE and ENU + 500 ppm ATR group were depressed throughout the study, the former being remarkable (statistical significant), the latter being slight (not statistical significant) (Fig. 1). There were no significant differences on food consumption between ENU + ATR groups and ENU alone group. All mice in the ENU + EE group showed persistent estrus in vaginal smear preparations, while no mice in the ENU alone and ENU + ATR groups disclosed any alteration of estrous cycles. The uterine weights (absolute weight: p<0.05; relative to the body weights: p<0.001) were significantly increased in the ENU + EE group, as compared to the corresponding ENU alone group (Fig. 2).

Microscopic examination

Table 1 summarizes the incidences of uterine endometrial proliferative lesions. Histopathologically, uterine proliferative lesions were classified into adenocarcinomas, atypical hyperplasias, and endometrial hyperplasias. The incidences of adenocarcinomas (Figs. 3 and 4) in the ENU alone, ENU + 5 ppm ATR, ENU + 50 ppm

Fig. 1. Mean body weight changes in mice treated with ATR or EE for 26 weeks after ENU initiation.
Effects of ATR on Uterine Tumors

ATR, ENU + 500 ppm ATR, and ENU + EE groups were 0, 0, 0, 0, and 37.5%, respectively, there being a significant difference between the ENU alone and ENU + EE groups (p<0.05). The incidences of atypical hyperplasias (Fig. 5) in the ENU alone, ENU + 5 ppm ATR, ENU + 50 ppm ATR, ENU + 500 ppm ATR, and ENU + EE groups were 0, 6.7, 0, and 18.8%, respectively, those of endometrial hyperplasias being 43.8, 35.3, 40.0, 13.3, and 31.3%, respectively. There were no increased incidences of histopathological changes that were attributable to the treatment of ATR, or EE in organs other than the uterus.

The PCNA positive indices for adenocarcinomas, atypical hyperplasias, and endometrial hyperplasias of the ENU + EE group were 41.0, 40.3, and 32.1%, respectively, while those for endometrial hyperplasias of the ENU alone and ENU + ATR groups were 31.1% and 31.8%, respectively. Values of the PCNA positivity for the luminal endometrium and endometrial glands of ICR mice in which no proliferative lesions were developed were 25.3 and 25.4% in the ENU alone group, 24.9 and 25.1% in the ENU + ATR group, and 25.5 and 25.0% in the ENU + EE group, respectively (Fig. 6).

The expression of ERα in the ENU + EE group was slight to moderate for the luminal epithelium, and moderate to marked for the glandular epithelium (Table 2). Only slight variation with the stage of estrus was evident in the ENU alone group. Mucosae of the lamina propria and the myometrium consistently showed slight expression in mice treated with ENU alone, ENU + 500 ppm ATR, or ENU + EE groups.

Discussion

Recently, the relation between ATR treatment and non-Hodgkin’s lymphomas has been pointed out in human beings27,28. In the present study, 6 animals died throughout the experimental period. The causes of death of 4 mice were...
thymic lymphomas (two of the ENU alone group, one of the ENU + 50 ppm ATR group, and one of the ENU + 500 ppm ATR group). However, the incidences of thymic lymphomas in the ENU + ATR groups were comparable with those in the ENU alone group. In addition, no lymphomas were developed in mice sacrificed at the terminal examination, suggesting that thymic lymphomas developed in the mice in the present study are not related to the ATR administration.

Estrogens are considered to be of essential importance for uterine carcinogenesis in humans and experimental animals, with hormone actions depending on binding to the ER, a specific nuclear receptor. Two ERs, ERα and ERβ, have been identified, and recently three subtypes of estrogen-related receptors (ERRs) such as ERRα, ERRβ, and ERRγ have been reported. Although ERβ is expressed in human breast cancer, its normal function and potential role in cancer progression have not yet been clearly defined. ERα, on the other hand, is a transcription factor that binds to response elements in the DNA of sensitive cells and induces or represses transcription of responsive genes. Maekawa et al. reported high productions of uterine endometrial adenocarcinomas in CD-1 mice given subcutaneous implantation of 17β-estradiol for 15 weeks after a single intra-uterine application of ENU. In our previous study in which ENU was intraperitoneally injected into heterozygous p53-deficient mice of the CBA strain (p53(+/–) mice), the incidences of endometrial stromal sarcomas and atypical hyperplasias of the endometrial glands were significantly increased in p53(+/–) mice as compared to their wild littermates (p53(+/+) mice). As the potential mechanism on the enhancement of uterine carcinogenesis of estrogen, it is well known that chemicals with estrogenic activity typically bind the estrogen response elements (ERE), and that transcription factors are recruited to the estrogen receptor-ligand/ERE complex, resulting in enhanced mRNA synthesis and gene expression of polypeptide growth factors such as epidermal growth factor (EGF) and transforming growth factor (TGF)-β, which can stimulate cell proliferation.

With regard to the carcinogenicity, ATR at a dose of 750 ppm induced mammary carcinomas and uterine endometrial adenocarcinomas in F344 rats over 24 months. In addition, chronic feeding of high dietary levels of ATR

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**Table 2. Immunohistochemical Intensity of ERα Expression in Uterine Epithelial Cells of Mice Treated with ATR or EE for 26 Weeks after ENU Initiation**

<table>
<thead>
<tr>
<th></th>
<th>ENU alone (n = 15)</th>
<th>ENU + 500 ppm ATR (n = 15)</th>
<th>ENU + 2.5 ppm EE (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice examined</td>
<td>D P E M</td>
<td>D P E M</td>
<td>D P E M</td>
</tr>
<tr>
<td>Endometrial epithelial cells</td>
<td>++ +++ 2 2</td>
<td>++ +++ 0 1</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Glandular epithelial cells</td>
<td>+++ +++ – ++</td>
<td>+++ +++ ++</td>
<td>++ +++</td>
</tr>
<tr>
<td>Mucosae of the lamina propria</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Myometrium</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + +</td>
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D: Diestrus, P: Proestrus, E: Estrus, M: Metestrus.
–: negative, +: slight, ++: moderate, +++: marked.

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**Fig. 5.** Uterine atypical hyperplasia in a mouse of the ENU + EE group, showing papillary proliferation of atypical cells. H.E. stain. × 140.

**Fig. 6.** Uterine endometrial hyperplasia in a mouse of the ENU + ATR 500 ppm group, showing focal proliferation of uterine glands. H.E. stain. × 70.
increased the number of days in estrus and fastened the onset of mammary tumors in female Sprague-Dawley rats. In our previous study, there was no modifying effect of ATR on uterine carcinogenesis. In our previous study in which female \( p53^{+/–} \) mice received an intraperitoneal injection of 120 mg/kg body weight of ENU followed by a diet containing 400 ppm ATR, no significant differences on the development of uterine proliferative lesions were observed as compared to the ENU alone group (unpublished data). These data may suggest the species difference between the rat and mouse. With regard to the mammary tumorigenesis, several previous studies showed that female SD rats administered ATR at doses close to the MTD had an increased incidence of mammary tumors or revealed their early induction. On the contrary, other studies with a similar experimental protocol of male SD rats, female or male Fischer 344 rats, or female or male CD-1 mice have failed to demonstrate an ATR treatment-related alteration in the mammary tumor incidence. These reports indicate the species and strain differences. Furthermore, the experimental period for 26 weeks in the present study may not be sufficient for the uterine tumor induction in mice. One question which arises is whether the no modification of uterine proliferative lesions in ICR mice of the ENU + ATR group might be attributable to hormone. It has been reported that SD rats given 400 ppm ATR in food to rats for 12 months had an increased number of days of vaginal estrous, increased levels of plasma estradiol, and decreased levels of plasma progesterone by the treatment for 9 to 12 months. In addition, the incidence of uterine adenocarcinomas has been shown to increase in F344 rats by the treatment of ATR over 24 months. Based on these reports, it is likely that uterine tumors are induced by ATR treatment over 26 weeks by prolonged-hormonal influence in the mouse model.

Connor et al. reported that ATR possesses estrogenic and/or antiestrogenic activities that are mediated by the ER. In the present study, though expression of ER\( \alpha \) in the endometrial glandular epithelium of mice given ENU + EE was increased by external EE treatment, along with the increased PCNA positive indices of uterine proliferative lesions, no alteration in the expression of ER\( \alpha \) in the luminal or glandular epithelium was evident between the ENU alone and ENU + ATR groups. The findings suggest that the expression of ER\( \alpha \) induced by ATR does not play a significant role on the uterine carcinogenesis, or ATR has no modifying effects on the mouse uterine carcinogenesis.

However, taking into account the findings that the results of our study using mice model were different from those in rats, further studies are essentially necessary to clarify the difference in uterine carcinogenesis of ATR between mice and rats.

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References

7. Short P and Colborn T. Pesticide use in the U.S. and policy


