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Effects of Tamoxifen on Endometrial Carcinogenesis in Mice

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Two experiments were conducted to determine the effect of tamoxifen (TAM) in mouse endometrium in comparison with that of 17β-estradiol (E_2). In a medium-term assay, TAM as well as E_2 treatment semi-dose-dependently increased the levels of *fos*/*jun* mRNA and their oncoproteins (Fos/Jun). The long-term effect of TAM on mouse endometrial carcinogenesis was also examined in the following model. A total of 150 female ICR mice, 12–13 weeks of age, were used. Of these, 125 mice received an injection of N-methyl-N-nitrosourea (MNU) solution (1 mg/100 g body weight) into their left uterine tube and saline into the right. One week later, they were divided into four groups: groups 1 (35 mice) and 2 (30 mice) were given 25 ppm and 5 ppm E_2-containing diet, respectively, while group 3 (30 mice) was fed 5 ppm TAM-containing diet. Group 5 (30 mice) was fed basal diet alone. The remaining 25 mice (group 4) received 5 ppm TAM-containing diet alone. At the termination of the experiment (30 weeks), endometrial carcinomas were confirmed to be present in the groups exposed to MNU. TAM increased the incidence of preneoplastic lesions of the endometrium, while E_2 enhanced the occurrence of the carcinoma. No carcinomas were found in the group given TAM alone. In the ovaries, corpora lutea were lacking in most of the mice exposed to TAM, suggesting that the animals were not cycling. Such findings indicate that TAM has an enhancing effect on endometrial carcinogenesis in mice, probably via a mechanism involving overexpression of Fos/Jun proteins.

Key words: Tamoxifen — Endometrial carcinogenesis — Fos/Jun — Polymerase chain reaction — Mice

Tamoxifen (TAM) has been employed for adjuvant chemotherapy in advanced breast cancer patients.[1)] Recently, the use of this agent has been proposed for prophylactic therapy in disease-free women with an increased risk of breast cancers.[2, 3)] The main anti-tumor effects of TAM are suggested to be attributed to anti-estrogenic activities through the competitive blockade of estrogen receptors.[4)] Although TAM is an anti-estrogen, it also acts as a weak estrogen agonist, especially in response to estrogen deficiency in postmenopausal women.[5, 6)] TAM is reported to exert some estrogenic effects in human endometrial epithelium[7)] and to promote development of endometrial hyperplasia and polyps.[8, 9)] Several clinical reports have implied that long-term TAM treatment increases the risk of endometrial cancers.[10, 11)] Furthermore, TAM has been shown to possess carcinogenic activity in rat liver.[12)] It is known that TAM induces overexpression of c-*fos* mRNA in human endometrial carcinoma cells in nude mouse,[13)] though no studies on the effects of TAM on the endometrium have been done in rodents. In the present study, the effects of TAM on endometrial carcinogenesis in mice were examined by using a medium-term assay and a long-term assay with N-methyl-N-nitrosourea (MNU).

MATERIALS AND METHODS

Animals and chemicals Female ICR mice, 10 weeks of age, were purchased from Japan SLC Co. (Shizuoka). The basal diet (Oriental MF, Oriental Yeast Co., Tokyo) and distilled water were available *ad libitum* throughout the experiment. 17β-Estradiol (E_2) and MNU were purchased from Sigma Chem. Co. (St. Louis, MO). TAM (*trans*-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine) was also purchased from Aldrich Chem. Co. (Milwaukee, WI).

Experimental protocol for medium-term assay Ovariectomized ICR mice, 12–13 weeks of age, were divided into five experimental groups (6 mice in each). Groups 1 and 2 were given the diet containing E_2 at the dose of 25 ppm or 5 ppm, respectively. The 5 ppm E_2-containing diet was similar to that used in our previous study.[14)] Groups 3 and 4 were fed the diet containing TAM at the dose of 25 ppm or 5 ppm, respectively. The 5 ppm TAM-containing diet was selected so that the dose of TAM based on the mean intake of the diet by the mice would be similar to the clinical dose in humans 20 mg/50 kg daily. Group 5

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served as an untreated control. Two weeks later, resected uteri were cut in half longitudinally. One half was quickly frozen in liquid nitrogen, and the other was subjected to pathological examination.

Reverse transcription-polymerase chain reaction (RT-PCR) Total RNA was isolated from the frozen tissues by a guanidinium thiocyanate-phenol-chloroform extraction method.[15] Total RNA (3 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RTase, 200 units, Gibco BRL, Gaithersburg, MO) in 20 μM Tris-HCl (pH 8.4), 50 μM KCl, 2.5 μM $MgCl_2$, 0.1 μg/ml bovine serum albumin, 10 μM dithiothreitol, and 0.5 μM deoxynucleotides to generate cDNAs using random hexamers (50 ng, Gibco BRL) at 37°C for 60 min. The RT reaction mixture was heated at 94°C for 5 min to inactivate MMLV-RTase. For c-*fos* or c-*jun* mRNA, forty cycles of PCR, consisting of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for extension, were carried out with reverse-transcribed cDNAs and 0.1 mM specific primers using an Iwaki TSR-300 thermal sequencer (Iwaki Glass, Tokyo) with Vent DNA polymerase (New England Biolabs, Beverly, MA) in 10 μM KCl, 20 μM Tris-HCl (pH 8.8), 10 μM $(NH_4)_2SO_4$, 2 μM $MgSO_4$, 0.1% Triton X-100, and 0.15 μM deoxynucleotide phosphates. Twenty cycles of PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a house-keeping gene) mRNA as an internal standard were performed similarly.

For quantitative PCR, a 50 μl reaction mixture, consisting of 2.5 μl of template, 25 pmol (0.25 μM) each of 3′- or 5′-primer, 2 μl of dNTPs (10 mM) and 2.5 IU of recombinant *Taq* DNA polymerase (Takara, Kyoto), was used. The following oligodeoxynucleotides were synthesized as specific primers according to the cited reports [cDNAs for c-*fos*,[16] c-*jun*[17] and GAPDH[18]]: sense for c-*fos*, 5′-CTTACGCCAGAGCGGGAATG-3′; anti-sense for c-*fos*, 5′-AAGCCTCAGGCAGACCTCCA-3′; sense for c-*jun*, 5′-AGAGCATGACCTTGAAC-3′; anti-sense for c-*jun*, 5′-CTGGGAAGCGTGTTCTGGCT-3′; sense for GAPDH, 5′-TGAAGGTCGGTGTGAACGGATTTGG-3′; antisense for GAPDH, 5′-CTCCTTGGAGGCCATGTAGGCCAT-3′.

Quantitative analysis of c-*fos*/*jun* mRNA expressions by Southern blot of PCR products PCR products were applied to 1.2% agarose gel for electrophoresis at 50–100 V. PCR products were capillary-transferred to Immobilon transfer membrane (Millipore Corp., Bedford, MA) for 16 h. The membrane was dried at 80°C for 30 min, and UV-irradiated to fix the PCR products. The products on the membrane were prehybridized in 1 M NaCl, 50 mM Tris-HCl (pH 7.6) and 1% sodium dodecyl sulfate at 42°C for 1 h, and hybridized at 65°C overnight in the same solution with biotinylated oligodeoxynucleotide probes corresponding to sequences between the specific individual primers of c-*fos* or c-*jun*. Specific bands hybridized with biotinylated probes on the membranes were detected with Plex Luminescent Kits (Millipore Corp.) after exposure to X-ray films at room temperature for 10 min. Southern blots were quantitated with a Bio image analyzer (Millipore Corp.). The intensity of specific bands was standardized with respect to that of *GAPDH* mRNA.

Immunohistochemical expression of Fos/Jun After having been fixed in 10% formalin, half portions of endometrial tissues were processed according to conventional methods. Briefly, staining with avidin-biotin-peroxidase complex was done using a Vectastain kit (Vector, Burlingame, CA). The primary antibodies used were directed against the proteins of c-*fos* and c-*jun* (anti-rabbit polyclonal, Oncogene Science, Inc., New York, NY), at 1:100 dilution. Staining intensity was assigned as follows: (+) positive; (+/−) minimally or randomly positive; (−) negative.

Experimental protocol for long-term assay One hundred and fifty female ICR mice, 12–13 weeks of age at the start of the experiment, were divided into five groups.

Mice in groups 1–3 and 5 underwent laparotomy under general anesthesia with diethyl ether and were injected with MNU solution (total volume: 0.1 ml) at a dose of 1 mg/100 g body weight into the left uterine tube and with normal saline into the right. One week after the MNU exposure, the animals were divided into four experimental groups. Group 1 (35 mice) was given 25 ppm E_2-containing diet, and group 2 (30 mice) was given 5 ppm E_2-containing diet. The level of E_2 in the diet was selected for the same reason as in the case of the medium-term assay. Group 3 (30 mice) and group 4 (25 mice) were fed with 5 ppm TAM-containing diet. The dose of TAM-containing diet was selected to match the clinically used dosage, as mentioned before. Group 5 (30 mice) served as a control, and was fed basal diet. The experiment was terminated 30 weeks after the MNU exposure. At the termination, all animals were killed and autopsied. All major organs, especially the reproductive organs, were grossly inspected. The uterus, ovaries, vagina and other lesions suspected of being neoplastic or hyperplastic were submitted to histological examination. Tissues were sectioned at 3 μm thickness and stained with hematoxylin and eosin (HE).

Histology of the uterine lesions According to the WHO criteria,[19] uterine endometrial lesions were divided into four lesions: a) endometrial hyperplasia, simple; b) endometrial hyperplasia, complex; c) atypical endometrial hyperplasia; d) adenocarcinoma. Uterine cervical lesions were basically diagnosed according to the criteria of Muñoz *et al*.[20]

Statistical analysis Statistical analysis was done by using the χ^2 test or Student's t test.

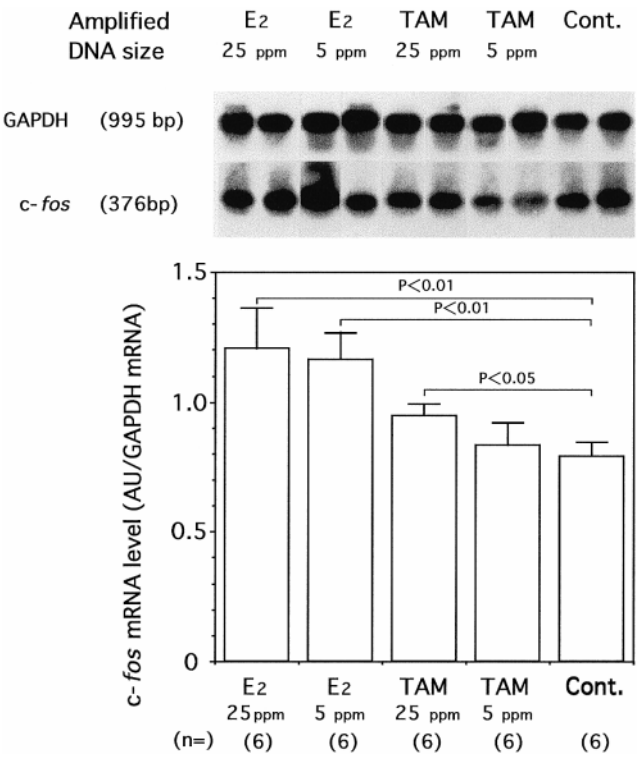

Fig. 1. Expression of c-*fos* mRNA in the uterus of ovariectomized mice treated continuously for 2 weeks with E_2 and TAM in the diet.

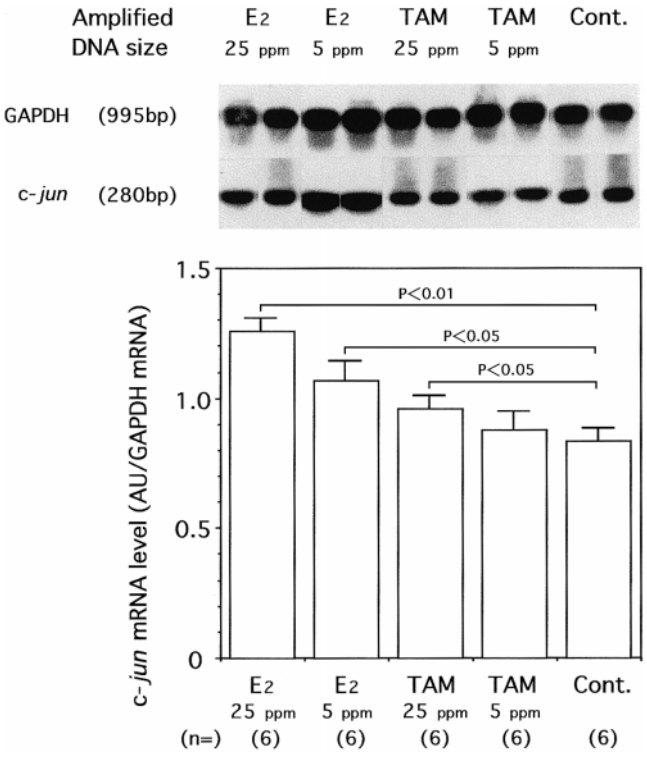

Fig. 2. Expressions of c-*jun* mRNA in the uterus of ovariectomized mice treated with E_2 and TAM in the same manner as described in the legend to Fig. 1.

RESULTS

Medium-term experiment The c-*fos* and c-*jun* mRNA levels are shown in Figs. 1 and 2, respectively. The levels of c-*fos* and c-*jun* mRNA in uteri of mice treated with TAM (25 ppm) were significantly higher than those of the control group ($P<0.05$). However, no significant differences were found between TAM (5 ppm)-treated groups and the control group. The levels 2 weeks after the start of feeding of E_2- or TAM-containing diet were semi-dose-dependently increased as compared with the control.

The results of immunohistochemical detection of Fos/Jun oncoproteins after 2 weeks' feeding of the diet containing E_2 and TAM are summarized in Table I. Representative examples of Fos/Jun oncoprotein staining are shown in Figs. 3 and 4. The expression of Fos oncoprotein was prominent in the glandular cells in the groups treated with E_2 (25 ppm) and TAM (25 ppm). The expression of Jun oncoprotein was also observed in glandular and luminal cells in the groups treated with TAM as well as E_2.

Table I. Immunohistochemical Analysis of Expression of Fos/Jun after 2 Weeks' Feeding of Diet Containing E_2 or TAM

Treatment	Fos			Jun		
	Glandular cells	Luminal cells	Stromal cells	Glandular cells	Luminal cells	Stromal cells
E_2 (25 ppm)	(+)	(+)	(+)	(+)	(+)	(+/–)
E_2 (5 ppm)	(+)	(+)	(+/–)	(+/–)	(+/–)	(–)
TAM (25 ppm)	(+)	(+)	(+)	(+)	(+)	(+/–)
TAM (5 ppm)	(+)	(+)	(+/–)	(+)	(+)	(+/–)
Control	(+/–)	(–)	(–)	(+/–)	(–)	(–)

Long-term experiment Five mice in group 1, six in group 2, three in group 3, and four in group 5 died within 15 weeks. No pathological abnormalities other than pneumonia were found. The remaining animals survived until the termination of the experiment and were counted as effective animals. The mean body weights are summarized in Table II. No significant differences were obtained among the five experimental groups.

Adenocarcinomas were recognized in the bilateral uterine corpora in the groups treated with MNU. Histological features of the endometrial adenocarcinomas and hyperplasia in this study were the same as those in our previous

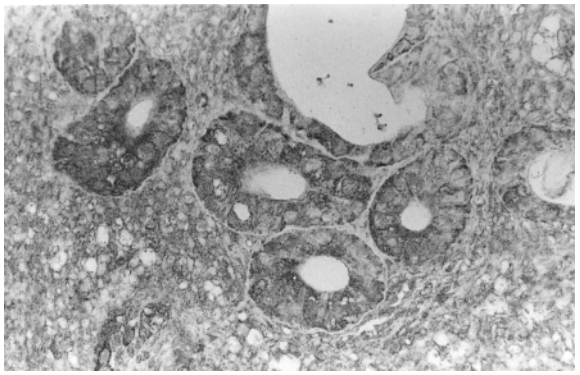

Fig. 3. Immunohistochemical Fos-staining of endometrium from an ovariectomized mouse after 2 weeks on a diet containing E_2 (sABC stain, ×110).

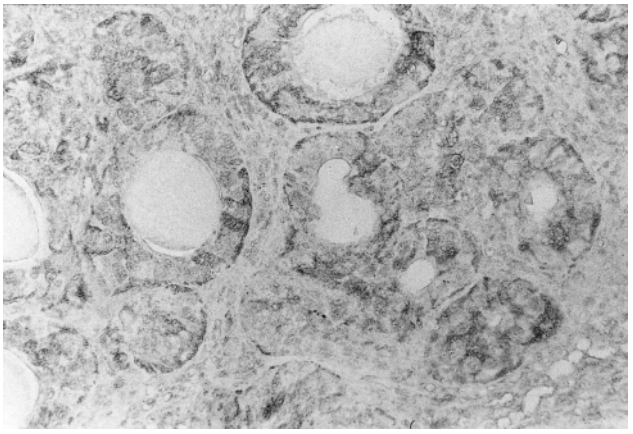

Fig. 4. Immunohistochemical Jun-staining of endometrium from an ovariectomized mouse after 2 weeks on a diet containing E_2 (sABC stain, ×110).

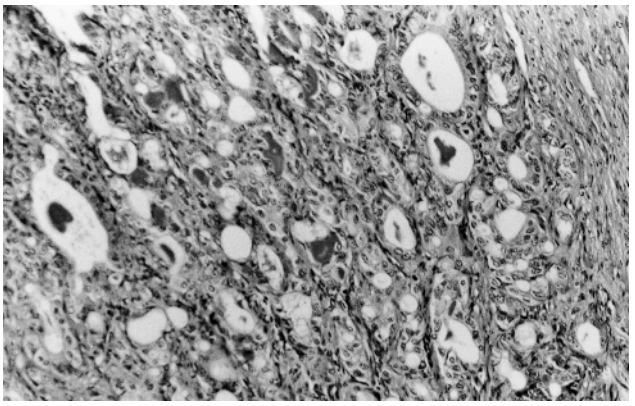

Fig. 5. Moderately differentiated adenocarcinoma of the endometrium in a mouse treated with MNU and E_2 (25 ppm) (HE, ×180).

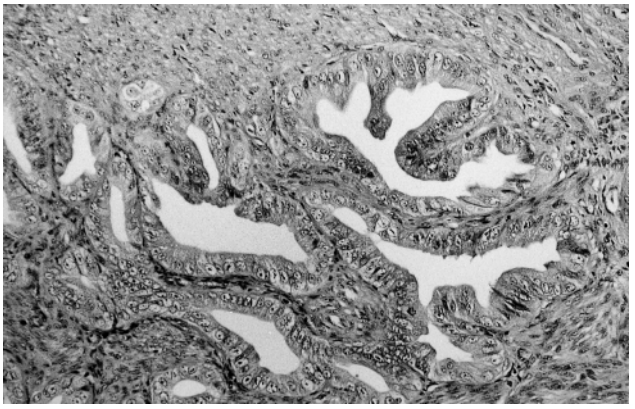

Fig. 6. Atypical endometrial hyperplasia of the endometrium in a mouse treated with MNU and E_2 (25 ppm) (HE, ×110).

reports.[14] Representative morphology of adenocarcinoma and atypical endometrial hyperplasia in this study is shown in Figs. 5 and 6. All adenocarcinomas arising in the endometria were well or moderately differentiated types. The incidence of preneoplastic and neoplastic lesions of the endometria is indicated in Fig. 7. While the incidence of carcinomas of the left (treated) uterine corpus in the groups given MNU and TAM was almost the same as in the group given MNU alone, that of preneoplastic endometrial lesions was higher. No carcinomas were found in the mice treated with TAM alone.

Pathological findings of ovary, oviduct and vagina in each group are summarized in Table III. Cystic ovaries were commonly seen in mice of groups 1–4. Corpora lutea were mostly absent in mice of groups 1, 3 and 4. No

Table II. Initial and Effective Numbers of Animals, and Mean Body Weights

Groups (treatment)	Initial number of animals	Effective number of animals[a)]	Mean body weights (g)
Group 1 [MNU/saline+E_2 (25 ppm)]	35	30	39.9±2.6
Group 2 [MNU/saline+E_2 (5 ppm)]	30	24	40.4±5.0
Group 3 [MNU/saline+TAM (5 ppm)]	30	27	42.2±3.9
Group 4 [TAM (5 ppm) alone]	25	21	38.1±2.5
Group 5 (MNU/saline alone)	25	25	45.5±3.8

a) Animals that survived more than 15 weeks.

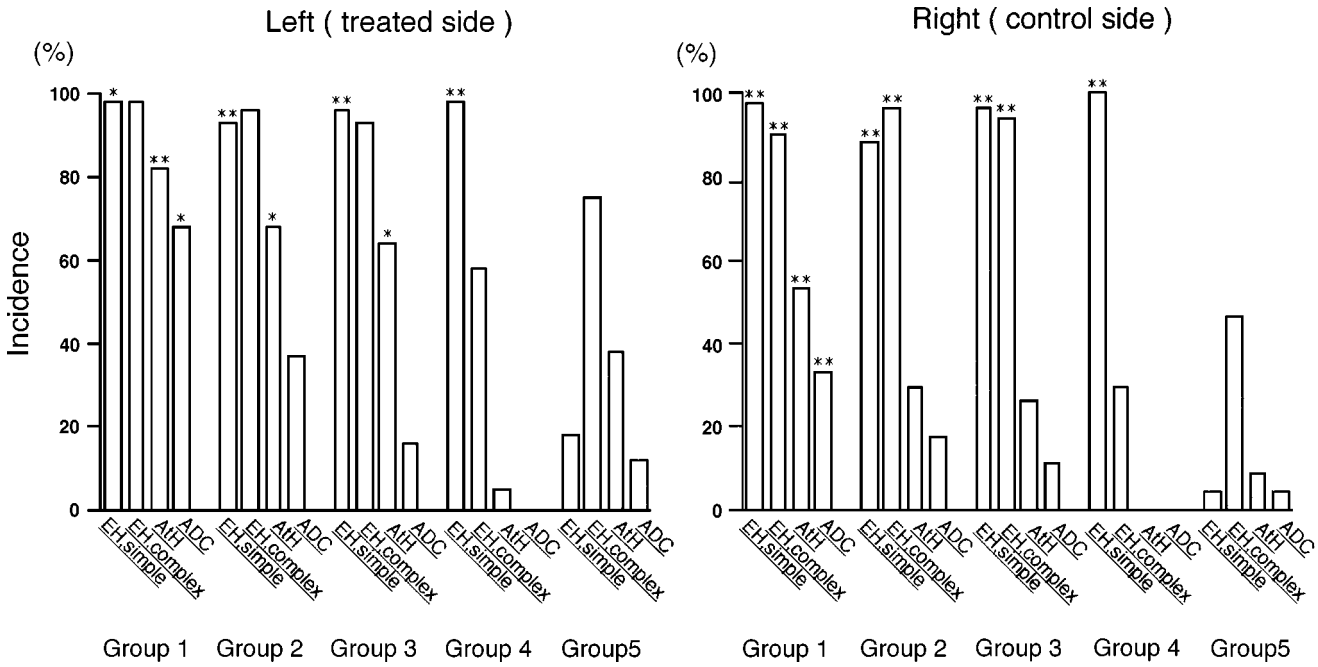

Fig. 7. Incidence of preneoplastic and neoplastic mouse endometrial lesions in each group. EH, simple: endometrial hyperplasia, simple. EH, complex: endometrial hyperplasia, complex. AtH: atypical endometrial hyperplasia. ADC: adenocarcinoma. * $P<0.01$, ** $P<0.05$.

Table III. Abnormalities of Ovary, Oviduct and Vagina in Each Group

Groups (Treatment)	Left side		Right side		Vagina
	Ovary	Oviduct	Ovary	Oviduct	
Group 1 [MNU/saline+E_2 (25 ppm)]	1/15 CL[a] (7%)* 5/15 Cystic (33%)	17/18 PPL[b] (94%)*	3/16 CL (19%)* 8/16 Cystic (50%)	16/18 PPL (89%)*	3/24 Pap[c] (13%)
Group 2 [MNU/saline+E_2 (5 ppm)]	16/17 CL (94%) 9/17 Cystic (53%)***	11/20 PPL (55%)**	11/12 CL (92%) 4/12 Cystic (33%)	11/16 PPL (69%)**	3/23 Pap (13%)
Group 3 [MNU/saline+TAM (5 ppm)]	2/17 CL (12%)* 4/17 Cystic (24%)	19/19 PPL (100%)*	1/16 CL (6%)* 4/16 Cystic (25%)	20/20 PPL (100%)*	6/6 Pap (100%)*
Group 4 [TAM (5 ppm) alone]	2/13 CL (15%)* 9/13 Cystic (69%)***	12/12 PPL (100%)*	2/13 CL (15%)* 8/13 Cystic (62%)	12/12 PPL (100%)*	12/14 Pap (86%)*
Group 5 (MNU/saline alone)	20/22 CL (91%) 3/22 Cystic (14%)	2/19 PPL (11%)	15/15 CL (100%) 3/15 Cystic (20%)	2/19 PPL (11%)	1/19 Pap (5%)

a) Corpora lutea.
b) Progressive proliferative lesion.
c) Papillary lesion.
* $P<0.001$, ** $P<0.01$, *** $P<0.05$ compared with the MNU/saline alone group.

tumors were present in any of the groups. Marked epithelial hyperplasia of the oviduct, diagnosed as "progressive proliferative lesion,"[21] was commonly observed in mice of groups 1–4 (Fig. 8). Papillary lesions were frequently seen in the vagina of mice treated with TAM (Fig. 9).

DISCUSSION

The transient expression of early genes, c-*fos*/*jun*, appears to be related to cellular proliferation and differentiation.[22] Acute administration of E_2 causes a transient increase in the expression of c-*fos*,[23] c-*jun*[24] and c-*myc*.[23] Among three natural estrogens (estrone, E_2 and estriol), E_2

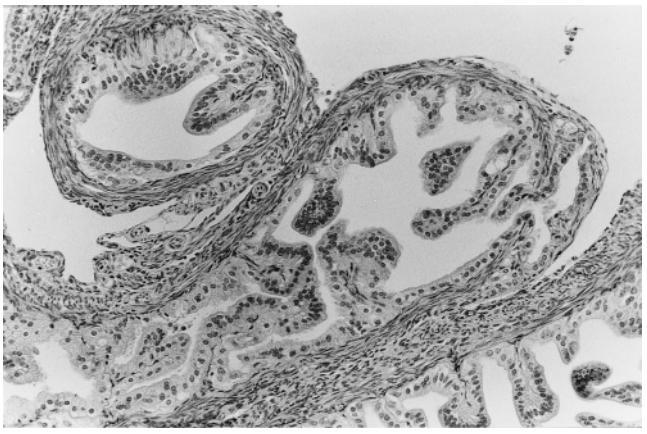

Fig. 8. Marked epithelial hyperplasia of a left oviduct from a mouse treated with TAM alone (HE, ×100). Note irregularities in the size and shape of mucosal folds.

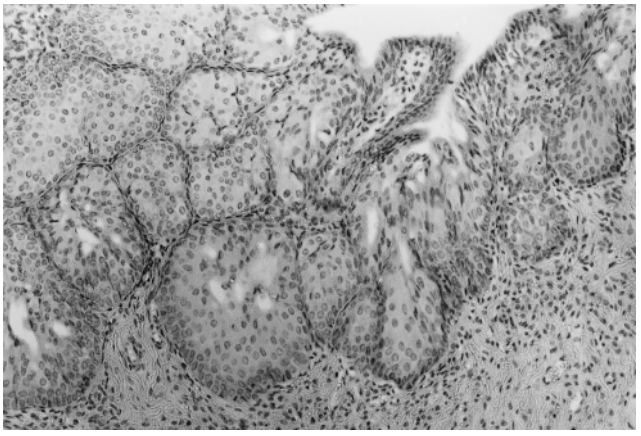

Fig. 9. Papillary lesion of the vagina in a mouse treated with TAM alone (HE, ×110).

is considered to exert the most prominent enhancing effect on MNU-induced endometrial carcinogenesis in mice.[14] Overexpression of c-*fos*/*jun* mRNA and their oncoproteins has been suggested to be related to the enhancing effects of the three natural estrogens.[25] Expression of the c-*fos* gene is induced transiently by various factors, such as platelet-derived growth factor,[26] epidermal growth factor,[27] nerve growth factor,[28] and even sex steroids,[23] and does not require any *de novo* protein synthesis. Consistent overexpression of Fos oncoprotein leads to cell transformation.[29] AP-1, a transcription regulation factor consisting of Jun-Jun or Fos-Jun protein dimers, binds to promoter sequences,[30, 31] to activate the transcription of target genes. In the present study, the enhancing effects of TAM on c-*fos*/*jun* mRNA expression, though similar to those of E_2, were weaker. The enhancing effects of TAM on c-*fos* mRNA expression are consistent with those found in a rat model.[32] Thus, the effects of TAM on endometrial carcinogenesis induced by MNU in mice may be related to the overexpressions of Fos/Jun oncoproteins.

Enhancing effects of TAM and E_2 on endometrial tumorigenesis in mice were seen in both uterine corpora. In this study, MNU was administered to the left uterine corpus, and normal saline to the right. It is possible that a small amount of the MNU solution flowed to the other uterine corpus from the injected side. This may be related to the different incidences of (pre)neoplastic lesions of the left and right uterine corpora. Such differences were also noted in our previous report.[14]

It is suggested that mutation of the rat *p53* gene is related to the hepatocarcinogenicity of TAM.[33] However, in our previous study, *p53* or *ras* gene mutation was rarely detected in the tissues of MNU and E_2-induced endometrial neoplasia or hyperplasia in mice.[34] Thus, such gene mutation may not be involved in MNU- and TAM-induced endometrial carcinogenesis in mice. Nevertheless, it is speculated that the enhancing effect of TAM on the tumorigenesis is related to overexpression of Fos/Jun oncoproteins via an estrogenic effect, even though the effect of TAM is weaker than that of E_2.

In the present study, the enhancing effect of TAM was recognized in the precancerous stage of endometrial tumorigenesis initiated by MNU. Recently, induction of uterine adenocarcinoma in mice treated neonatally with TAM alone was reported.[21] No adenocarcinomas, however, were found in the endometrium after treatment with TAM alone for 30 weeks in this study. The differences may be mainly due to the different ages at which TAM was administered. It is known that TAM is not hepatocarcinogenic in mice,[35] but is in rats.[35–37] This may be related to the lower level of DNA adducts induced by TAM in mice.[35, 37] Although E_2 is carcinogenic to the mouse endometrium,[38] TAM may be less estrogenic and less carcinogenic.

In the present study, the effect of TAM-exposure was seen in other tissues of the reproductive tract. The incidences of ovaries lacking corpora lutea in groups 1, 3 and 4 were significantly lower than in other groups. From the data in the present study, it is assumed that TAM affects ovarian functions more strongly than E_2. Although ovarian tumors were not detected in any of the groups, cysts of the ovaries were commonly noted in all groups. Furthermore, hyperplastic epithelium in the oviducts[21] was commonly observed in groups 1, 3 and 4, suggesting that TAM influences the oviducts more than E_2 does. These findings are consistent with the results of studies on neonatal exposure to TAM.[21]

TAM has been used as an adjuvant chemotherapy for breast cancer[1)] and is considered to be beneficial for prevention of breast cancer.[2, 3)] However, the results of this study indicate an enhancing effect of TAM on MNU-induced endometrial carcinogenesis in mice. Accordingly, it is suggested that a periodic check-up of the endometrium is necessary in patients taking TAM.

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