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Inhibitory Effects of Toremifene on *N*-Methyl-*N*-nitrosourea and Estradiol-17β-induced Endometrial Carcinogenesis in Mice

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Short- and long-term experiments were designed to determine the effects of toremifene (TOR) on estrogen-related endometrial carcinogenesis in mice. In the short-term experiment, a single low dose of TOR (0.2 mg/30 g body weight) decreased expression of c-*fos*, interleukin (IL)-1α, estrogen receptor (ER)-α mRNAs and corresponding proteins induced by estradiol-17β (E_2), in the uteri of the ovariectomized mice. Expression of ER-β mRNA was increased by the TOR treatment, compared with the control. In the long-term experiment, 106 female ICR mice were given *N*-methyl-*N*-nitrosourea (MNU) into their uterine corpora. The animals were divided into four groups as follows: group 1, E_2 diet (5 ppm) plus TOR (0.2 mg/30 g body weight, subcutaneously, every four weeks); group 2, E_2 diet alone; group 3, basal diet plus TOR. Group 4 served as the control. TOR treatment decreased the incidence of MNU and E_2-induced endometrial adenocarcinoma and atypical hyperplasia at the termination of the experiment (30 weeks after the start). These results suggest that TOR exerts preventive effects against estrogen-related endometrial carcinogenesis in mice, through the suppression of c-*fos* as well as IL-1α expression induced by E_2. Such suppressive effects of TOR may be related to the decreased ER-α and increased ER-β expressions.

Key words: Toremifene — Inhibition — Cytokines — Estrogen receptors — Endometrial carcinogenesis

Tamoxifen (TAM) and toremifene (TOR), which are structurally related polyphenylethylene anti-estrogens used for the treatment of breast cancer, are selective estrogen receptor modulators (SERMs). SERMs, however, have different biological effects in various target tissues. It is known that TAM induces endometrial cancers in patients treated for breast cancers, whereas TOR does not.[1)] TAM is reported to increase the incidence of preneoplastic lesions in the mouse endometrium, possibly through overexpression of c-*fos*/*jun*.[2)] In the rat liver, TAM exerts an initiating activity in carcinogenesis, but TOR does not.[3)] Further, only TAM enhances the formation of an endogenous adduct when TAM or TOR are chronically administered to rat uteri.[4)]

The transient expression of immediate early genes, c-*fos* and c-*jun*, appears to be related to cellular proliferation and differentiation.[5–7)] The acute administration of estradiol-17β (E_2) causes a transient increase of c-*fos*[6)] and c-*jun*[7)] expressions, followed by DNA replication. Among three natural estrogens (estrone, E_2 and estriol), E_2 is considered to exert the most prominent enhancing effect on mouse endometrial carcinogenesis initiated with *N*-methyl-*N*-nitrosourea (MNU).[8, 9)] The overexpression of c-*fos*/*jun* mRNA in uterine corpora of ovariectomized mice is closely related to such activities of estrogens.[8, 10, 11)]

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There is evidence that internal cytokines, such as interleukin (IL)-1α and tumor necrosis factor (TNF)-α, are important factors for promotion and progression in chemical carcinogenesis.[12–15)] However, no information on the effects of TOR on endometrial carcinogenesis in relation to the expression of such internal cytokines is available.

Estrogens and anti-estrogens are known to exert their biological effects via estrogen receptors (ERs), involving a variety of transcription factors. Two ER-α and -β isoforms, which are distributed differentially in various tissues,[16, 17)] are suggested to influence biological interactions with SERMs.[17, 18)]

This study was designed to determine if TOR exerts inhibitory effects on mouse endometrial carcinogenesis induced by MNU and E_2 or not. Furthermore, the effects of TOR on the expressions of c-*fos*/*jun*, IL-1α, TNF-α, ER-α and ER-β mRNAs, and corresponding proteins, induced by estrogen in mouse uteri, were also examined by reverse transcription-polymerase chain reaction (PCR)/Southern blot analysis and immunohistochemical methods. In addition, the levels of proliferating cell nuclear antigen (PCNA) were measured in histologically normal glandular cells of the endometrium to clarify the effects of TOR.

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 filtered tap water were available *ad libitum* throughout the experiment. E_2 and MNU were purchased from Sigma Chem. Co. (St. Louis, MO). TOR was purchased from Nihon Kayaku Co. (Tokyo).

Experimental protocol for short-term effects of TOR Female ICR mice, 12 weeks of age, were ovariectomized under general anesthesia with diethylether. Two weeks later, the ovariectomized mice were divided into four experimental groups (6 mice in each group). Groups 1 and 2 were given the diet containing E_2 (5 ppm). The 5 ppm-E_2-containing diet has an enhancing effect on mouse endometrial carcinogenesis.[9–11, 15] Mice of group 1 were subcutaneously injected with TOR at the dose of 0.2 mg/30 g body weight 24 h prior to resection of the uteri on the 13th day. The dose of TOR (0.2 mg/30 g body weight) was calculated from the clinical dose (40 mg/50 kg body weight/day). Mice of group 3 were subcutaneously injected with TOR. Group 4 served as a control. Resected uteri were cut longitudinally in half. One half was quickly frozen in liquid nitrogen for the following experiment, and the other was submitted to pathological examination.

Reverse transcription-PCR (RT-PCR) Total RNA was isolated from frozen tissue by a guanidium thiocyanate-phenol-chloroform extraction method.[19] Total RNA (3 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RTase, 200 units, GibcoBRL, Gaitherburg, 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 hexameters (50 ng, GibcoBRL) 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* (320 bp) and TNF-α (369 bp) mRNA expressions, we used 30 cycles of PCR (1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1.5 min at 72°C for extension). For c-*jun* (257 bp) and IL-1α (401 bp) mRNA expressions, 25 cycles of PCR were performed, consisting of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for extension. For ER-α (250 bp) mRNA expression, 30 cycles of PCR reaction were performed, consisting of 1 min at 95°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for extension. For ER-β (203 bp) mRNA expression, 35 cycles of PCR reaction were performed, consisting of 1 min at 94°C for denaturation, 1 min at 57°C for annealing, and 1 min at 72°C for extension. The PCR reaction was carried out with reverse-transcribed cDNAs and 0.1 μM specific primers using an Iwaki thermal sequencer TSR-300 (Iwaki Glass, Tokyo) with Vent DNA polymerase (New England Biolabs, Beverley, 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 simultaneously.

The following oligodeoxynucleotides were synthesized as specific primers in PCR according to the published information [cDNAs for c-*fos*,[20] c-*jun*,[21] IL-1α,[22] TNF-α,[23] and ER-α,[24] ER-β[24] and GAPDH[20]]: sense for c-*fos*, 5′-GCTTCTATAAAGGCGCCAGCTGA-3′; anti-sense for c-*fos*, GACAGGAGAGCCCATGCTGGAG-3′; sense for c-*jun*, 5′-GGAGTGGGAAGGACGTGGCGC-3′; anti-sense for c-*jun*, 5′-TCCCAGCCCTCCCTGCTTTGTG-3′; sense for IL-1α, 5′-GATGGCCAAAGTTCCTGACTTG-3′; anti-sense for IL-1α, GCCTGACGAGCTTCATCA-GTTT-3′; sense for TNF-α, 5′-AGGCAGGTTCTGTCCC-TTTCA-3′; anti-sense for TNF-α, 5′-TCCACTTGGTGG-TTTGCTACG-3′; sense for ER-α, 5′-GAGAAAGGAAA-CATGATCATGGA-3′; anti-sense for ER-α, 5′-TTCATC-ATGCCCACTTGGTAAC-3′; sense for ER-β, 5′-AAAGC-CAAGAGAACCAGTGGGCAC-3′; anti-sense for ER-β, 5′-GCCAATCATGTGCACCAGTTCCTT-3′; and sense for GAPDH, 5′-CAAGGTCATCCCAGAGCTGAA-3′; anti-sense for GAPDH, 5′-GCAATGCCAGCCCCGGCA-TCG-3′.

Semi-quantitative analysis of c-*fos*/*jun*, IL-1α, TNF-α, ER-α and ER-β mRNA expressions by Southern blot of PCR products PCR products were applied to 1.5% agarose gel electrophoresis performed 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 tightly fix the products, prehybridized in 1 *M* NaCl, 50 μM Tris-HCl (pH 7.6) and 1% sodium dodecyl sulfate (SDS) at 42°C for 1 h, and hybridized in the same solution with biotinylated oligodeoxynucleotide probes synthesized from the sequences between the specific individual primers at 65°C overnight. Specific bands hybridized with biotinylated probes were detected with Plex Luminescent Kits (Millipore Corp.) on the membrane after exposure to X-ray films at room temperature for 10 min. The semi-quantification of Southern blot was carried out with a Bio Image analyzer (Millipore Corp.). The intensity of specific bands was standardized with that of GAPDH mRNA.

Immunohistochemical expressions of c-*fos*/*jun*, IL-1α, TNF-α, ER-α and ER-β protein After having been fixed in 10% formalin, half of the uterine corpus was processed for conventional staining methods. Briefly, the avidin-biotin-peroxidase complex (ABC) was applied using a Vestain Kit (Vector, Burlingame, CA). The primary antibodies against c-*fos*, c-*jun*, IL-1α and TNF-α (anti-rabbit polyclonal, Oncogene Science, Inc., New York, NY), ER-α (anti-rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), and ER-β (anti-rabbit polyclonal, Santa Cruz Biotechnology) were used at 1:100 dilution.

Staining intensity for c-*fos*/*jun*, IL-1α and TNF-α was assigned as follows[11]: (+), positive; (+/−), minimally or randomly positive; (−), negative. Sections stained with ER-α and -β were scored according to the intensity and proportion of cells highlighted as proposed by Giri *et al*.[25] The staining intensity was scored as follows: 0=no staining; 1=weak nuclear staining; 2=moderate nuclear staining; 3=strong nuclear staining. The proportion of cells stained in each category was noted and H-score was calculated as follows: (3×% strong nuclear staining)+(2×% moderate nuclear staining)+(1×weak nuclear staining), with the score ranging from 0 to 300.[26] Immunohistochemical findings were analyzed by two independent investigators counting more than 200 cells and discordant results were reviewed jointly.

Experimental protocol for long-term effects of TOR A total number of 106 female ICR mice, 10 weeks of age, underwent laparotomy under general anesthesia with diethylether. MNU solution (total volume, 0.1 ml) at a dose of 1 mg/100 g body weight was injected into the left uterine tube and normal saline into the right. One week after the MNU exposure, the animals were divided into the following three experimental groups. Groups 1 (23 mice) and 2 (30 mice) were given a diet with 5 ppm E_2 throughout the experiment, and mice in group 1 were injected with TOR subcutaneously at the dose of 0.2 mg/30 g body weight every four weeks throughout the experiment. The doses of E_2 and TOR were the same as those in the short-term experiment. The 5 ppm E_2-containing diet was considered to be an adequate dose, as shown in our previous studies.[9–11, 15] Group 3 (23 mice) was given TOR subcutaneously. Group 4 (30 mice) was given basal diet alone. Thirty weeks after the MNU exposure, all animals were killed and autopsied. All major organs, especially the reproductive organs, were inspected carefully. The uterus, ovaries, vagina, and other lesions suspected of being neoplastic and hyperplastic were submitted to histological examination. Each corpus uteri was weighed. Tissues were sectioned at 3 μm and stained with hematoxylin and eosin.

Histology of the uterine and ovarian lesions According to the WHO criteria,[27] uterine endometrial lesions were divided into four lesions: (i) endometrial hyperplasia (EH), simple; (ii) EH, complex; (iii) atypical endometrial hyperplasia (Aty Hyp); (iv) adenocarcinoma (ADC).

For the assessment of hormonal condition in each group, the ovarian cyst and corpus lutea were evaluated.

Immunohistochemical staining for PCNA The PCNA-labeling index was evaluated in pathologically normal endometrial glandular cells in several mice of each group in the long-term experiment by use of the ABC method. After deparaffinization, endometrial sections were treated sequentially with normal goat serum, anti-PCNA antibody (DAKO Japan Co., Ltd., Tokyo), biotin-labeled goat anti-rabbit IgG (1:400) and ABC. The peroxidase binding sites were demonstrated by the diaminobenzidine method. PCNA-positive nuclei were counted under a microscope and expressed as a proportion of over 200 pathologically normal endometrial glandular cells in five mice of each group.

Statistical analyses Statistical analysis was done according to the κ^2 test, Fisher's exact probability test, or Student's *t* test.

RESULTS

Short-term experiment Measurement of the expression

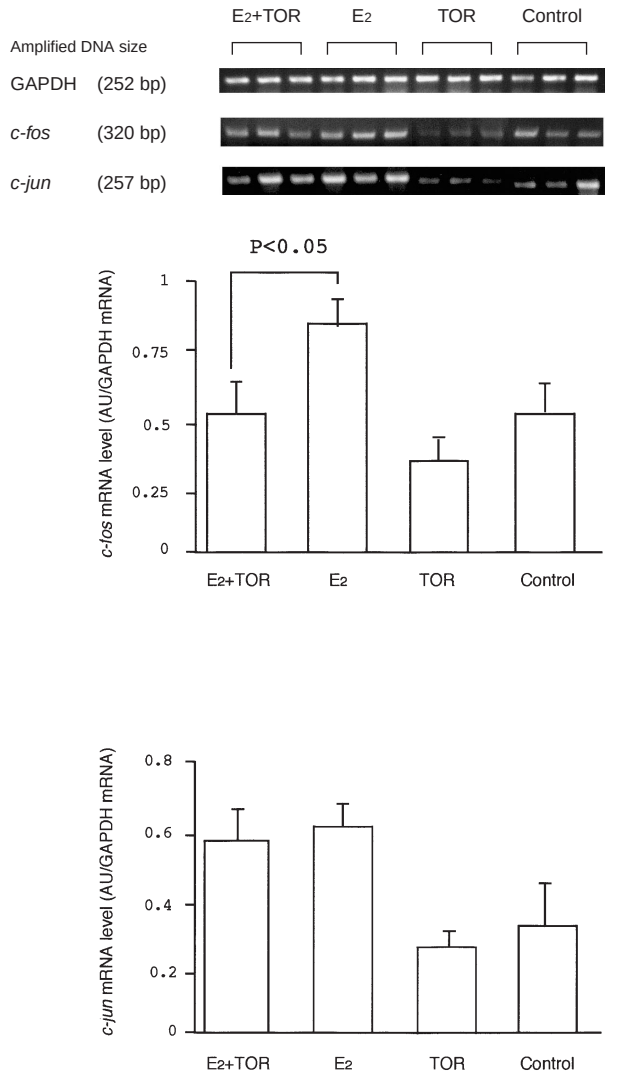

Fig. 1. Expression of c-*fos* and c-*jun* mRNA in the uteri of ovariectomized mice treated continuously for 2 weeks with E_2 and TOR injection 24 h prior to resection of the uteri, E_2 alone, TOR alone and no treatment control.

of mRNA was done with the whole uteri of the animals in each group (5–6 animals in each). Representative bands in each group are shown in Figs. 1–3. The levels of c-*fos* and c-*jun* mRNA expressions are presented in Fig. 1. TOR exposure significantly decreased the level of c-*fos* mRNA induced by E_2 treatment ($P<0.05$). TOR also tended to decrease the c-*jun* mRNA levels. The levels of IL-1α and TNF-α mRNA expressions are indicated in Fig. 2. TOR exposure significantly decreased the expression of E_2-induced IL-1α mRNA ($P<0.001$). ER-α and ER-β mRNA expressions are shown in Fig. 3. TOR also significantly decreased the expression of ER-α mRNA generated by E_2 treatment ($P<0.05$). Conversely, TOR increased the expression of ER-β mRNA when compared with the control group ($P<0.001$). The ER-β mRNA/ER-α mRNA ratio is indicated as follows: E_2 plus TOR, 1.3±0.2; E_2 alone, 1.1±0.2; TOR alone, 2.5±0.6; non-treatment control, 0.5±0.1. Although the ratio of the group with E_2 and TOR showed only a relatively small increase when compared with the group with E_2 alone, TOR treatment alone significantly increased the ratio in comparison with the control ($P<0.001$).

Immunohistochemical staining for c-*fos* oncoprotein is shown in Fig. 4, A) and B). The expression of c-*fos* onco-

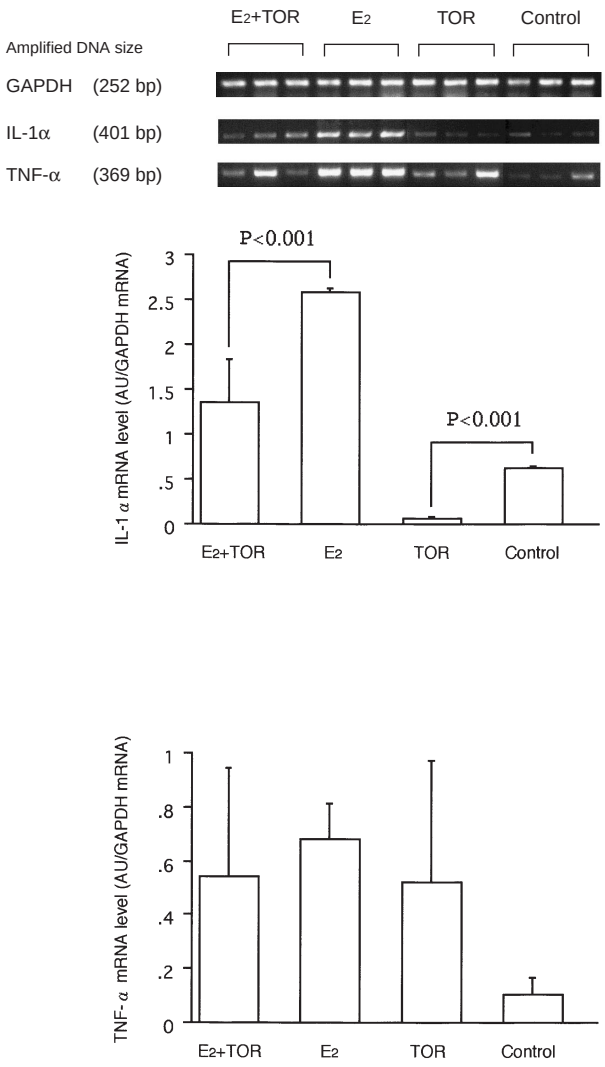

Fig. 2. Expression of TNF-α and IL-1α mRNA in the uteri of ovariectomized mice treated continuously for 2 weeks with E_2 and TOR injection 24 h prior to resection of the uteri, E_2 alone, TOR alone and no treatment control.

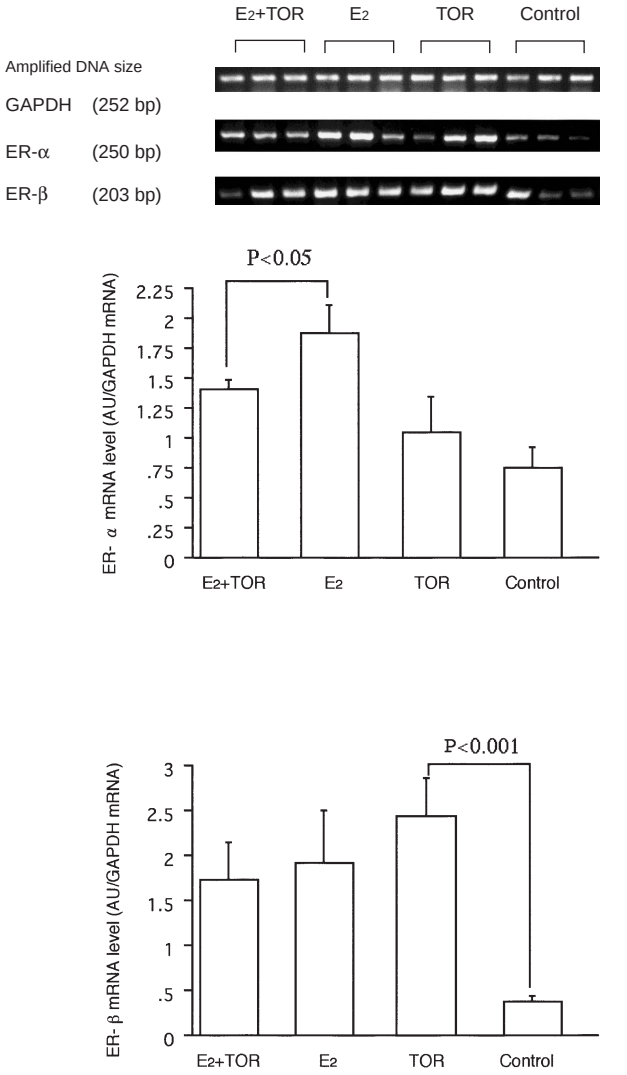

Fig. 3. Expression of ER-α and ER-β mRNA in the uteri of ovariectomized mice treated continuously for 2 weeks with E_2 and TOR injection 24 h prior to resection of the uteri, E_2 alone, TOR alone and no treatment control.

protein was prominent in glandular and luminal cells treated with E_2, and the expression was decreased by TOR. The expression of c-*fos* and c-*jun* oncoproteins is summarized in Table I. The expressions tended to decrease on TOR treatment. Immunohistochemical staining for IL-1α is shown in Fig. 4, C) and D). The expression of IL-1α, which was in the same place as c-*fos*, was also decreased by TOR. The expression of IL-1α and TNF-α is also shown in Table I. The expressions tended to be decreased by TOR treatment. Immunohistochemical staining for ER-α is shown in Fig. 5, A) and B). The expression of ER-α, which was present predominantly in the glandular cells treated with E_2, was also decreased by TOR. However, that of ER-β was increased by TOR [Fig. 5C)], in comparison with the no treatment control [Fig. 5D)]. The H-scores of ER-α and -β of the three kinds of cells are indicated in Table II. As all H-scores of ER-α were over 75 in the three types of cells in each group,[26] ER-α were considered to be positive. Meanwhile, as the H-scores of ER-β were under 50 except for stromal cells in the group treated with TOR alone, ER-β of these cells was considered to be negative. The expression of ER-β, especially in stromal cells, tended to be increased by TOR treatment.

Long-term experiment Four mice in group 1, 6 in group 2, 3 in group 3 and 4 in group 4 died within 15 weeks. No pathological abnormalities other than pneumonia were found in these animals. The remaining animals survived more than 15 weeks and were enrolled as effective animals (Table III). No significant difference in the mean body weight was found among groups. Under E_2 treatment, the mean wet weight of the left or right uterine corpora of group 1 was smaller than that of group 2 ($P<0.01$). The mean wet weights of both uterine corpora of group 3 were significantly smaller than those of group 4 ($P<0.01$).

Histopathology of EHs and ADCs was the same as described in the previous reports.[9–11, 15] All ADCs developed in the endometria were well or moderately differentiated types. No significant differences of histopathological features of ADCs among the four groups were recognized. All ADCs in the endometria in the mice treated with E_2 were accompanied with EH, simple and/or complex.

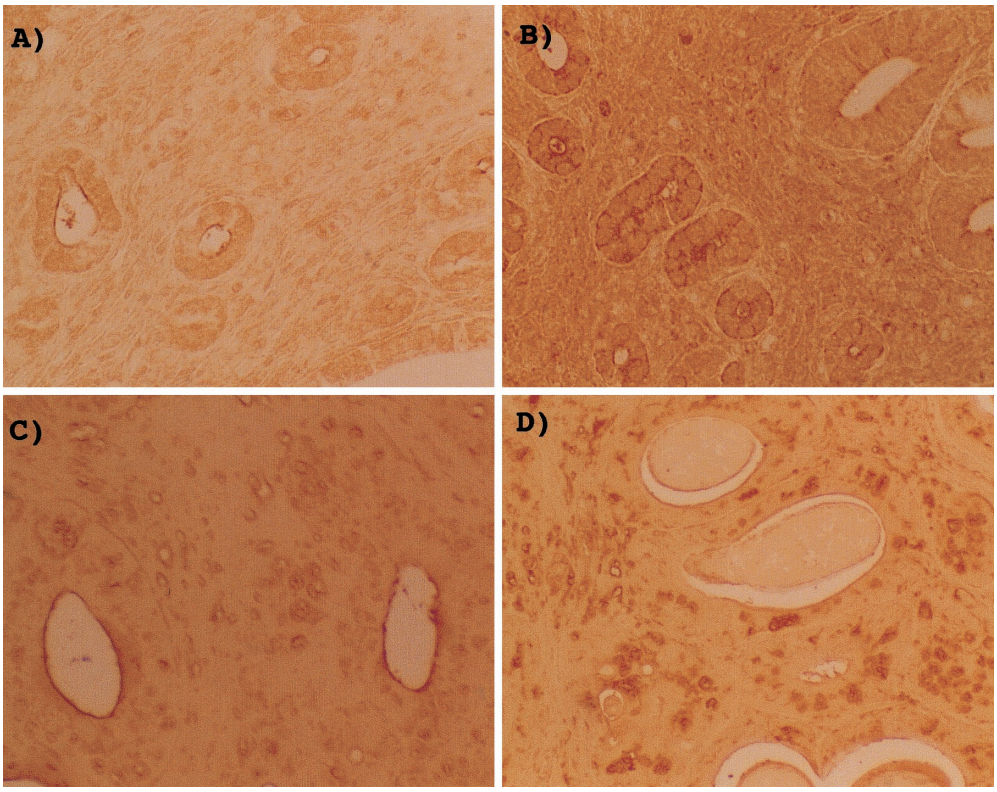

Fig. 4. The expressions of c-*fos* (A and B) and IL-1α (C and D) in the uteri of ovariectomized mice treated orally for 2 weeks with E_2 and TOR injection 24 h prior to resection of the uteri (A and C) (sABC stain, ×350), and with E_2 alone (B and D) (sABC stain, ×350). The expressions with E_2 and TOR were predominantly present in the glandular cells, and both were weaker than those in the case of E_2 alone.

Table I. Immunohistochemical Expressions of c-*fos*, c-*jun*, IL-1α and TNF-α of Ovariectomized Mouse Uterus after 2 Weeks Feeding with E_2 Diet Alone, E_2 plus TOR Injection, or TOR Injection Alone

Treatment	c-*fos*			c-*jun*			IL-1α			TNF-α		
	G[a)]	L	S	G	L	S	G	L	S	G	L	S
Group 1 (E_2+TOR)	+/−[b)]	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−
Group 2 (E_2 alone)	+	+	+/−	+	+	+/−	+	+/−	+/−	+	+	+/−
Group 3 (TOR alone)	+/−	−	−	+/−	−	−	+/−	−	−	+/−	−	−
Group 4 (no treatment)	+/−	−	−	+/−	−	−	+/−	−	−	+/−	−	−

a) G, glandular cells; L, luminal cells; S, stromal cells.
b) +, positive; +/−, minimally or randomly positive; −, negative.

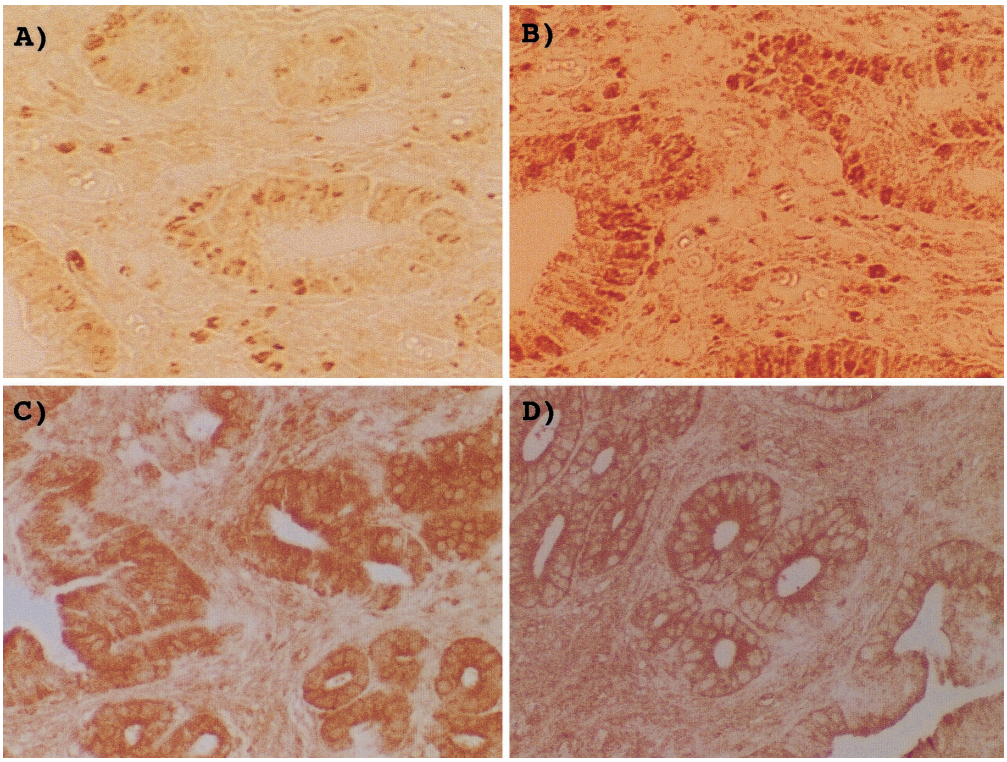

Fig. 5. The expression of ER-α (A and B) in the uteri of ovariectomized mice treated orally for 2 weeks with E_2 and TOR injection 24 h prior to resection of the uteri (A) (sABC stain, ×350), and with E_2 alone (B) (sABC stain, ×350). The expression with E_2 and TOR was also prominent in the glandular cells, and was weaker than that in the case of E_2 alone. The expression of ER-β (C and D) in the uteri of ovariectomized mice treated with TOR injection 24 h prior to resection of the uteri (C) (sABC stain, ×350), and no treatment control (D) (sABC stain, ×350). The expression with TOR injection was also prominent in the glandular cells, and was more prominent than that in the no treatment control.

Table II. Immunohistochemical H-scores of ER-α and ER-β of Ovariectomized Mouse Uterus after 2 Weeks Feeding with E_2 Diet Alone, E_2 plus TOR Injection, TOR Injection Alone

Treatment	ER-α			ER-β		
	Glandular cells	Luminal cells	Stromal cells	Glandular cells	Luminal cells	Stromal cells
Group 1 [E_2+TOR, (n=5)]	145.5±6.7[a)]	122.1±9.0	77.2±29.6	19.9±5.5	23.9±10.9	33.6±15.8[b)]
Group 2 [E_2 alone, (n=4)]	187.9±15.9	175.4±24.3	126.2±33.7	20.1±10.9	17.2±4.4	10.8±3.7
Group 3 [TOR alone, (n=4)]	146.5±18.2	136.5±23.4	109.7±18.5	28.4±11.4	31.4±10.4	56.9±12.7[b)]
Group 4 [no treatment, (n=4)]	96.0±16.4	98.0±30.1	79.7±40.7	10.7±5.3	17.6±12.5	21.5±13.9

a) Mean±SD.
b) Significant differences are present from each control group (P<0.05).

Table III. Mean Body Weight, and Mean Weight of Left (Treated) and Right (Control) Uterine Corpora of Mice in Each Group

Group (treatment)	Initial number of animals	Effective number of animals[a)]	Body weight (g)	Wet weight of uterine corpora (g)	
				Left	Right
1 (MNU/saline+TOR+E_2)	23	19	35.1±3.5[b)]	0.14±0.06[*]	0.13±0.04[*]
2 (MNU/saline+E_2)	30	24	42.8±5.0	0.71±0.25[*]	0.35±0.16
3 (MNU/saline+TOR)	23	20	39.5±4.8	0.10±0.04[*]	0.11±0.03[*]
4 (MNU/saline alone)	30	26	48.0±5.9	0.47±0.33	0.33±0.20

a) Animals that survived more than 15 weeks.
b) Mean±SD.
∗ P<0.01 compared with each control group.

Table IV. Incidence of Preneoplastic and Neoplastic Mouse Endometrial Lesions in Each Group

Group (treatment)	Left side				Right side			
	EH, simple[a)]	EH, complex	AtH	ADC	EH, simple	EH, complex	AtH	ADC
1 (MNU/saline+TOR+E_2)	18/19 (95%)	15/19 (79%)	4/19[*] (21%)	1/19[**] (5%)	15/19 (79%)	14/19 (74%)	2/19 (11%)	1/19 (5%)
2 (MNU/saline+E_2)	22/24 (92%)	23/24 (96%)	16/24 (67%)	8/24 (33%)	21/24 (88%)	23/24 (96%)	7/24 (29%)	4/24 (17%)
3 (MNU/saline+TOR)	2/20 (10%)	14/20 (70%)	7/20 (35%)	1/20 (5%)	3/20 (15%)	4/20 (20%)	5/20 (25%)	1/20 (5%)
4 (MNU/saline alone)	5/26 (19%)	20/26 (77%)	7/26 (27%)	3/26 (12%)	1/26 (4%)	12/26 (46%)	2/26 (8%)	1/26 (4%)

a) EH, simple: endometrial hyperplasia, simple; EH, complex: endometrial hyperplasia, complex; AtH: atypical endometrial hyperplasia; ADC: adenocarcinoma.
∗ P<0.05, ∗∗ P<0.01 compared with each control group.

Meanwhile, all ADCs in the endometria of the animals without E_2 did not have other preneoplastic lesions. The incidence of preneoplastic as well as neoplastic lesions of the endometria is summarized in Table IV. The incidence of atypical hyperplasia and ADC of the treated side of the uterine corpus of group 1 (treated with E_2 plus TOR) was significantly lower than that of group 2 (treated with E_2 alone) (P<0.05 and P<0.01, respectively).

Table V. PCNA-labeling Index of Histologically Normal Glandular Cells in the Endometrium in Each Group

Group (treatment)	Number of animals examined	PCNA-labeling index
1 (MNU/saline+TOR+E_2)	5	35.2±1.6[a)]
2 (MNU/saline+E_2)	5	39.7±6.7
3 (MNU/saline+TOR)	5	25.8±5.7
4 (MNU/saline alone)	5	31.4±11.8

a) Mean±SD.

Table VI. Pathological Findings of Ovary (Left Side) in Each Group

Group (treatment)	Ovary	
1 (MNU/saline+TOR+E_2)	17/19 CL[a)] (89%)	2/19 Cyst (11%)
2 (MNU/saline+E_2)	16/17 CL (94%)	9/17 Cyst (53%)[b)]
3 (MNU/saline+TOR)	21/21 CL (100%)	2/21 Cyst (10%)
4 (MNU/saline alone)	20/22 CL (91%)	3/22 Cyst (14%)

a) CL: corpora lutea.
b) Significantly different from the other three groups, $P<0.05$.

Data for PCNA-positive cells per 200 normal glandular cells are indicated in Table V. In histologically normal glandular cells, the PCNA-labeling index in mice treated with TOR tended to be smaller than that without TOR, although no significant difference was observed in any group.

Pathological findings of the ovary in each group are summarized in Table VI. The incidence of cystic ovaries in group 2 was significantly higher than that in the other three groups ($P<0.05$). Corpora lutea were mostly present in each group. No tumors were present in any of the groups.

DISCUSSION

It is reported that estrogens and SERMs, such as TOR or TAM are usually able to bind to genes possessing an AP-1 site, apart from classical estrogen receptor elements.[28)] The transcription factors, Fos and Jun, are known to require binding of ER to the AP-1 site. In this study, TOR suppressed estrogen-induced expression of c-*fos* mRNA and its oncoprotein in the uterine corpora of ovariectomized mice. Since c-*fos* and c-*jun* fluctuate with the estrus cycle in response to estrogens,[29)] ovariectomized mice were used to avoid hormonal effects in the short-term assay. The inhibitory effect of TOR on the endometrial carcinogenesis was prominent in the groups treated with estrogen, suggesting that TOR has an inhibitory effect, possibly through suppression of estrogen-related carcinogenesis.

It is well known that development of various tumors, especially hormone-dependent tumors, in animal models is influenced by the nutritional condition of the hosts. In the present long-term study, mean body weights in groups 1 and 3 were somewhat lower than those in groups 2 and 4, respectively, although the differences were not significant. Body weight loss has been reported in long-term experiments with SERMs, such as TAM and TOR.[30, 31)] This is considered to be due to decreased consumption of diet, although the decrease of consumed diet containing TAM or TOR has not been clarified.[30, 31)] Thus, the increased incidence of (pre)neoplastic lesions may not be related to the nutritional condition in this study.

We found that TOR suppressed the expression of IL-1α mRNA and the corresponding protein. TNF-α and IL-1α are known to stimulate tumor promotion and progression of initiated and preneoplastic cells in chemical carcinogenesis.[12–15, 32–34)] Thus, the inhibitory effects of TOR on mouse endometrial carcinogenesis may be related to the inhibition of IL-1α expression.

ER-α mRNA level was increased in the uteri of mice by E_2 treatment, whereas it was decreased by TOR treatment. Furthermore, expression of ER-β mRNA in the uteri of mice was increased by TOR without E_2. Such an increase in the expression of ER-α by E_2 is consistent with the previous report.[35)] The ER-β mRNA expression in the stroma was decreased by E_2 treatment in the previous report.[35)] In this study, however, ER-β mRNA expression was increased in the whole uterus, whereas ER-β protein expression (H-score) of the three types of cells was differently affected by E_2 in this study. This discrepancy may be related to differences in the proportions of epithelial (glandular and luminal) and stromal cells, and amounts of internal hormone, due to the age of animals or castration.

It is known that expressions of ER-α and ER-β are regulated by estrogens and anti-estrogenic compounds.[36)] Such transcriptions are under the control of two pathways: estrogen response elements and AP-1 sites. The AP-1 pathway is suggested to be the major route for target tissue growth and differentiation *in vivo*.[37)] The treatment of estrogens as well as SERMs increased the ligand activation profile of ER-α at an AP-1 element. In an ER-α knockout mice model, it was shown that functional ER is necessary for the proliferative response to estrogens in the uterus.[35)] In contrast to the results seen with ER-α, data on ER-β at an AP-1 element clarified a difference between estrogens and SERMs.[16)] Although the precise role of ER-β has not been fully clarified, it is suggested that ER-β plays a role in modulation of the effects of ER-α. Further-

more, ER-β has an anti-proliferative effect in the immature uterus.[35] In this study, the expression of ER-β in the uteri of mice treated with TOR was increased compared to the control level. The ER-β/ER-α mRNA ratio was also increased by treatment with TOR alone. ER-β may exert an inhibitory effect against ER-α synthesis, as shown in breast cancer.[38–40] In the long-term experiment of this study, the PCNA-labeling index in the histologically normal glandular cells in the endometria tended to be decreased by TOR treatment. Accordingly, decreased expression of ER-α and increased expression of ER-β by TOR may be related to the inhibitory effects of this agent on endometrial carcinogenesis in mice.

The hormonal milieu of animals is considered to be very important in endometrial carcinogenesis. In the present study, pathological examinations of the ovary were done to investigate the hormonal milieu. Corpora lutea were frequently seen in each group. In our previous report, TAM-exposure generated significantly decreased frequency of loss of corpora lutea.[2] It is assumed that effect of TOR on ovarian function is weaker than that of TAM. Although ovarian tumors were not detected in any of the groups, cyst formation was noted in ovaries. The frequency of the ovarian cysts in group 2 was significantly higher than in the other three groups. Although the reasons for the difference are not yet clear, it may be that the effects of TOR on the ovary are less than those of E_2.

In conclusion, TOR had an inhibitory effect on estrogen-related endometrial carcinogenesis in mice, presumably through the suppression of c-*fos* and IL-1α expressions, and an increased ER-β/ER-α ratio.

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