Inhibitory effects of Hochu-ekki-to on endometrial carcinogenesis induced by N-methyl-N-nitrosourea and 17ß-estradiol in mice

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Abstract. An evaluation of the effects of a traditional Chinese herbal medicine, Hochu-ekki-to (Bu-zong-yi-qi-tang) on endometrial carcinogenesis was performed in experiments with female mice. In the short-term experiment, dietary exposure of Hochu-ekki-to (0.2% for 2 weeks) decreased the estradiol-17ß (E_2)-stimulated expression levels of c-*jun* ($P<0.001$), tumor necrosis factor (TNF)-α ($P<0.005$), estrogen receptors (ER)-α ($P<0.001$) and ER-ß ($P<0.005$), as determined by reverse transcription-polymerase chain reaction and a Southern blot analysis in the uteri of the ovarectomized mice. In the long-term experiment, the mice were given N-methyl-N-nitrosourea (MNU) solution (1 mg/100 g body weight) and normal saline (as controls) into their left and right uterine corpora, respectively, and then were divided into four groups. Group 1 (25 mice) was given a diet with Hochu-ekki-to and 5 ppm E_2. Group 2 (25 mice) was given a diet with E_2 alone. Group 3 (25 mice) was given a diet with Hochu-ekki-to alone. Group 4 (25 mice) was kept on the basal diet alone and treated as a control. The incidence of uterine endometrial cancer in the group with Hochu-ekki-to treatment was substantially lower than of the control group. The inhibitory effect of Hochu-ekki-to on endometrial carcinogenesis is thus suggested to decrease the expressions of c-*jun*, TNF-α, ER-α and -ß.

Introduction

Kampo (Japanese and Chinese traditional herbal) medicine, a form of complementary or alternative medicine, is considered to be potentially effective in both clinical as well as scientific medical usage. Especially, clinical and basic pharmacological research on Hochu-ekki-to has been extremely active in Japan since the beginning of the 1990's. Hochu-ekki-to is composed of crude herbs (*Astragali radix*, *Atractylodis lanceae rhizoma*, *Ginseng radix*, *Angelicae radix*, *Bupleuri radix*, *Zizyphi fructus*, *Aurantii nobilis pericarpium*, *Glycyrrhizae radix*, *Cimicifugae rhizoma* and *Zingiberis rhizoma*) as shown in Table I. Hochu-ekki-to is a tonic formula in Kampo Medicine, mainly consisting of Shikunshi-to. Shikunshi-to is composed of four crude herbs (*Atractylodis lanceae rhizoma*, *Ginseng radix*, *Glycyrrhizae radix* and *Holen*), and it is mainly used for improving the depression of 'Ki' (a concept that encompasses the mental nervous function), particularly the appetite for food and the actual process of digesting and absorbing nutrients (1). We previously reported an inhibitory effect of *Glycyrrhizae radix* on endometrial carcinogenesis in mice, related with the suppression of c-*fos* and c-*jun* expressions (2). On the other hand, *Astragali radix* in Hochu-ekki-to is reported to inhibit the growth of gastric cancer cells and this effect is suggested to be related to the cytostastic mechanism or the induction of apoptosis (3). Whereas, Hochu-ekki-to is known to enhance concomitant anti-tumor immunity through the augmentation of natural killer (NK) cells (4). More importantly, Hochu-ekki-to does not increase the activity of splenic NK cells, although it appears that Hochu-ekki-to induces a cytotoxic capability in NK cells (4).

There have been several reports describing that Hochu-ekki-to activates the macrophage activity (5) while enhancing the host immune response in virus-infected mice (6). Hochu-ekki-to is reported to suppress the azoxymethane-induced formation of aberrant crypt foci, which are regarded as putative preneoplastic lesions of the colon (7). This traditional medicine is also regarded as a beneficial treatment of infectious diseases in immunocompromised patients (8).

The transient expression of the immediate early genes, c-*fos* and c-*jun*, is suggested to be related to both cellular proliferation and differentiation (9-12). The acute administration of estradiol-17ß (E_2) causes a transient increase in the expression of c-*fos* (9) and c-*jun* (10), followed by DNA replication. We previously demonstrated that estrogens have a potent enhancing effect on endometrial carcinogenesis by N-methyl-N-nitrosourea (MNU) in ICR mice (11,12) and the effects are closely related to the estrogen-induced over-expression of c-*fos* and c-*jun* (13,14). There is evidence that internal cytokines, such as interleukin (IL)-1α and TNF-α,

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Abbreviations: E2, estradiol-17ß; IL-1α, interleukin-1α; TNF, tumor necrosis factor; ER, estrogen receptor

Key words: endometrial carcinogenesis, N-methyl-N-nitrosourea, 17ß-estradiol

Table I. The ingredients and botanical origins of Hochu-ekki-to.

Ingredients	Botanical origin	Representative defined compounds	Ratio
Astragali radix	*Astragalus membranceus*	Formononetin	4.0
Atractylodis rhizoma	*Atractylodes japonica*	Atractylon	4.0
Ginseng radix	*Panax ginseng*	Ginsenodeide Ro	4.0
Angelicae radix	*Angelica acutiloba*	Ligustilide	3.0
Bupleuri radix	*Bupleurum falcatum*	Saikosaponic	2.0
Zizyphi fructus	*Zizyphus jujuba*	Oleanolic acid, betulinic acid	2.0
Aurantil nobilis pericarpium	*Citrus unshiu*	Limonene, hesperidin	2.0
Glycyrrhizae radix	*Glycyrrhiza uralesis*	Glycyrrhizin, formononetin	1.5
Cimicifugae rhizoma	*Cimicifuga simplex*	Cimigenol, ß-sitosterol	1.0
Zingiberis rhizoma	*Zingiber officinale roscoe*	Zingibero, curcumene	0.5

are important factors for progression as well as promotion and in chemical carcinogenesis (15-18). Both estrogens and anti-estrogens are known to exert their biological effects via estrogen receptors (ERs), thus involving a variety of transcription factors. ER-α and ER-ß isoforms are known to be expressed differently by some chemicals, such as selective estrogen receptor modulators (18,19). This study was designed to determine whether Hochu-ekki-to exerts a modifying effect on endometrial carcinogenesis in mice induced by MNU and E_2. Furthermore, the effects of Hochu-ekki-to on the expressions of c-*fos*, c-*jun*, IL-1α, TNF-α, ER-α and ER-ß mRNAs, which were induced by E_2 in mice uteri, were also examined by reverse transcripton-polymerase chain reaction (PCR) and a Southern blot analysis.

Materials and methods

Animals and chemicals. Female ICR mice, 10 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. All animals were housed in plastic cages (four or five mice/cage) with access to normal drinking water. The basal diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and filtered distilled water were available *ad libitum* throughout the experiment. E_2 and MNU were purchased from Sigma Chemicals Co. (St. Louis, MO). Hochu-ekki-to (Bu-zong-yi-qi-tang) was purchased from Tsumura Co. (Tokyo, Japan). The ingredients of Hochu-ekki-to are shown in Table I.

Experimental protocol for the short-term effects of Hochu-ekki-to. Female ICR mice, 12 weeks of age, were ovariectomized under general anesthesia with a diethylether. Two weeks later, the ovariectomized mice were divided into four experimental groups (6 mice in each group). The dose of 0.2% Hochu-ekki-to in the diet was equivalent to the clinical dose (7.5 g/50 kg daily) (11). Group 1 was given a diet with 0.2% Hochu-ekki-to and 5 ppm E_2. Group 2 was given a diet containing 5 ppm E_2 alone. Group 3 was given the diet with 0.2% Hochu-ekki-to alone. Group 4 was kept on the basal diet alone and treated as a control. Two weeks later, the mice uteri were resected and cut in half longitudinally. One half was quickly frozen in liquid nitrogen for the following experiments, while the other half was subjected to pathological examinations.

Reverse transcripton-PCR (RT-PCR). Total-RNA was isolated from frozen tissue specimens by a guanidium thiocyanate-phenol-chloroform extraction method (20). Total-RNA (3 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RTase, 200 units, Gibco BRL, 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, Gibco BRL) at 37˚C for 60 min. The RT reaction mixture was heated at 94˚C for 5 min to inactive MMLV-RTase. For the 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 the 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 the 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 the ER-ß (203 bp) mRNA expression, 35 cycles of PCR reaction were performed, consisting of 1 min 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, Japan) 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 glyceraldehydes-3-phosphate dehydrogenase (GAPDH, a housekeeping gene) mRNA as an internal standard were simultaneously performed.

The following oligodeoxynucleotides were synthesized as specific primers in PCR according to the published information [cDNAs for c-*fos* (21), c-*jun* (22), IL-1α (23),

Table II. DNA sequences for specific primers.

Primers	DNA sequences	Citation
c-*fos*		
Sense	5'-GCTTCTATAAAGGCGCCAGCTAG-3'	21
Anti-sense	5'-GACAGGAGAGCCCATGCTGGAG-3'	
c-*jun*		
Sense	5'-GGAGTGGGAAGGACGTGGCGC-3'	22
Anti-sense	5'-TCCCAGCCCTCCCTGCTTTGTG-3'	
IL-1α		
Sense	5'-GATGGCCAAAGTTCCTCCTGACTTG-3'	23
Anti-sense	5'-GCCTGACGAGCTTCATCAGTTT-3'	
TNF-α		
Sense	5'-AGGCAGGTTCTGTCCCTTTCA-3'	24
Anti-sense	5'-TCCACTTGGTGGTTTGCTACG-3'	
ER-α		
Sense	5'-GAGAAAGGAAACATGATGGA-3'	25
Anti-sense	5'-TTCATCATGCCCACTTGTAAC-3'	
ER-ß		
Sense	5'-AAAGCCAAGAGAACCAGTGGGCAC-3'	25
Anti-sense	5'-GCCAATCATGTGCACCAGTTCCTT-3'	
GAPDH		
Sense	5'-CAAGGTCATCCCAGAGCTGAA-3'	21
Anti-sense	5'-GCAATGCCAGCCCCGGCATCG-3'	

TNF-α (24), ER-α (25), ER-ß (25) and GAPDH (21)] as shown in Table II.

Semi-quantitative analysis of c-fos/jun, IL-1α, TNF-α, ER-α and ER-ß mRNA expressions by Soutern blot of PCR products. PCR products were applied to 1.5% agarose gel and then electrophoresis was performed at 50-100 V. The PCR products were capillary-transferred to an Immobilon transfer membrane (Millipore Co., Bedford, MA) for 16 h. The membrane was then dried at 80˚C for 30 min, and UV-irradiated to tightly fix the products, prehybridized in 1 M NaCl, 50 M 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 specfic individual primers at 65˚C overnight. Specific bans hybridized with biotinylated probes were detected with Plex Luminescent Kits (Millipore Co.) on the membrane after exposure to X-ray film at room temperature for 10 min. The semi-quantification of Southern blotting was carried out using a Bio-Image analyzer (Millipore Co.). The intensity of the specfic bands was standardized with that of GAPDH mRNA.

Experimental protocal for the long-term effects of Hochu-ekki-to. An experimental protocal is shown in Fig. 1. A total number of 100 female ICR mice, 10 weeks of age, underwent a laparotomy under general anesthesia with a diethylether. The MNU soulution (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 was injected into the right one. One week after the MNU exposure, the animals were divided into the following four experimental groups: Group 1 (25 mice) was given a diet containing 0.2% Hochu-ekki-to and 5 ppm E_2. Group 2 (25 mice) was given a diet containing 5 ppm E_2 alone. Group 3 (25 mice) was given a diet containing 0.2% Hocchu-ekki-to alone. Group 4 (25 mice) was kept on the basal diet alone and treated as a control. At 30 weeks after exposure to MNU, all animals were sacrificed and autopsied. All major organs, particularly the reproductive organs, were carefully inspected. The uterus, ovaries, vagina and any other lesions suspected of being neoplastic and hyperplastic underwent a histological examination. Each corpus uteri was weighed. The tissue specimens were sectioned at 3 μm and stained with hematoxylin and eosin.

Histology of the uterine. Uterine endometrial lesions were divided into 4 lesions according to the WHO criteria (26): a) endometrial hyperplasia (EH), simple; b) (EH), complex; c) atypical endometrial hyperplasis (Aty Hyp); d) adeno-carcinoma (ADC).

Results

Short-term experiment. The levels of c-*fos*, c-*jun*, IL-1α, TNF-α, ER-α and ER-ß mRNA expression are shown in Figs. 2-4. Hochu-ekki-to treatment clearly decreased the E_2-induced

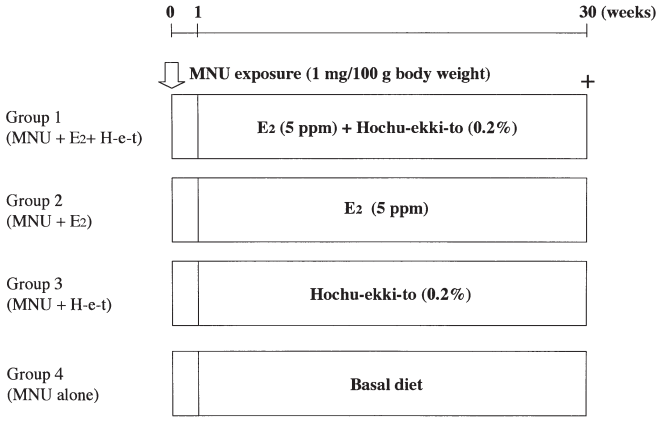

Figure 1. Experimental design: ⇓, MNU solution at a dose of 1 mg/100 g body weight was injected into the left uterine tube, and normal saline was injected into the right uterine tube. +, Resection of uteri.

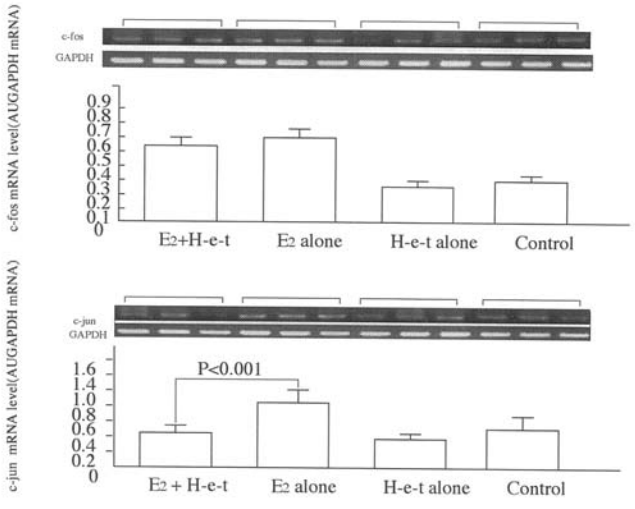

Figure 2. Expression of c-*fos* and c-*jun* mRNA in the uteri of the ovarectomized mice treated continuously for 2 weeks with E2 plus Hochu-ekki-to, E2 alone or Hochu-ekki-to alone in the diet.

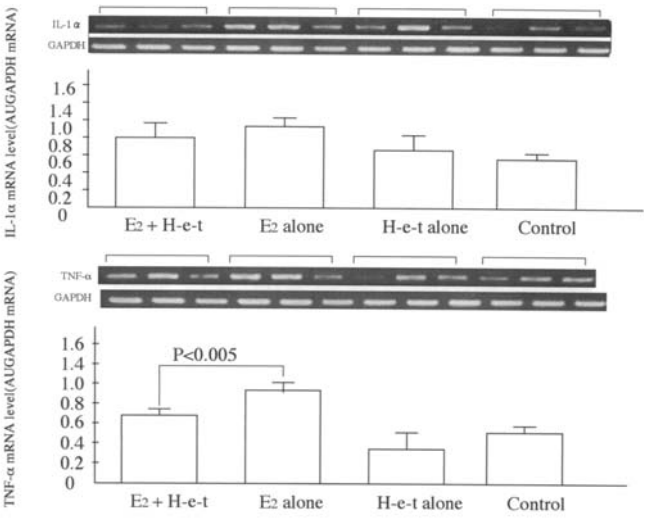

Figure 3. Expression of IL-1α and TNF-α mRNA in the uteri of the ovarectomized mice treated continuously for 2 weeks with E2 plus Hochu-ekki-to, E2 alone or Hochu-ekki-to alone in the diet.

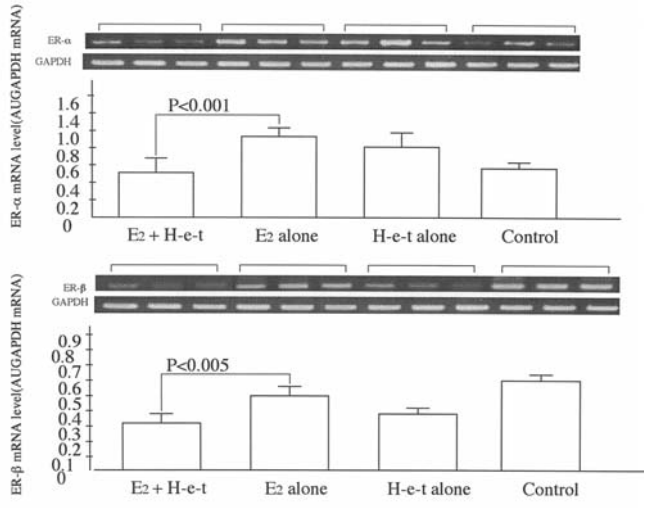

Figure 4. Expression of ER-α and ER-ß mRNA in the uteri of the ovarectomized mice treated continuously for 2 weeks with E2 plus Hochu-ekki-to, E2 alone or Hochu-ekki-to alone in the diet.

expression of c-*jun* (P<0.001), whereas no change was found for the expression of c-fos in any group (Fig. 2). Hochu-ekki-to treatment also decreased the E2-induced expression of TNF-α (P<0.005), but no change was obtained for IL-1α in any group (Fig. 3). Furthermore, the treatment of Hochu-ekki-to decreased the E2-induced expression of ER-α (P<0.001) and ER-ß (P<0.005) in each group (Fig. 4).

Long-term experiment. Two mice in group 1 and one in group 4 died within 15 weeks, and no pathological abnormalities other than pneumonia were found. Other animals survived until the termination of the experiment and were counted as effective animals. The mean body weights of the mice were as follows: group 1 [MNU plus E2 plus Hochu-ekki-to (n=23)], 44.4±6.2 g; group 2 [MNU plus E2 (n=25)], 40.6±4.2 g; group 3 [MNU plus Hochu-ekki-to (n=25)], 53.7±5.8 g; group 4 [MNU alone (n=24)], 49.1±7.7 g. The mean body weights of groups 1 and 3 were significantly more than those of groups 2 and 4, respectively.

The incidence of neoplastic and preneoplastic endometrial lesions in each group are shown in Fig. 5 {group 1 [MNU plus E2 plus Hochu-ekki-to (n=23)], ADC 22%; AtH 61%; EH, complex 96%; EH simple 96%; group 2 [MNU plus E2 (n=25)], ADC 40%; AtH 68%; EH, complex 96%; EH, simple 100%; group 3 [MNU plus Hochu-ekki-to (n=25)], ADC 4%; AtH 8%; EH, complex 40%; EH, simple 24%; group 4 [MNU alone (n=24)], ADC 4%; AtH 8%; EH, complex 54%; EH, simple 25%}. The incidence of ADC in the uterine corpus of group 1 (treated with MNU plus E2 plus Hochu-ekki-to) was substantially less frequent than that of group 2 (treated with MNU plus E2).

Discussion

We previously reported that Glycyrrhizae radix, one of the main ingredients in Shikunshi-to of Hochu-ekki-to demonstrates a

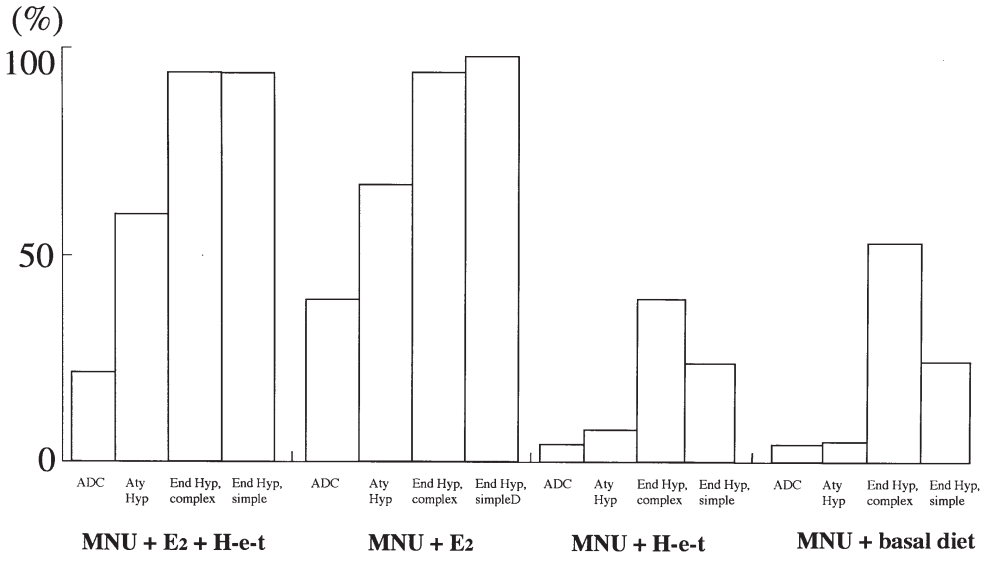

Figure 5. Incidence of prenoplastic and neoplastic mouse endometrial lesions on the treated side of the uterine corpus in each group.

preventive effect on endometrial carcinogenesis in mice (2). The administration of Glycyrrhizae radix decreased the level of c-*fos* and c-*jun* in the endometria (2). The exposure of Glycyrrhizae radix and Hocyu-ekki-to generate the same tendency of the expression of c-*fos* and c-*jun*. A decreased expression of c-*fos* and c-*jun* may be related to a decreased tendency of endometrial carcinomas in mice.

In this study, Hochu-ekki-to significantly suppressed the estrogen-induced expression of c-*jun*, TNF-α, ER-α and ER-ß mRNA. The modifying effect of Hochu-ekki-to on the expression of the early genes or transcription factors was slightly different from that of Juzen-taiho-to and Shimotsu-to. This difference may be related to the fact that the preventive effect of Hochu-ekki-to on the endometrial carcinogenesis is substantially less than of Juzen-taiho-to and Shimotsu-to (11,27). The anti-metastatic as well as anti-carcinogenic effects of Juzen-taiho-to in animal models have been reported (28-30). The mechanism(s) for the anti-metastatic effects are speculated to be related to the activation of macrophages and/or T cells in the host immune system (28). It is probable that the activation of macrophages and/or T cells is concerned with the present modifying effect of Hochu-ekki-to on ednometrial carcinogenesis.

We previously reported that Juzen-taiho-to and Shimotsu-to, as well as Shikunshi-to, inhibited the growth of Ishikawa cells through the suppression of ER-α expression (31). It is known that Juzen-taiho-to mainly consists of Shimotsu-to and Shikunsi-to. We proved that Shimotsu-to thus has an inhibitory effect on the expression of cyclooxygenase (COX)-2, c-*fos*, IL-1α and TNF-α in the endometrium on MNU-and E2-induced endometrial carcinogenesis in mice, thus suggesting that Shimotsu-to in Juzen-taiho-to is a key formula for the prevention of carcinogenesis (11,27).

We also previously reported that toremifene, one of the selective estrogen receptor modulators (SERMs), inhibits endometrial carcinogenesis while also suppressing the expression of ER-α (13). In the present study, the treatment of Hochu-ekki-to decreased the expression of ER-α and -ß induced by E2. These findings suggest that Hochu-ekki-to is therefore a natural SERM. More studies are necessary to more fully elucidate the modifying effects of Hochu-ekki-to on endometrial tumorigenesis.

References

1. Saiki I: A kampo medcine 'Juzen-taiho-to'. Prevention of malignant progression and metastasis of the tumor cells and the mechanism of action. Biol Pharm Bull 23: 677-688, 2000.
2. Niwa K, Hashimoto M, Morishita S, Yokoyama Y, Mori H and Tamaya T: Preventive effects of Glycyrrhzae radix extract on estrogen-related endometrial carcinogesis in mice. Jpn J Cancer Res 90: 726-732, 1999.
3. Lin J, Dong HF, Oppenheim JJ and Howard OM: Effects of astragal radix on the growth of different cancer cell lines. World J Gastroenterol 9: 670-673, 2003.
4. Cho JM, Sato N and Kikuchi K: Prophylactic anti-tumor effect of Hochu-ekki-to (TJ-41) by enhancing natural killer cell activity. In Vitro 5: 389-391, 1991.
5. Kataoka T, Akagawa KS, Tokunaga T and Nagao S: Activication of macrophages with Hochu-ekki-to. Jpn J Cancer Chemother 16: 1490-1493, 1989.
6. Mori K, Kido T, Daikuhara H, Sakakibara I, Sakata T, Shimizu K, *et al*: Effect of Hochu-ekki-to (TJ-41), a Japanese herbal medicine, on the survival of mice infected with influenza virus. Antivir Res 44: 103-111, 1999.
7. Oshima M, Takahashi M, Oshima H, Tsutsumi M, Yazawa K and Takeo M: Effects of docosahexaenoic acid (DHA) on intestinal poly development in Apcd7 16 knockout mice. Carcinogenesis 16: 2605-2607, 1995.
8. Kido T, Mori K, Daikuhara H, Tsuchiya H, Ishige A and Sasaki H: The protective effect of hochu-ekki-to, a Japanese herbal medicine, against HSV-1 infection in Mitomycin C-treated mice. Anticancer Res 20: 4109-4113, 2000.
9. Weitz A and Bresciania F: Estrogen induces expression of c-fos and c-myc protooncogenes in the rat uterus. Mol Endocrinol 2: 816-824, 1998.
10. Weitz A, Cicatiell L, Persiot E, Scalona M and Bresciant F: Estrogen stimulation of transcripton of c-jun protooncogene. Mol Endocrinol 4: 1031-1350, 1990.
11. Niwa K, Hashimoto M, Morishita S, Lian Z, Tagami K, Mori H, *et al*: Preventive effects of Juzen-taiho-to on N-methyl-N-nitrisourea and estadiol-17ß-induced endometrial carcinogenesis in mice. Carcinogenesis 22: 587-591, 2001.
12. Lian Z, Niwa K, Gao J, Tagami K, Hashimoto M, Yokoyama Y, *et al*: Shimotsu-to is the agent in Juzen-taiho-to responsible for the prevention of endometrial carcinogenesis in mice. Cancer Lett 182: 19-26, 2002.

13. Niwa K, Hashimoto M, Lian Z, Gao J, Tagami K, Yokoyama Y, *et al*: Inhibitory effects of toremifene on N-methyl-N-nitrisourea and estadiol-17ß-induced endometrial carcinogenesis in mice. Jpn J Cancer Res 93: 626-635, 2002.
14. Morishita S, Niwa K, Ichigo S, Hori M, Murase T, Tamaya, T, *et al*: Overexpression of c-fos/jun mRNA and their oncoproteins (Fos/Jun) in the mouse uterus treated with three natural estrogens. Cancer Lett 97: 225-231, 1995.
15. Komori A, Yatsunami J, Suganuma M, Okabe S, Abe S, Sakai A, *et al*: Tumor necrosis factor acts as a tumor promoter in BALB/3T3 cell transformation. Cancer Res 53: 1982-1985, 1993.
16. Yoshimi N, Sato S, Makita H, Wang A, Hirose Y, Tanaka T, *et al*: Expression of cytokines, TNF-α and IL-1α, in MAM acetate and 1-hydroxyanthraquinone-induced colon carcinogenesis of rats. Carcinogenesis 15: 783-785, 1994.
17. Moore RJ, Owens DM, Stamp G, Arnott C, Burke F, East N, *et al*: Mice deficient in tumor necrosis-alpha are resistant to skin carcinogenesis. Nat Med 5: 828-831, 1999.
18. Lian Z, Niwa K, Tagami K, Hashimoto M, Gao J, *et al*: Preventive effects of isoflavones, genestein and daidzein, on estradiol-17ß-related endometrial carcinogenesis in mice. Jpn J Cancer Res 92: 726-734, 2001.
19. Bardin A, Boulle N, Lazennec G, Vignon F and Pujol P: Loss of ERß expression as a common step in estrogen-dependent tumor progression. Endocrine-related Cancer 11: 537-551, 2004.
20. Chomczynski P and Sacchi N: Single-step method of RNA isolation by acid guanidium thiocyanate-chloroform extraction. Anal Biochem 162: 156-159, 1987.
21. Van Beveren C, van Straaten F, Curran T, Muller R and Verma I: Analysis of FBJ-MuSV provirus and c-fos (mouse) gene reveals that viral and cellular gene products have different carboxy termini. Cell 32: 1241-1255, 1983.
22. Lamph WW, Wamsley P, Sassone-Corsi P and Verma I: Induction of protoncogene jun/AP-1 by serum and TPA. Nature 334: 629-631, 1988.
23. Lumedico PT, Gubler U, Hellmann CP, Dukovich M, Giri JG and Yu-Ching EP, *et al*: Cloning and expression of murine interleukin-1 cDNA in *Escherichia coli*. Nature 312: 458-462, 1984.
24. Fransen L, Muller R, Marmenout A, Travernier J, van der Heyden J, Kawashima E, *et al*: Molecular cloning of mouse tumor necrosis cDNA and eukaryotic expression. Nucleic Acids Res 13: 185-191, 1985.
25. Hiroi H, Momoeda M, Inoue S, Tsuchiya F, Matsumi H, Tsutsumi O, *et al*: Stage specific expression of estrogen receptor subtypes and estrogen responsive finger protein in preimplantational mouse embryos. Endocr J 46: 153-158, 1999.
26. Silverberg SG and Wilkinson EL (eds): Uterine corpus. Epithelia tumors and related lesions. In: Histological Typing of Female Genital Tract Tumours. 2nd edition. WHO, Geneva, pp13-18, 1994.
27. Tagami K, Niwa K, Mori H and Tamaya T: Preventive effect of Juzen-taiho-to on endometrial carcinogenesis in mice is based on Shimotsu-to constituent. Biol Pharm Bull 27: 156-161, 2004.
28. Ohnishi Y, Fujii H, Kimura F, Mishima T, Murata J, Tazawa K, *et al*: Inhibitory effect of a traditional Chinese Medicine, Juzen-taiho-to on progressive growth of weakly malignant clone cells derived from murine fibrosarcoma. Jpn J Cancer Res 87: 1039-1044, 1996.
29. Ohnishi Y, Fujii H and Hayakawa Y: Oral administration of a Kampo (Japanese herbal) medicine Juzen-taiho-to inhibits liver metastasis of colon 26-L5 carcinoma cells. Jpn J Cancer Res 89: 206-213, 1998.
30. Ohnishi Y, Yamamura T, Tauchi K, Tsukada K, Nomura S and Saiki I: Expression of the anti-metastatic effect induced by Juzen-taiho-to is based on the content of Shimotsu-to-constituents. Biol Pharm Bull 21: 761-765, 1988.
31. Lian Z, Niwa K, Onogi K, Mori H, Harrigan RC and Tamaya T: Anti-tumor effects of herbal medicines on endometrial carcinomas via estrogen receptor-alpha-related mechanism. Oncol Rep 15: 1133-1136, 2006.