

Anti-tumor effects of herbal medicines on endometrial carcinomas via estrogen receptor- α -related mechanism

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Abstract. This study was performed to examine the relationship between the anti-tumor effects of herbal medicine and endometrial carcinoma with ER-related mechanisms. An endometrial cancer cell line (Ishikawa) was used for this study. The cell viability and expression of estrogen receptors (ER) were determined by MTT and RT-PCR. A dose-dependent decrease of viability and apoptosis of the cancer cells was generated by exposure to the herbal medicines, Juzen-taiho-to or Shimotsu-to. The expression of ER- α mRNA, but not ER- β mRNA was suppressed by Juzen-taiho-to or Shimotsu-to in an endometrial cancer cell line. The anti-tumor effect of these herbal medicines against endometrial carcinoma might be correlated to the ER- α related mechanism.

Introduction

We have reported that Juzen-taiho-to has a preventive effect on endometrial carcinogenesis in mice (1). In our subsequent study, the main component of the agent, Shimotsu-to, was found to relate to the suppression of expression of estrogen-stimulated genes in mice uteri (2,3). Originally, Juzen-taiho-to, which contained Shimotsu-to, Shikunshi-to and two other oriental drugs have been reported to relate to the induction of cytokine (4), antibody (5) and anti-tumor activity (6-8). Juzen-taiho-to is reported to show an anti-metastatic effect in an animal model (9). Another agent, Shikunshi-to, is described as a modulator of hematopoiesis and immune response *in vitro* (10).

Meanwhile, it is known that estrogen usually exerts its biological function via estrogen receptor (ER)-related

mechanism(s). The two isoforms of ERs, ER- α and ER- β , are distributed differentially in various tissues (11,12). It is suggested that they act as co-activators of estrogen binding with the estrogen-responsive element (ERE) included in estrogen-responsive genes, thereby influencing the transcriptional effects of target genes. In our previous studies, the long-term (29 weeks) exposure of E2 (5 ppm) and N-methyl-N-nitrosourea induced a high incidence of endometrial carcinoma in mice. The expression of c-fos/jun genes was overexpressed by estrogen exposure. The preventive effects of Juzen-taiho-to and Shimotsu-to on E2-related endometrial carcinogenesis were reported to relate to decreased expression of c-fos/jun. We used an *in vitro* study to confirm the ER-mediated mechanism for the preventive effect of Juzen-taiho-to, Shimotsu-to and Shikunshi-to.

Materials and methods

Chemicals. 17 β -E2 was purchased from Sigma Chemical Co. (St. Louis, MO), and Juzen-taiho-to, Shimotsu-to and Shikunshi-to were purchased from Tsumura Co. (Tokyo, Japan). For the *in vitro* study, agents were dissolved in Eagle's minimal essential medium and passed sequentially through a 0.45 μ m filter sterilization, then 10% fetal bovine serum was added. Five doses (0.25, 0.5, 1.0, 2.5 and 5.0 mg/ml) were used for the exposure of each agent to the cell culture.

Cell line and cell culture. An endometrial cancer cell line of Ishikawa cells was used. Cells were cultured in MEM for 24 h, treated with the herbal medicines and maintained at 37°C in a humidified 5% CO₂/95% air atmosphere. After 12, 24, 48 and 72 h culture, the cells were washed twice with phosphate-buffered saline (PBS) and used for the following experiments.

Cancer cell viability. The 50% inhibition concentration (IC₅₀) and cell viability were determined by MTT assay. After different treatments with herbal medicines, the cells (1x10⁴) were washed with PBS and incubated in 10 μ l of 5 mg/ml MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] dissolved in PBS for 4 h. One hundred μ l of the dissolved solution (34 μ l of HCl with 9.966 ml isopropanol)

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Table I. Sequences of the primers and PCR conditions.

Primers	Nucleotide sequences	PCR conditions
ER- α	Sense 5'-TGTGCAATGACTATGCTTCA-3' Anti-sense 5'-GCTCTTCCTCCTGTTTTTA-3'	1 min at 94°C, 1 min at 54°C and 1.5 min at 72°C, 35 cycles
ER- β	Sense 5'-GTCCATCGCCAGTTATCACATC-3' Anti-sense 5'-GCCTTACATCCTTCACACGA-3'	45 sec at 94°C, 45 sec at 56°C and 1.5 min at 72°C, 30 cycles
β -actin	Sense 5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3' Anti-sense 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'	1 min at 95°C, 1 min at 63°C and 2 min at 72°C, 35 cycles

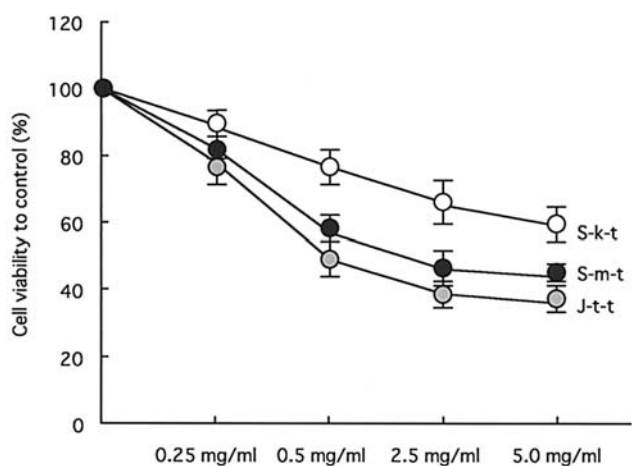


Figure 1. Viability of the Ishikawa cells. The growth of Ishikawa cells was significantly inhibited by J-t-t and S-m-t. The value of the cell viability is mean \pm SD of five determinations.

was then added to the cells. Absorbance of the dissolved solution was measured using the Easy Reader EAR 400 (SLT-Labin Struments, Austria) at 550 and 620 nm. The absorbance without treatment was designated as 100%.

Apoptosis assay. As described in our previous study, 1×10^5 cells were exposed to the herbal medicines for 48 h, then collected and centrifuged at $400 \times g$ for 5 min (13). The sediment was dissolved in $100 \mu\text{l}$ of cell fixing solution (10% glutaraldehyde) for 30 min, then mixed with 1 ml PBS and centrifuged. The sediment was stained with $20 \mu\text{l}$ PBS and $4 \mu\text{l}$ staining solution (1 mM Hoechst 33258 in PBS). The nuclear morphology of cells in one drop on a glass slide was observed under a fluorescence microscope.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from cells by a guanidium thiocyanate-phenol-chloroform extraction method (14). Total RNA ($3 \mu\text{g}$) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RTase, 200 units; Gibco BRL, Gaithersburg, MD) in $20 \mu\text{M}$ Tris-HCl buffer (pH 8.4) with

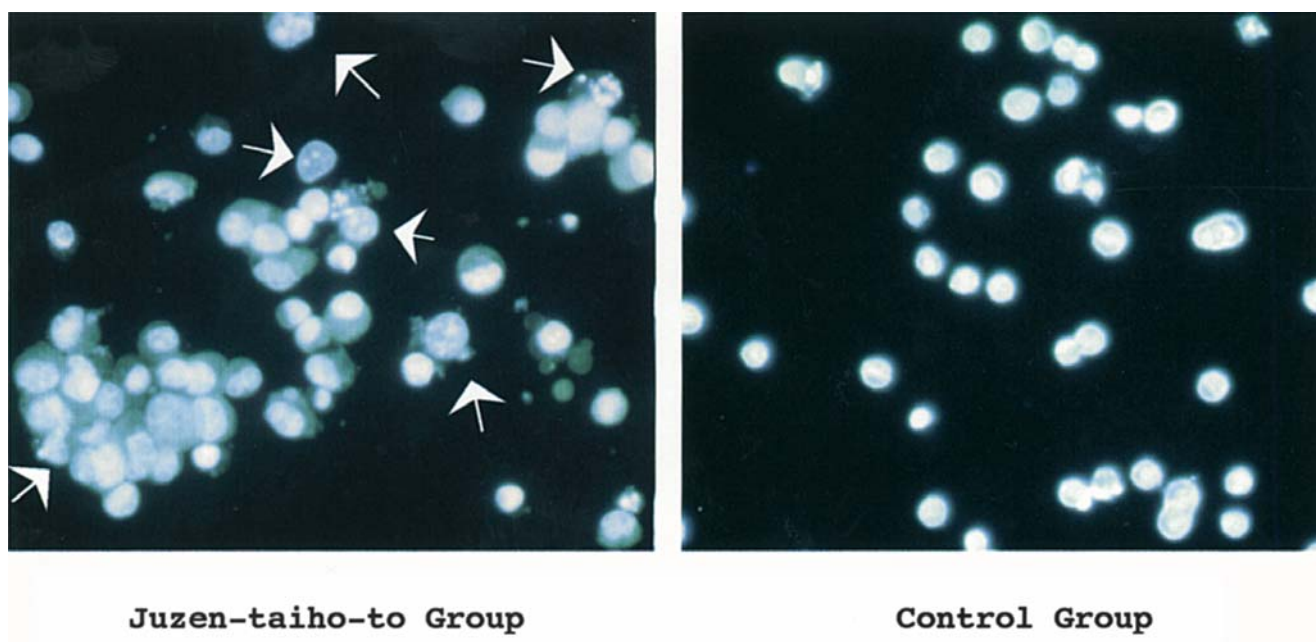


Figure 2. Cancer cell apoptosis. Apoptotic cells were detected by immunohistochemical staining method in the J-t-t group and compared with the control group.

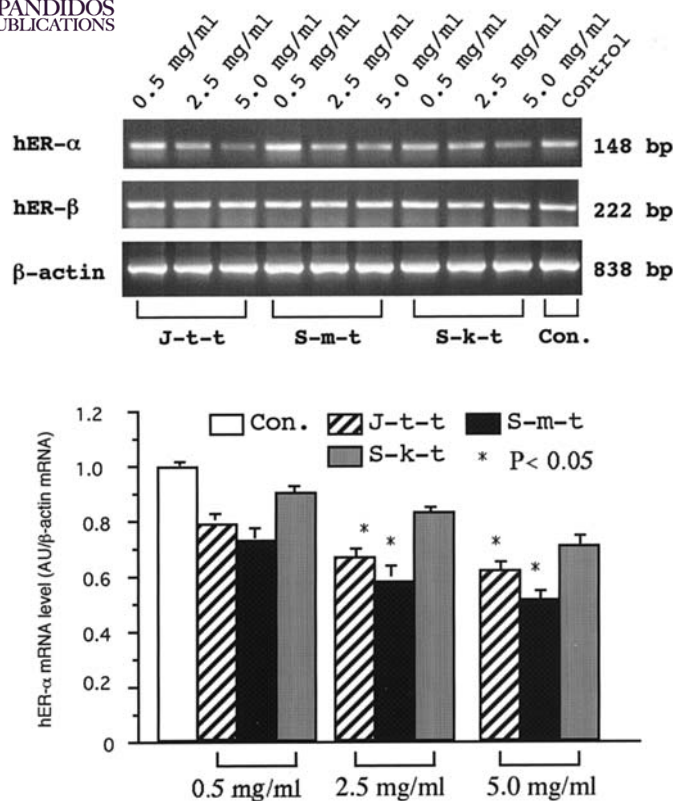


Figure 3. Expression of hER- α and hER- β RNA in Ishikawa cells are shown at various doses. The bands are representative of five determinants. The hER- α mRNA level (AU/ β -actin mRNA) was calculated from each of the five determinations. At doses of 2.5 and 5.0 mg/ml, both J-t-t and S-m-t significantly suppressed the expression of ER- α ($p < 0.05$), but S-K-t did not. Meanwhile, hER- β expression was not affected by the herbal medicines.

50 μ M KCl, 2.5 μ M MgCl₂, 0.1 μ g/ml bovine serum albumin, 10 μ M dithiothreitol, and 0.5 μ M deoxynucleotides to generate cDNAs, using random hexamers (50 ng; Gibco BRL) at 37°C for 60 min. RT reaction was heated at 94°C for 5 min to inactivate MMLV-RTase. The PCR conditions and primer sequences of hER- α , hER- β (15) and β -actin (13) are described in Table I and were carried out in reverse transcribed cDNAs with 0.1 mM specific primers described below, using an Iwaki thermal sequencer TSR-300 (Iwaki Glass, Tokyo) with Vent DNA polymerase (New England Biolabs, Beverly, MA) in 20 μ M Tris-HCl buffer (pH 8.8) with 10 μ M KCl, 10 μ M (NH₄)₂SO₄, 2 μ M MgSO₄, 0.1% Triton X-100, and 0.15 μ M deoxynucleotide phosphates.

Statistical analysis. The analysis was performed according to the χ^2 test or Student's *t*-test.

Results

Cell viability and apoptosis. As shown in Fig. 1, viability of Ishikawa cells was decreased dose-dependently by the addition of herbal medicines. IC₅₀ of Juzen-taiho-to, Shimotsu-to and Shikunshi-to on day 3 were 0.49 \pm 0.02, 0.53 \pm 0.01 and 0.65 \pm 0.03 mg/ml, respectively. Apoptosis of Ishikawa cells was confirmed with a fluorescence microscope after exposure to Juzen-taiho-to (Fig. 2).

Suppression of ER- α mRNA and protein. After 72 h treatment with Juzen-taiho-to and Shimotsu-to (2.5 and 5 mg/ml), the mRNA expression of hER- α , but not hER- β in Ishikawa cells was significantly suppressed ($p < 0.05$), while Shikunshi-to had no effect (Fig. 3).

Discussion

It was shown that Juzen-taiho-to and Shimotsu-to have a preventive effect on E2-related endometrial carcinogenesis in mice (1-3). Thus, chemopreventive effects by Juzen-taiho-to or Shimotsu-to on endometrial carcinogenesis are suggested to relate to the suppression of ER- α (18).

We have also reported that the inhibitory effects of herbal medicines like Juzen-taiho-to or Shimotsu-to on *c-fos/jun*, recognized as estrogen-responsive genes, may be related to the suppression of ER- α . Therefore, the preventive effects of Shimotsu-to and Juzen-taiho-to on E2-induced mouse endometrial carcinogenesis may occur via the ER-mediated pathway.

In this study, Juzen-taiho-to, Shimotsu-to and Shikunshi-to suppressed the expression of ER- α and inhibited the growth of Ishikawa cells. Such anti-tumor effects of these agents is suggested to relate to the generation of cellular apoptosis. Juzen-taiho-to and Shimotsu-to induced cancer cell apoptosis and suppressed the expression of ER- α *in vitro*, suggesting that such herbal medicines exert anti-tumor effects on endometrial carcinoma via the ER- α related mechanism.

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