Effects of selective estrogen receptor modulators and genistein on the expression of ERα/β and COX-1/2 in ovarectomized mouse uteri

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Summary

This study was performed to examine the effects of selective estrogen receptor modulators [tamoxifen (TAM) and toremifene (TOR)] and pure anti-estrogen, ICI-182780 (ICI, Faslodex) and soybean isoflavone, genistein (GE) on the expression of estrogen-stimulated c-fos/jun, ERα/β and COX-1/2 in the uteri of ovarectomized mice. TAM, TOR, ICI and GE treatment significantly decreased the levels of estradiol (E_2)-induced c-fos. ICI and GE treatment significantly decreased the levels of E_2-induced c-jun and ERα expressions. High doses of TOR treatment significantly increased the E_2-induced ERβ expression. In contrast, ICI and GE treatment significantly decreased the levels of E_2-induced COX-2 expression, thus suggesting that TOR and GE might prevent E_2-related endometrial carcinogenesis.

Key words: SERMs; Genistein; c-fos/jun; ERα/β; COX-1/2.

Introduction

The endometrium is a major target organ of ovarian sex steroid hormones, estradiol and progesterone, which mediate diverse physiological functions via interaction with their nuclear receptors [1]. Recent studies have noted a small but significant increase in the incidence of endometrial cancer in postmenopausal women using hormone replacement therapy [2], while an increased risk of endometrial cancer has also been observed, particularly in postmenopausal women treated with selective estrogen receptor modulators (SERMs) such as tamoxifen (TAM) in a chemopreventive setting for breast cancer [2].

TAM and TOR are structurally related polyphenylethylene anti-estrogens (Figure 1). SERMs, however, have different biological effects on various target organs. Only TAM enhances the formation of an endogenous adduct when TAM or TOR are chronically administered to rat uteri [3]. We have previously examined the effects of TAM [4] and toremifene (TOR) [5] on estradiol (E_2)-related endometrial carcinogenesis in mice. TAM was shown to have an enhancing effect [4], meanwhile TOR was found to have a preventive effect on E_2-related endometrial carcinogenesis [5].

The transient expression of immediate early genes, c-fos and c-jun, appears to be related to cellular proliferation and differentiation [6-8]. The acute administration of E_2 causes a transient increase of both the c-fos [7] and c-jun [8] expressions, followed by DNA replication. The over-expression of c-fos/jun mRNA in the uterine corpus of ovarectomized mice is therefore closely related to the activities of estrogens [9, 10].

Estrogens and anti-estrogens are known to exert their biological effects via estrogen receptors (ERs), while involving a variety of transcription factors. Two ERα and ERβ isoforms, which are distributed differentially in various tissues [11, 12], are suggested to influence the biological interactions with SERMs and isoflavones [13]. Recent data suggest ERβ to be a possible tumor-suppressor in estrogen-dependent tumor progression [14, 15].

Meanwhile, it is known that cyclooxygenase (COX), an enzyme activating producer of prostaglandins from arachidonic acid, has two isoforms (1 and 2). The function of inducible COX-2 is associated with endometrial carcinogenesis in humans [16, 17] and mice [18].

These circumstances prompted us to examine the effects of SERMs (TAM and TOR) and genistein on the expression of c-fos/jun, ERα/β and COX-1/2 in ovarectomized mice uteri.

Materials and Methods

Animals and chemicals

Female ICR mice, ten weeks of age, were purchased from Japan SLC Co. (Shizuoka Japan). They were maintained at the Animal Facility of Gifu University School of Medicine according to the Institutional Animal Care Guidelines. Oriental MF (Oriental Yeast Co., Tokyo, Japan) was used as a basal diet. The Diet and filtered tap water were available *ad libitum* throughout the experiment. All animals were housed in plastic cages (four or five mice/cage). Estradol-17β (E_2), tamoxifen (TAM), toremifene (TOR), ICI-182780 (ICI, Faslodex) and genistein (GE) were purchased from Aldrich Chem. Co. (Milwaukee, WI), and their chemical structures are shown in Figure 1.

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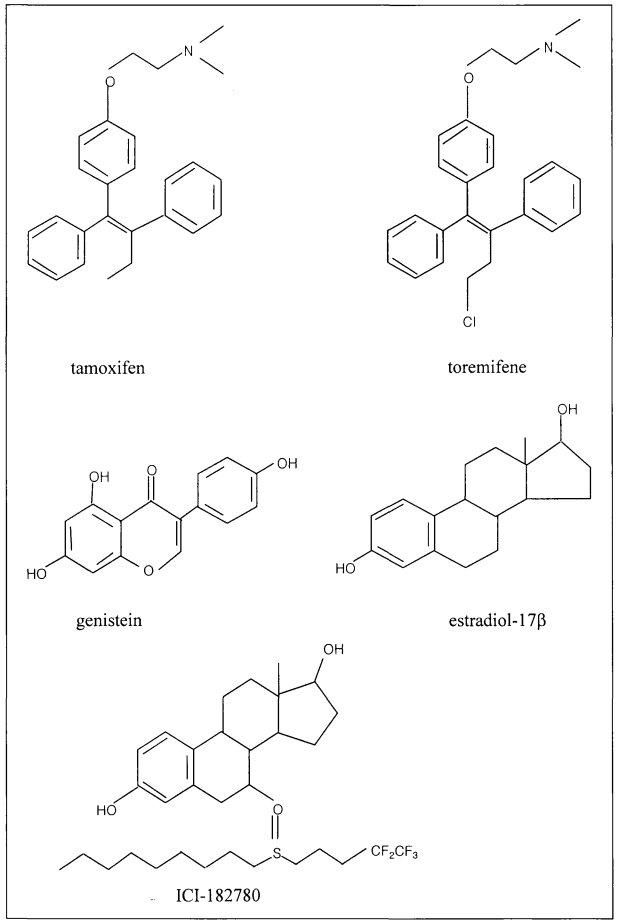

Figure 1. — The structure formula of tamoxifen, toremifene, genistein, estradiol-17β and ICI-182780.

Experimental protocol

According to a previous study [4, 5], ovariectomized mice, from 12-13 weeks of age, were divided into nine experimental groups (5 mice in each). Groups 1-8 were given a daily diet containing E_2 (5 ppm) for two weeks. Groups 1-7 received a single subcutaneous (SC) injection of TAM, TOR, ICI and GE, 24 hours prior to the resection of the uteri on the 13th day. Group 1 received an SC injection of high-dose TAM (1 mg/30 g/body wt) while group 2 received an SC injection of low-dose TAM (0.1 mg/30 g/body wt). Group 3 was given an SC injection of high-dose TOR (1 mg/30 g/body wt) and group 4 received an SC injection of low-dose TOR (0.1 mg/30 g/body wt). Group 5 received an SC injection of high-dose ICI (1 mg/30 g/body wt) and group 6 received an SC injection of low-dose ICI (0.1 mg/30 g/body wt). Group 7 received an SC injection of GE (1 mg/30 g/body wt). The low dose of SERMs was calculated to be half of that described in our previous report [5]. The high dose was determined to be ten times that of the low dose. TAM, TOR, ICI and GE were dissolved in ethanol, and mixed with sesame oil. Groups 8 and 9 were given a mixture of ethanol and sesame oil alone as a non-treatment control. After two weeks of treatment, the mice uteri were resected and then the uteri were cut in half longitudinally. One half was quickly frozen in liquid nitrogen, while the other was subjected to pathological and immunohistochemical examinations.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the frozen tissues using a guanidinium thiocyanate phenol-chloroform extraction method [9]. The total RNA (3 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RTase, 200 units, Gibce 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 the MMLV-RTase. The PCR conditions of c-fos [7], c-jun [8], ERα/β [20], COX-1,2 [21] and GAPDH [7] were performed as described previously [4, 5, 18]. The DNA sequences for specific primers are shown in Table 1. For the c-fos (320 bp) mRNA expression, 35 cycles of PCR reaction were performed, consisting of 1 min at 94°C for denaturation, 1 min at 53°C for annealing, and 1.5 min at 72°C for extension. For the expression of ERα (250 bp) and c-jun (257 bp) mRNA, we used 30 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. For ERβ (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 2 min at 72°C for extension. For COX-1 (450 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. For COX-2 (583 bp) mRNA expression, 35 cycles of PCR reaction were performed, consisting of 15s at 94°C for denaturation, 1 min at 55°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 a TSR-300 thermal sequencer

Table 1. — *Sequences of primers.*

Primers	Nucleotide sequences	Citation
c-fos sense	5'-GCTTCTATAAAGGCGCCAGCTGA-3'	7
c-fos antisense	5'-GACAGGAGAGCCCATGCTGGAG-3'	
c-jun sense	5'-GGAGTGGGAAGGACGTGGCG-3'	8
c-jun antisense	5'-TCCCAGCCCTCCCTGCTTTGTG-3'	
ERα sense	5'-TGTGCAATGACTATGCTTCA-3'	20
ERα antisense	5'-GCTCTTCCTCCTGTTTTTA-3'	
ERβ sense	5'-GTCCATCGCCAGTTATCACATC-3'	20
ERβ antisense	5'-GCCTTACATCCTTCACACGA-3'	
COX-1 sense	5'-TGCATGTGGCTGTGGATGTCATCAA-3'	21
COX-1 antisense	5'-CACTAAGACAGACCCGTCATCTCCA-3'	
COX-2 sense	5'-ACTCACTCAGTTTGTTGAGTCATTC-3'	21
COX-2 antisense	5'-TTTGATTAGTACTGTAGGGTTAATG-3'	
GAPDH sense	5'-CAAGGTCATCCCAGAGCTGAA-3'	7
GAPDH antisense	5'-GCAATGCCAGCCCCGGCATCG-3'	

Table 2. — *Immunohistochemical H-score of ERα and ERβ of the ovarectomized mouse uteri after 2 weeks of feeding with E_2 diet alone E_2 plus SERMs or genistein.*

	ERα		ERβ	
Treatment	Glandular cells	Stromal cells	Glandular cells	Stromal cells
E_2 + TAM-H	164.7 ± 18.1[a]	118.3 ± 27.5	35.0 ± 10.8	20.2 ± 5.8
E_2 + TAM-L	158.1 ± 18.3	110.1 ± 27.7	34.7 ± 10.8	19.5 ± 6.6
E_2 + TOR-H	145.5 ± 18.7	108.5 ± 18.2	56.2 ± 12.2[b]	30.3 ± 11.3[b]
E_2 + TOR-L	133.5 ± 18.1[b]	102.1 ± 18.0	58.5 ± 12.8[b]	29.2 ± 10.9[b]
E_2 + ICI-H	90.5 ± 11.6[c]	76.3 ± 23.6[b]	10.2 ± 0.3	11.7 ± 2.3
E_2 + ICI-L	91.7 ± 12.6[c]	75.2 ± 24.0[b]	15.2 ± 0.8	9.2 ± 0.8
E_2 + GE	120.8 ± 13.8[b]	96.4 ± 34.0	47.5 ± 9.6[b]	22.7 ± 5.2
E_2	190.7 ± 15.4	128.2 ± 33.5	20.7 ± 9.8	15.2 ± 4.3
Control	94.0 ± 14.1	77.9 ± 40.4	21.5 ± 12.8	11.7 ± 4.3

[a] Mean ± SD; [b, c] Significantly different from the E_2 group (b, $p < 0.05$; c, $p < 0.01$).

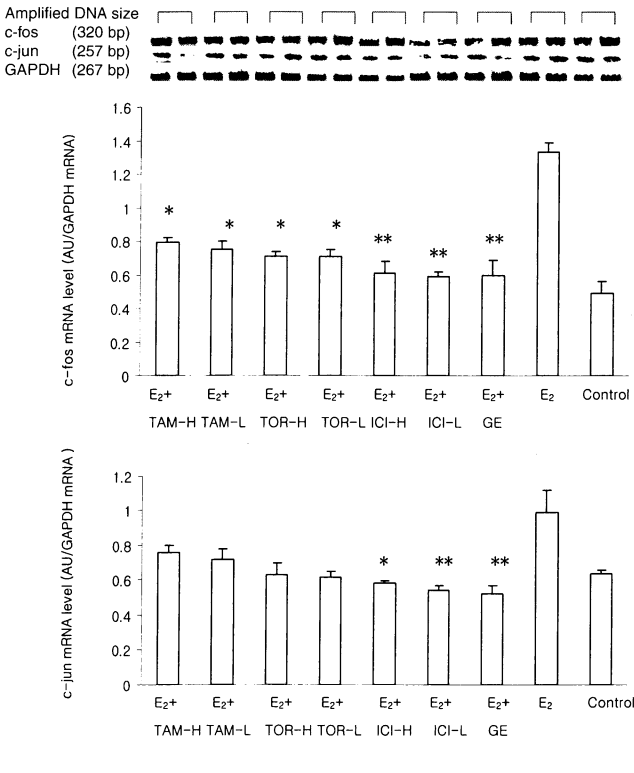

Figure 2. — Expression of c-fos/jun mRNAs in the uteri of ovarectomized mice treated continuously for two weeks with either E_2 or E_2 plus tamoxifen, toremifene, ICI-182780 and genistein. *p < 0.05, **p < 0.01 compared with E_2 alone. TAM, tamoxifen; TOR, toremifene; ICI, ICI-182780; GE, genistein.

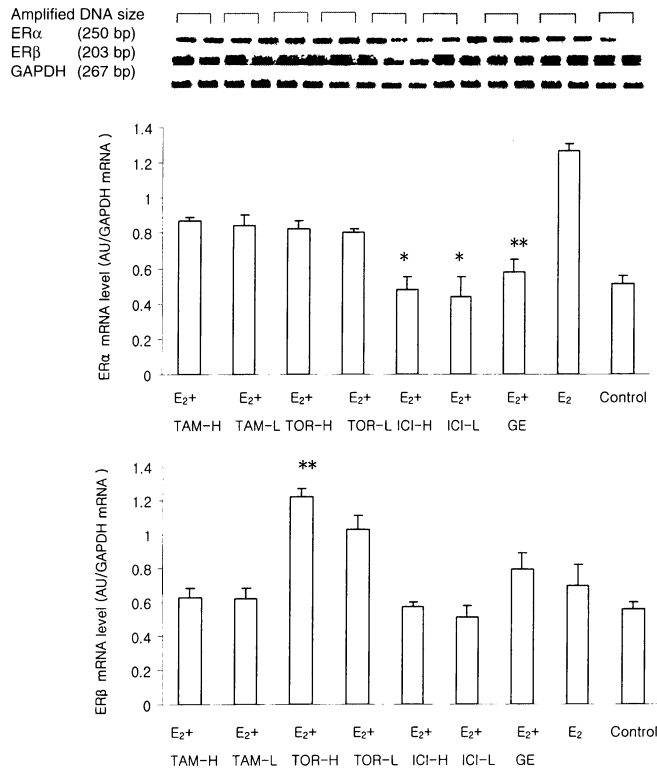

Figure 3. — Expression of ERα/β mRNAs in the uteri of ovarectomized mice, treated continuously for two weeks with either E_2 or E_2 plus tamoxifen, toremifene, ICI-182780 and genistein. *p < 0.01, **p < 0.05 in comparison to E_2 alone. TAM, tamoxifen; TOR, toremifene; ICI, ICI-182780; GE, genistein.

(Iwaki Glass, Tokyo, Japan) with Vent DNA polymerase (New England Biolabs, Beverly, MA) in 10 μM KC1, 0.20 μM Tris-HCI (pH 8.8), 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, and 0.15 μM deoxynucleotide phosphates. Twenty cycles of PCR for glyceraldhyde-3-phosphate dehydrogenase (GAPDH, a housekeeping gene) mRNA (267 bp) as an internal standard were performed at the same time.

For quantitative PCR, a 50 μl reaction mixture, consisting of a 2.5 μl of template, 25 pmol (0.25 μM) each of 3' - or 5' - primer, 2 μl of dNTPs (10 μM) and 2.5 IU of recombinant Taq DNA polymerase (Takara Co., Kyoto, Japan), was used.

Semi-quantitative analysis of the c-fos/jun, ERα/β and COX-1/2 mRNA expressions in the mouse uterine corpus based on the PCR products

The PCR products were applied to 1.5% agarose gel for electrophoresis at 50-100 V. Quantification of the products was carried out using the Bio image software program (Nihon Millipore Corp., Tokyo, Japan). Intensity of the specific bands was standardized with that of GAPDH mRNA.

Immunohistochemical expression of ERα/β and COX-1/2 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 ERα (1:100, anti-rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), ERβ (1:50, anti-rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), COX-1 (1:250, anti-mouse monoclonal, Cayman Chemial, Ann Arbor, MI) and COX-2 (1:200, anti-mouse monoclonal, Alexis Biochem, Carlsbad, CA) were used.

Sections stained with ERα and β were scored according to intensity and proportion of the cells highlighted as proposed by Giri *et al.* [22]. Staining intensity was scored as follows: 0 = no staining; 1 = weak nuclear staining; 2 = moderate nuclear staining; 3 = strong nuclear staining. The proportion of the cells stained in each category was noted and the 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 [23].

Table 3. — *Immunohistochemical expression of COX-1 and COX-2 in ovarectomized mouse uteri.*

	COX-1		COX-2	
Treatment	Glandular cells	Stromal cells	Glandular cells	Stromal cells
E_2 + TAM-H	2.5 ± 0.8[a)]	2.0 ± 0.7	7.6 ± 2.1	6.0 ± 2.0
E_2 + TAM-L	3.3 ± 0.4	1.8 ± 0.5	6.7 ± 1.2	5.0 ± 1.0
E_2 + TOR-H	2.0 ± 0.6	1.7 ± 0.2	5.3 ± 1.5	4.6 ± 1.2
E_2 + TOR-L	2.1 ± 0.7	1.2 ± 0.3	5.0 ± 1.7	4.3 ± 1.5
E_2 + ICI-H	1.7 ± 0.2	1.0 ± 0.1	3.7 ± 1.5[b)]	2.6 ± 1.2[b)]
E_2 + ICI-L	1.7 ± 0.2	1.0 ± 0.0	3.0 ± 1.7[b)]	2.3 ± 0.6[b)]
E_2 + GE	2.4 ± 0.9	1.6 ± 1.0	4.8 ± 1.6	4.0 ± 0.9
E_2	2.2 ± 1.1	1.5 ± 0.4	8.6 ± 2.5	6.3 ± 2.3
Control	1.9 ± 0.1	1.4 ± 0.1	2.3 ± 0.6	1.7 ± 0.6

[a)] Mean ± SD; [b)] Significantly different from the E_2 group ($p < 0.05$).

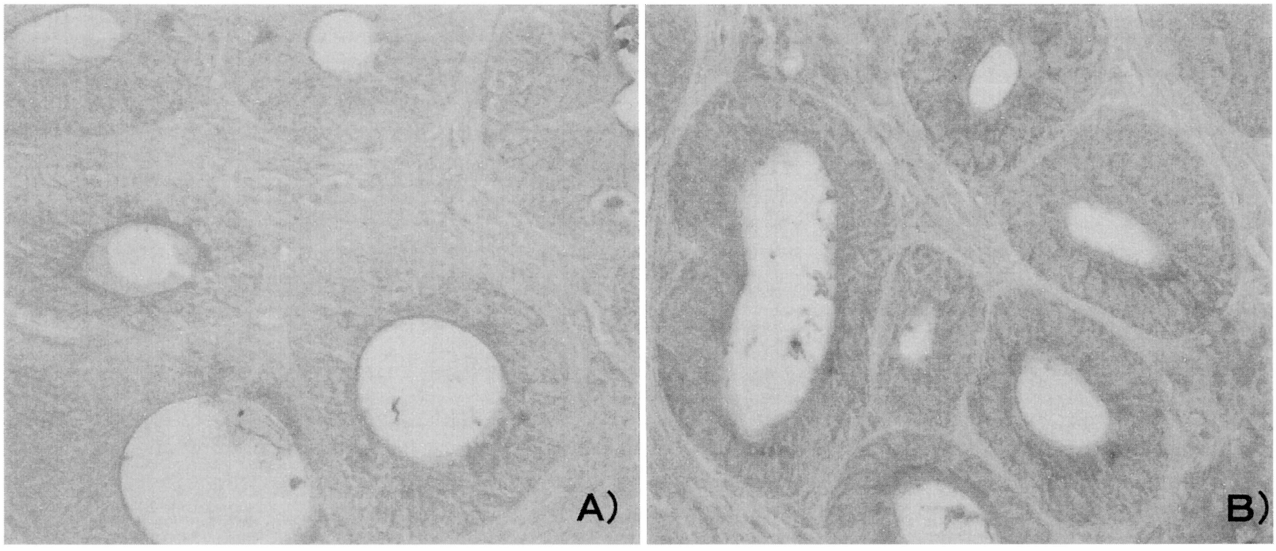

Figure 4. — Expression of ERb in the uteri of ovarectomized mice treated with E_2 alone (A) and with E_2 and TOR (B) (sABC stain, x 350). The expression in the mice treated with E_2 and TOR was stronger than that in those treated with E_2 alone.

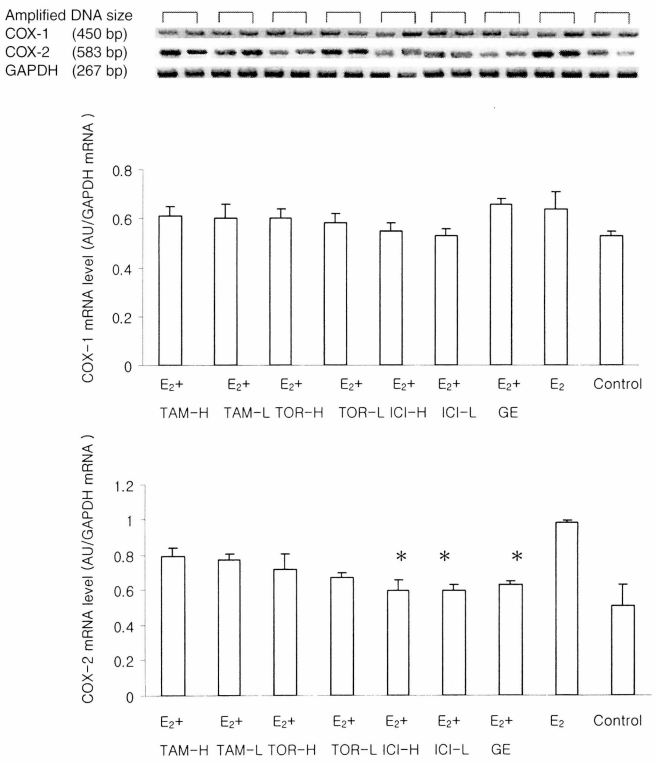

Figure 5. — Expression of COX-1/2 mRNAs in the uteri of ovarectomized mice treated continuously for two weeks with either E_2 or E_2 plus tamoxifen, toremifene, ICI-182780 and genistein. *$p < 0.05$ in comparison to E_2 alone. TAM, tamoxifene; TOR, toremifene, ICI, ICI-182780; GE, genistein.

Immunohistochemical COX-1 and -2 expressions in glandular and stromal cells were basically scored separately according to the criteria of Krajewska *et al.* [24]. The scoring methods were modified by Fujiwaki *et al.* [25]. Namely, the percentage of COX-1 and -2 immunostaining in the glandular and stromal cells were graded as follows: 0, no staining; 1, 1-25%; 2, 26%-50%; 3, 51%-75%; and 4, 76%-100%. Intensity of immunostaining was rated as follows: 0, none; 1, weak; 2, moderate; 3, intense. Therefore, the immunohistochemical COX scores ranged from 0 to 12 [24]. Immunohistochemical findings were analyzed by two independent investigators who counted more than 200 cells and the discordant results were reviewed jointly.

Statistical analysis

The statistical analysis was done by using the chi-square test or Student's t-test.

Results

Measurement of expression of mRNA was done with the whole uteri of the animals in each group (4-5 animals in each). The representative bands in each group are shown in Figures 2, 3 and 5.

Uterine mRNA expressions of c-fos and c-jun are shown in Figure 2. The expression of c-fos mRNA in E_2 plus TAM, TOR, ICI or GE group significantly decreased in comparison to the E_2 treatment group. In addition, the expression of c-jun in E_2 plus ICI or GE also significantly decreased in comparison to the E_2 treatment group. E_2 plus TAM or TOR treatment also showed a decreased tendency compared with the E_2 treatment group, but the difference was not significant.

Uterine mRNA expressions of ERα/β are shown in Figure 3. The expression of ERα mRNA in E_2 plus ICI or GE significantly decreased in comparison to the E_2 treatment group. The expression of ERα mRNA in E_2 plus TAM or TOR showed a decreased tendency in comparison to the E_2 treatment group, but the difference was not significant. The expression of ERβ mRNA in the E_2 plus high dose of TOR significantly increased in comparison to the E_2 treatment group. The expression of ERβ mRNA in E_2 plus low dose of TOR or GE showed a decreased tendency in comparison to the E_2 treatment group, but the difference was not significant. Immunohistochemical staining for ERβ is shown in Figure 4, A and B). The expression was more prominent in the glandular cells than in the stromal cells treated with E_2 alone. The TOR treatment increased the ERβ expres-

sion. The immunohistochemical H-score of ERα/β is shown in Table 2. The ERα score of the glandular cells in E_2 plus high dose of TAM, low dose of TOR, ICI or GE significantly decreased in comparison to the E_2 treatment group. The ERα score of stromal cells in E_2 plus ICI significantly decreased compared with the E_2 treatment group. The ERβ score of the glandular cells in E_2 plus a high dose of either TOR or GE significantly increased in comparison to the E_2 treatment group. The ERβ score of stromal cells in E_2 plus TOR significantly increased in comparison to the E_2 treatment group.

Uterine mRNA expressions of COX-1/2 are shown in Figure 4. The expression of COX-1 mRNA was not affected by adding TAM, TOR, ICI or GE. The immunohistochemical score of COX-2 in E_2 plus ICI significantly decreased in comparison to the E_2 treatment group. The expression of COX-2 mRNA in both the glandular and stromal cells in E_2 plus TAM or TOR significantly decreased in comparison to the E_2 treatment group. The expression of COX-2 mRNA in both the glandular and stromal cells in E_2 plus either TOR or GE tended to decrease in comparison to the E_2 treatment group.

Discussion

In this study, the expressions of c-fos and c-jun stimulated by E_2 were confirmed in endometrial carcinogenesis in mice as demonstrated in our previous study [9, 10], while the inhibition of those expressions was related to the prevention of mouse endometrial cancer [5]. The present study demonstrated that an inhibitory effect of TOR, ICI or GE on estrogen-stimulated genes seemed to be stronger than that of TAM. Those inhibitory effects were thought to be related to the preventive effects of TOR or GE on endometrial carcinogenesis [5, 26].

The anti-tumor effects of some herbal medicines on endometrial carcinoma cells are reported to be related to the ERα-related mechanism [27]. The increased ERα expression induced by E_2 was strongly inhibited by GE as well as ICI in this study. This effect may be related to the inhibitory effect of GE [26].

More recently, ERβ is thought to act as a suppressor during hormone-dependent cancer [14, 15]. ERβ expression treated with E_2 plus TOR or GE was significantly increased. This may be related to the preventive effects of TOR or GE on endometrial carcinogenesis [5, 26].

In conclusion, TOR as well as GE could therefore exert a preventive effect on estrogen-related endometrial carcinogenesis through the decreased expression of c-fos/jun, ERα and COX-2, and the increased expression of ERβ.

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