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Preventive Effects of *Glycyrrhizae radix* Extract on Estrogen-related Endometrial Carcinogenesis in Mice

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Short- and long-term experiments were conducted to examine the effects of *Glycyrrhizae radix* (*Gl radix*) extract on mouse endometrial carcinogenesis. *Gl radix* treatment (2 weeks) decreased the levels of c-fos/jun mRNA and the corresponding oncoproteins induced by estradiol-17β (E_2) in castrated mice uteri, as determined by reverse transcription-polymerase chain reaction and Southern blot analysis, and immunohistochemical methods, respectively. For the long-term assays, 98 female ICR mice were given N-methyl-N-nitrosourea (MNU) solution (1 mg/100 g body wt.) and normal saline (as controls) into their left and right uterine corpora, respectively. They were divided into four groups as follows: group 1 was given 0.625% *Gl radix*- and 5 ppm E_2-containing diet; group 2, 5 ppm E_2-containing diet; group 3, 0.625% *Gl radix*-containing diet; and group 4, the basal diet alone. *Gl radix* treatment significantly decreased uterine weights and the incidences of uterine endometrial atypical hyperplastic and malignant lesions. It is suggested that *Gl radix* has inhibitory effects on E_2-related endometrial carcinogenesis in mice, through suppression of estrogen-induced c-fos/jun-expressions.

Key words: Fos/jun — MNU — Endometrial carcinoma — Prevention — Mice

Glycyrrhizae radix (*Gl radix*) is used in approximately 74% of traditional Chinese herbal medicines.[1)] Glycyrrhizin (GL) is a major constituent of *Gl radix*, and other components include glabric acid, flavonoids such as liquiritin, licoricone, licoflavone, licoricidin, and formononetin, as well as putrescine, glycyrol, isoglycyrol, glycyrin, glycycoumarin and deoxoglycyrrhetol.[1)] GL has been shown to possess several beneficial pharmacological effects including anti-inflammatory activity,[2)] corticosteroid effects[3)] and others. GL has anti-estrogenic[4)] as well as estrogenic effects.[5)] In general, anti-estrogenic effects work protectively against estrogen-dependent cancers. This has been confirmed for uterine endometrial cancer in animals.[6)] There are some reports of chemopreventive effects of GL on skin carcinogenesis,[7, 8)] although the role of the anti-estrogenic action was not established.

Among the transiently expressed immediate early genes, c-fos/jun appears to be related to cellular proliferation and differentiation.[9)] It is noteworthy that acute administration of estradiol-17β (E_2) causes a transient increase in expressions of c-fos,[10)] c-jun[11)] and c-myc,[10)] 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).[12, 13)] Recently, the overexpression of c-fos/jun mRNA in castrated mouse uterine corpora was shown to be closely related to estrogenic activity.[12–14)] Elevated c-fos/jun expression was reported in estrogen-induced hamster kidney tumors.[15)] Expression of c-jun in human endometrial carcinomas was also reported to be a prognostic indicator.[16)]

Therefore, the present study was undertaken to assess whether administration of *Gl radix* exerts suppressive effects on mouse uterine endometrial carcinogenesis induced by MNU and E_2, and whether expression of fos/jun mRNA and the corresponding proteins is associated with the mechanism of estrogenic action.

MATERIALS AND METHODS

Animals and chemicals Female ICR mice 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 was purchased from Sigma Chemical Co. (St. Louis, MO). *Gl radix* extract was purchased from Tsumura Co. (Tokyo).

Experimental protocol for assay of short-term effects of *Gl radix* Female ICR mice, 12 weeks of age, were ovariectomized by laparotomy under general anesthesia with diethyl ether. Two weeks later, castrated mice were divided into three experimental groups (6 mice in each). Group 1 was given the diet containing E_2 (5 ppm) and *Gl radix* (0.625%). The dose of 0.625% *Gl radix* extract in the diet proved to be enough to inhibit the estrogenic

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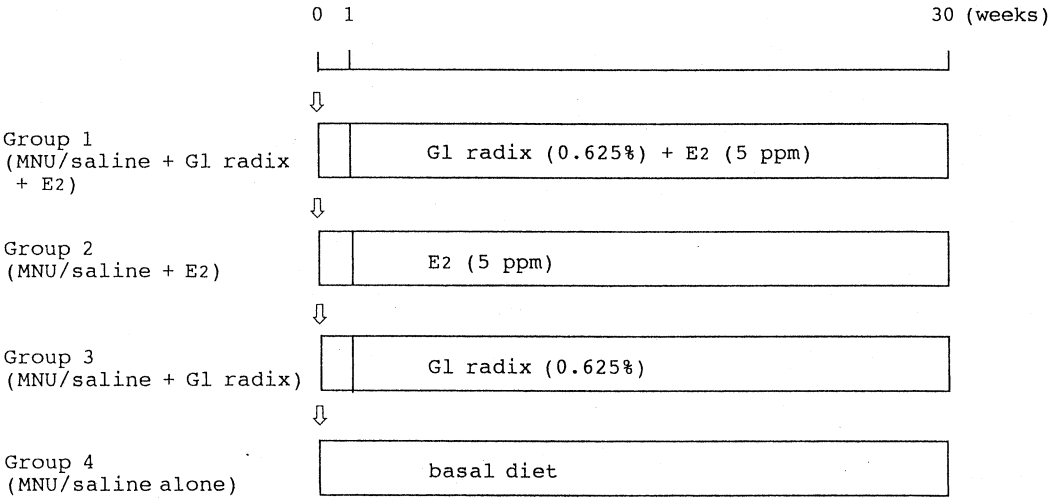

Fig. 1. Experimental design. ⇩: MNU solution at a dose of 1 mg/100 g body weight was injected into the left uterine tube, and normal saline into the right.

action of 5 ppm E_2.[5] Group 2 was fed the diet containing E_2 (5 ppm). Group 3 served as controls. Two weeks later, resected uteri were cut longitudinally in half. One half was quickly frozen in liquid nitrogen for the following experiment, and the other was subjected to pathological examination.

Reverse transcription-polymerase chain reaction (RT-PCR) Total RNA was isolated from frozen tissues by a guanidium thiocyanate-phenol-chloroform extraction method.[17] 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 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 expression, 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 using reverse-transcribed cDNAs and 0.1 mM specific primers in an Iwaki thermal sequencer TSR-300 (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.

The following oligodeoxynucleotides were synthesized as specific primers for PCR according to published information [cDNAs for c-fos,[18] c-jun,[19] and GAPDH[20]]: sense for c-fos, 5′-CTTACGCCAGAGCGGGAATG-3′; antisense for c-fos, 5′-AAGCCTCAGGCAGACCTCCA-3′; sense for c-jun, 5′-AGAGCATGACCTTGAAC-3′; antisense for c-jun, 5′-CTGGGAAGCGTGTTCTGGCT-3′; sense for GAPDH, 5′-TGAAGGTCGGTGTGAACGGATTTGG-3′; antisense for GAPDH, 5′-CTCCTTGGAGGCCATGTAGGCCAT-3′.

Semi-quantitative analysis of c-fos/jun mRNA expression 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. PCR products on the membrane were prehybridized in 1 M NaCl, 50 mM 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 sequences between the specific individual primers of c-fos or c-jun at 65°C overnight. Specific bands hybridized with biotinylated probes were detected with Plex Luminescent Kits (Millipore Corp.) on the membranes after exposure to X-ray films at room temperature for 10 min. The quantification of Southern blots was carried out with a Bio image analyzer (Millipore Corp.). The intensity of specific bands was standardized with respect to that of GAPDH mRNA.

Immunohistochemical expression of c-fos and c-jun proteins After having been fixed in 10% formalin, a half of the uterine corpus was processed for conventional staining. Briefly, the avidin-biotin-peroxidase complex was applied to the sections using a Vectastain kit (Vector, Burlingame, CA). The primary antibodies against the c-fos and

c-jun proteins (anti-rabbit polyclonal, Oncogene Science, Inc., New York, NY) were used at 1:100 dilution. Staining intensity was assigned as follows: +, positive; +/−, minimally or randomly positive; −, negative.

Experimental protocol for assay of long-term effects of *Gl radix* A total number of 98 female ICR mice, 10 weeks of age, underwent laparotomy under general anesthesia with diethyl ether. 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 four experimental groups. Group 1 (20 mice), diet with 0.625% *Gl radix* and 5 ppm E_2; group 2 (30 mice), diet with 5 ppm E_2; group 3 (18 mice), diet with 0.625% *Gl radix*; group 4 (30 mice), basal diet. In 30 weeks after the MNU exposure, 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 and hyperplastic were submitted to histological examination. Tissues were sectioned in 3 μm thickness and stained with hematoxylin and eosin.

Histology of the uterine lesions According to the WHO criteria,[21] uterine endometrial lesions were divided into four lesions: a) endometrial hyperplasia, simple; b) endometrial hyperplasia, complex; c) atypical endometrial hyperplasia; d) adenocarcinoma.

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

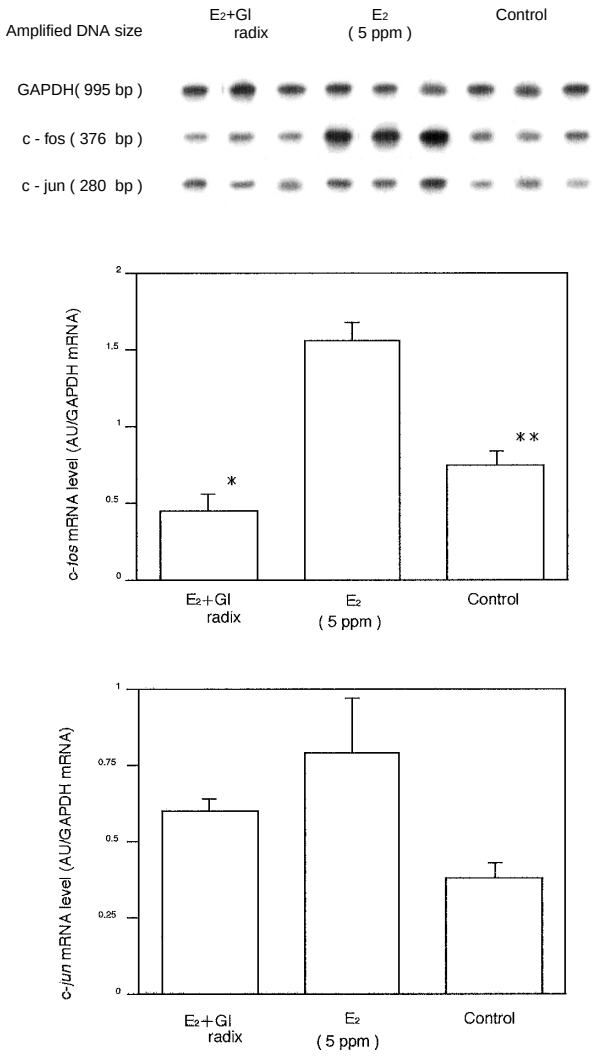

Fig. 2. Expressions of c-fos and c-jun mRNA in the uterus of ovariectomized mice treated continuously for two weeks with E_2 alone and *Gl radix* plus E_2 in the diet. E_2, estradiol-17β; *Gl radix*, *Glycyrrhizae radix*. * $P<0.01$, ** $P<0.05$.

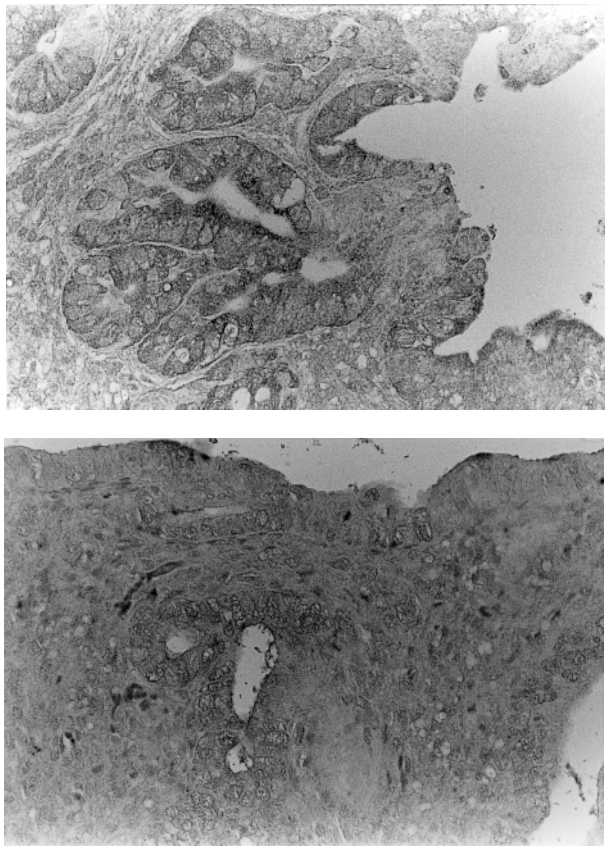

Fig. 3. A. The expression of c-fos in the uterus of a mouse orally given E_2. The expression was most prominent in the glandular cells (sABC stain, ×340). B. The expression of c-fos in the uterus of a mouse orally given E_2 plus *Gl radix*. The expression was weaker than that in the case of E_2 alone (sABC stain, ×340).

RESULTS

Short-term experiment The mean wet weights of the bilateral uterine corpora were as follow: E_2 alone (n=6), 0.14±0.03 g; E_2 plus *Gl radix* (n=6), 0.13±0.02 g; no treatment (n=6), 0.06±0.03 g. No significant differences were found between mice with E_2 alone and E_2 plus *Gl radix*. Expression levels of c-fos and c-jun mRNA are shown in Fig. 2. *Gl radix* significantly decreased the c-fos level induced by E_2-diet (P<0.01). *Gl radix* also tended to decrease the c-jun mRNA level induced by E_2-diet.

Histologically, endometrial glands in mice treated with E_2 resembled complex endometrial hyperplasia. *Gl radix* treatment tended to decrease hyperplastic glandular and luminal cells (Fig. 3, A and B).

Immunohistochemical expression of c-fos and c-jun oncoproteins is summarized in Table I. The expression of c-fos and c-jun oncoproteins was prominent in the glandular cells in the groups treated with E_2, but this was decreased by the treatment with E_2 and *Gl radix*.

Long-term experiment Three mice in group 1, six in group 2, three in group 3, and four in group 4 died within 15 weeks, though no pathological abnormalities other than pneumonia were found. The remaining animals survived until the termination of the experiment and were enrolled as effective animals (Table II). No significant difference in mean body weights was found among the four groups. The mean wet weight of the left uterine corpus in *Gl radix*-treated groups 1 and 3 was significantly smaller than that of non-treated groups 2 and 4, respectively (P<0.01).

Histological examinations revealed adenocarcinomas in the bilateral uterine corpora in the groups treated with MNU. Histological appearance of endometrial adenocarcinoma and hyperplasia in the present study was the same as that described in our previous reports.[12] All adenocarcinomas observed in the endometria were well or moderately differentiated. The incidence of preneoplastic and neoplastic lesions of the endometria is summarized in Fig. 4. The incidence of atypical hyperplasia and adenocarcinoma of the treated side of the uterine corpus in group 1 (treated with E_2 plus *Gl radix*) was significantly smaller than that in group 2 (treated with E_2, P<0.01). The inci-

Table I. Immunohistochemical Assay of Expression of c-fos and c-jun in Mouse Uterus after Two Weeks' Feeding of Diet with E_2 or E_2 Plus *Gl radix* Extract

Treatment	c-fos			c-jun		
	Glandular cells	Luminal cells	Stromal cells	Glandular cells	Luminal cells	Stromal cells
Group 1 (E_2 + *Gl radix*)	+/−	+/−	+/−	+/−	+/−	+/−
Group 2 (E_2 alone)	+	+/−	+/−	+	+/−	+/−
Group 3 (no treatment)	+/−	−	−	+/−	−	−

+, positive; +/−, minimally or randomly positive; −, negative.

Table II. Mean Body Weight, and Mean Weight of Left (Treated) and Right 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
Group 1 (MNU/saline + *Gl radix* + E_2)	20	17	45.2±5.2[b)]	0.30±0.12*	0.21±0.10**
Group 2 (MNU/saline + E_2)	30	24	42.8±5.0	0.71±0.25	0.35±0.16
Group 3 (MNU/saline + *Gl radix*)	18	15	47.2±6.0	0.28±0.20**	0.20±0.09
Group 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<0.001, ** P<0.05 compared with each control group.

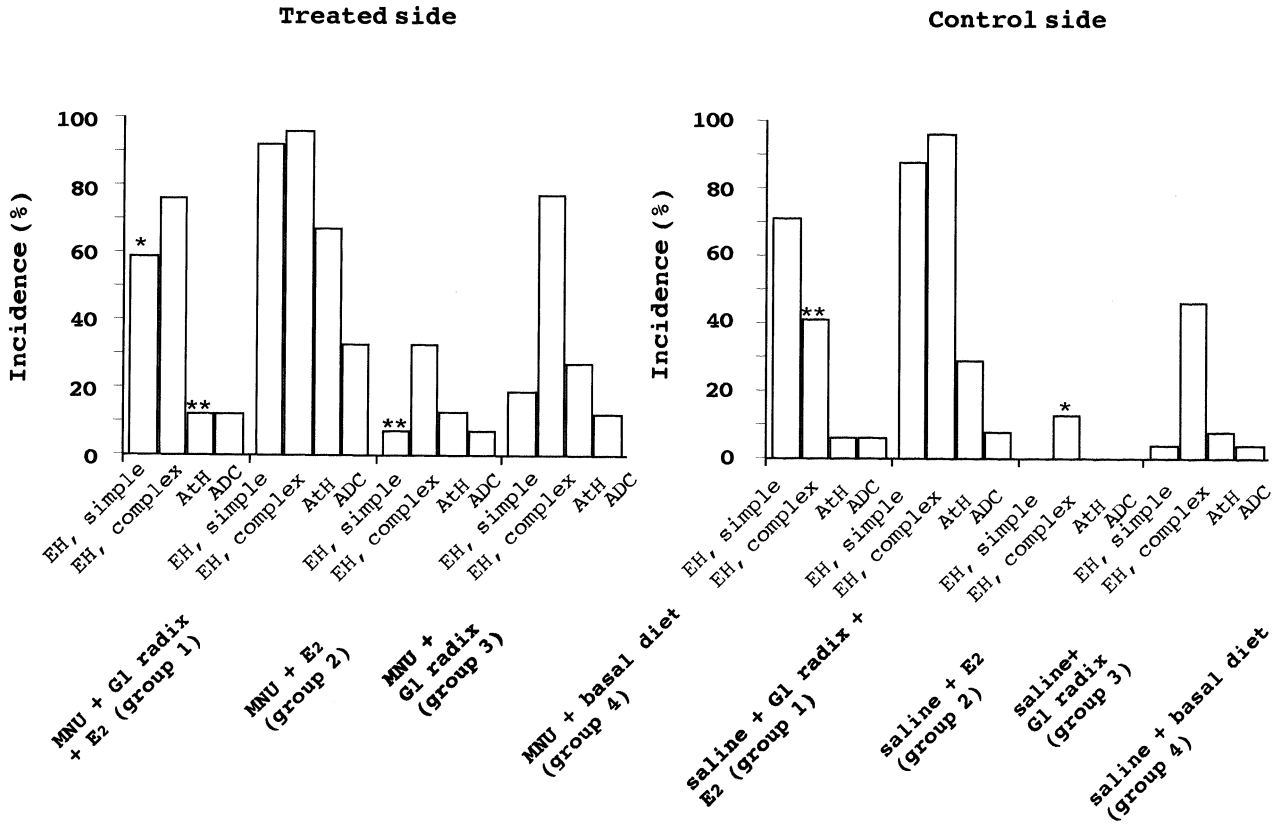

Fig. 4. Incidence of preneoplastic and neoplastic mouse endometrial lesions in each group. EH, simple: simple endometrial hyperplasia. EH, complex: complex endometrial hyperplasia. AtH: atypical endometrial hyperplasia. ADC: adenocarcinoma. * $P<0.05$, ** $P<0.01$.

dence of simple endometrial hyperplasia of the treated side of the uterine corpus in group 1 was significantly smaller than that in group 2 ($P<0.05$). In the right (control) uterine corpus, the incidence of complex endometrial hyperplasia in group 1 was significantly smaller than that in group 2 ($P<0.01$), yet *Gl radix* tended to decrease other endometrial preneoplastic lesions and adenocarcinoma in group 3 (treated with *Gl radix*).

Pathological examinations of ovary, oviduct and vagina were also done to investigate the hormonal conditions in each group. Cystic ovaries were commonly seen in mice treated with E_2 (group 1, 53%, $P<0.05$; group 2, 55%, $P<0.05$; group 3, 36%; group 4, 16%; each in left ovary). Corpora lutea were frequently observed in mice of each group (group 1, 94%; group 2, 95%; group 3, 93%; group 4, 93%; each in left ovary). No tumors were present in any of the groups. Marked epithelial hyperplasia of the oviduct, diagnosed as "progressive proliferative lesion,"[22] was commonly observed in mice of groups 1 (88%), 2 (100%) and 3 (93%), compared with group 4 (12%, $P<0.001$). Papillary lesions[14] were sometimes seen in the vagina of groups 1 (12%), 2 (13%), 3 (13%) and 4 (8%).

DISCUSSION

Gl radix treatment decreased expression of estrogen-induced c-fos/jun mRNA and the corresponding oncoproteins in the uterine corpora of castrated mice. Furthermore, *Gl radix* decreased the uterine weight overgrowth induced by estrogen in the long-term experiment, although such a decrease of overgrowth was not seen in the short-term experiment. These results suggest that *Gl radix* has anti-estrogenic effects at the dose used in the present study, and the administration term of *Gl radix* for 2 weeks seems to be too short to affect the uterotropic effects, such as uterine weight, in the short-term experiment.

In the present study, the effects of *Gl radix* on other tissues of the reproductive tract were examined. In all groups, corpora lutea were frequently seen in the ovaries, in contrast to the ovaries lacking corpora lutea found after treatment with tamoxifen in our previous study.[14] Estrogenic effects of *Gl radix* could not be found at the dose used in this study.

In the present study, inhibitory effects of *Gl radix* on endometrial carcinogenesis were seen in the bilateral uter-

ine corpora, especially of the E_2-treated mice. GL is reported to possess inhibitory effects on skin carcinogenesis in mice.[7, 8] The mechanism of anti-tumor activity might involve components of GL acting as inhibitors of carcinogen metabolism and DNA adduct formation.[8] The present study clarified the inhibitory effects of *Gl radix* on endometrial carcinogenesis induced by MNU in mice with estrogen-dominance, and the inhibition of estrogen-induced c-fos and c-jun expression in mouse uterus. *Gl radix* is known to contain triterpenoids such as GL and flavones such as formononetin.[1] GL has a steroid structure resembling those of glucocorticoid and mineralcorticoid, which bind to the cognate receptor[23] to exert their biological effects.[2, 24] It exhibits anti-estrogenic actions.[3] GL binds minimally to sex hormone-binding globulin (SHBG) and estrogen receptor.[23] In the presence of GL, estrogen bound to SHBG can be displaced and quickly metabolized, whereas GL binding to estrogen receptor blocks the estrogenic effect.

Flavones, and especially isoflavones such as formononetin, are classified as phytoestrogens.[25] Genistein, a metabolite of formononetin, is reported to exert anti-estrogenic effects and to inhibit cell proliferation by modulation of estrogen receptor binding and estrogen-regulated effects.[26] Isoflavones exhibit anti-carcinogenic activity *in vivo*[27] and reduce the proliferation of cells, including those in estrogen-sensitive breast cancer cell lines, and other tumors.[28] There is evidence that isoflavones exhibit anti-carcinogenic activity *in vitro*, and inhibit angiogenesis[29] and cell cycle progression,[30] as well as aromatase activity.[31]

In summary, we suggest that *Gl radix*, most likely its phytoestrogenic components, exerts anti-tumorigenic effects via estrogen-related action(s), and *Gl radix* is thus a promising agent for prevention of human endometrial cancer.

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