Abstract. We have previously reported on the inhibitory effect of Glycyrrhiza radix (Gl radix) on mouse endometrial carcinogenesis. The present study was performed to clarify the effects of Gl radix and glycyrrhizin (GL), the main part of Gl radix, on estradiol (E2)-related endometrial carcinogenesis. Both Gl radix and GL exerted a significant decrease in the COX-2, IL-1 and TNF-α mRNA expressions. GL generated a significant decrease in the incidence of endometrial adenocarcinoma. Accordingly, the preventive effects of Gl radix may be attributable to GL, thus being related with the suppression of COX-2, IL-1α and TNF-α. Gl radix and GL could therefore be a promising formula for the chemoprevention of human endometrial cancer.

Introduction

Glycyrrhizin (GL), a major constituent of Glycyrrhizae radix (Gl radix) which is ~10% of the dry weight of Gl radix extract, is the calcium and potassium salt of glycyrrhizinic acid. GL glycoside loses its sweet taste and upon hydrolysis is then converted to aglycone glycyrrhetinic acid plus two molecules of glucuronic acid. GL has an anti-estrogenic as well as an estrogenic effect (1). There is some evidence that GL has a protective effect on the skin (2,3) or liver carcinogenesis (4,5). It is known that Gl radix is widely used as a Kampo medicine in Asian countries and ~75% of traditional Japanese and Chinese medicines contain this agent. We have previously reported that Gl radix has a preventive effect on endometrial carcinogenesis in mice, and this effect is related to the inhibition of the expression of estrogen-stimulated genes c-fos/jun mRNA and proteins (6). We also proved that the herbal complex containing Gl radix suppresses cell proliferation in a chemo-endocrine resistant cancer cell line (7). In general, the anti-estrogenic effects are considered to have a protective effect against the occurrence of estrogen-dependent cancers. Such effects are confirmed in animal models of uterine endometrial cancer (8).

Cyclooxygenase (COX), an enzyme that activates the production of prostaglandins from arachidonic acid, has two isoforms. COX-2 has attracted considerable attention in connection with carcinogenesis in organs such as the large bowel, mammary glands and breast carcinogenesis (9-11). There is evidence that the COX activity is related to the promotion and progression in the tumorigenesis of the prostatic gland, stomach and lung (12-14). It remains unclear whether COX-2 is involved in E2-related mouse endometrial carcinogenesis, although a high expression level of COX-2 has been confirmed in human endometrial carcinomas (15). In this study, we examined the possible association of the preventive effects of GL on E2-induced mouse endometrial carcinogenesis with COX-2 activity.

We have reported that the expression of internal cytokines, interleukin (IL)-1α and tumor necrosis factor (TNF)-α, increase after E2 treatment (16). Such an increase of the internal cytokines is suggested to contribute to both tumor promotion and progression (17-19). Such circumstances prompted us to clarify the effects of GL on endometrial carcinogenesis in mice. Therefore, the expression of IL-1α and TNF-α were examined to indentify any further mechanism(s) of the effects of Gl radix and GL in the uteri of ovariectomized mice.

Materials and methods

Animals and chemicals. Female ICR mice were purchased from Japan SLC Co. (Shizuoka, Japan). As a basal diet, Oriental MF (Oriental Yeast Co., Tokyo, Japan) was used. Both the basal diet and filtered tap water were available ad libitum throughout the experiment. E2 and GL were purchased from Sigma Chemical Co. (St. Louis, MO), and
buffer (pH 8.4) with 50 μM KCl, 2.5 μM MgCl₂, 0.1 μg/ml 200 units, Gibco BRL, Gaithersburg, MO) in 20 μM Tris-HCl. Murine leukemia virus reverse transcriptase (MMLV-RTase, (20). Total RNA (3 μg) was reverse transcribed with Moloney guanidium thiocyanate-phenol-chloroform extraction method.

Total-RNA was isolated from frozen tissue specimens by the reverse transcription-polymerase chain reaction (RT-PCR). After two weeks feeding with the diet containing the above materials, one half was quickly frozen in the liquid nitrogen for dually. One half was quickly frozen in the liquid nitrogen for the following experiments, while the other specimen was submitted to a pathological examination.

The following oligodeoxynucleotides were synthesized as specific primers in PCR according to the published information (cDNA for COX-1/2 (21), IL-1α (22) TNF-α (23) and GAPDH (24)) as shown in Table I.

Semi-quantitative analysis of the COX-1, COX-2, IL-1α and TNF-α mRNA expressions by PCR products. PCR products were applied on 15% agarose gel electrophoresis at 50-100 V. The quantification of the products was carried out using Bio image (Millipore Corp.). The intensity of specific bands was standardized with that of GAPDH mRNA.

Immunohistochemical expression of COX-1/2, IL-1α and TNF-α protein. After being fixed in 10% formalin, half the uterine corpus was processed by conventional staining methods. Briefly, the avidin-biotin-peroxidase complex was applied on the sections using a Vestain kit (Vector, Burlingame, CA). The primary antibodies used were against the proteins of COX-1 (1:250, anti-mouse monoclonal, Cayman Chemical, Ann Arbor, MI) and COX-2 (1:200, anti-mouse monoclonal, Alexis Biochem., Carlsbad, CA). IL-1α (1:200, anti-rabbit polyclonal, Santa Cruz Biotech Inc., Santa Cruz, CA) and TNF-α (1:100, anti-goat polyclonal, Santa Cruz Biotech Inc.).

Immunohistochemical COX-1/2 expressions in glandular and stromal cells were scored separately according to the criteria of Krajewska et al (25). The scoring methods were modified by Fujiwaki et al (26). 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%. The intensity of immunostaining was rated as: 0, none; 1, weak; 2, moderate; 3, intense. As a result, the immunohistochemical COX scores

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**Table I. Sequences of primers.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide sequences</th>
<th>Citation</th>
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<tbody>
<tr>
<td>COX-1 sense</td>
<td>5'-TGCAATGTTGGCTTGAGTGATCATCA-3'</td>
<td>21</td>
</tr>
<tr>
<td>COX-1 antisense</td>
<td>5'-CCTAAAGACAGGCCATTCA-3'</td>
<td></td>
</tr>
<tr>
<td>COX-2 sense</td>
<td>5'-ACCTACAAGAGTTGGAGTACCTAC-3'</td>
<td>21</td>
</tr>
<tr>
<td>COX-2 antisense</td>
<td>5'-TGGATTAGTACCTAGGGTAATG-3'</td>
<td></td>
</tr>
<tr>
<td>IL-1α sense</td>
<td>5'-GATGGCCAAGTGCTGAGTCTG-3'</td>
<td>22</td>
</tr>
<tr>
<td>IL-1α antisense</td>
<td>5'-GCTCAGACAGCTCA-3'</td>
<td>23</td>
</tr>
<tr>
<td>TNF-α sense</td>
<td>5'-AGGACGTTTGCTGCCCTTTCA-3'</td>
<td></td>
</tr>
<tr>
<td>TNF-α antisense</td>
<td>5'-TCCACTTGGTTGCTGCTACG-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH sense</td>
<td>5'-CAAGGTATCCAGAGCTGAA-3'</td>
<td>24</td>
</tr>
<tr>
<td>GAPDH antisense</td>
<td>5'-GCATGTCAGCCCGCCATCG-3'</td>
<td></td>
</tr>
</tbody>
</table>

Gl radix (powder of the crude extract) was purchased from Tsumura Co. (Tokyo, Japan), respectively.

Experimental protocol for short-term experiment. Female (12 weeks old) ICR mice were ovariectomized at laparotomy under general anesthesia with diethylether. Two weeks later, the ovariectomized mice were divided into 6 experimental groups (6 mice in each). Group 1 was given daily 0.625% Gl radix and 5 ppm E₂; Group 2 was fed with 0.0625% of GL and 5 ppm E₂; Group 3 was exposed to 5 ppm E₂-containing diet alone; Group 4 was given daily 0.625% Gl radix alone; Group 5 was fed 0.0625% GL alone. Group 6 was given the basal diet only as a control. The dose of 0.625% Gl radix in the diet has been proven to be sufficient to inhibit the estrogenic action of 5 ppm E₂ (1,2), and the concentration, at the GL diet was calculated to be 10% of the 0.625% Gl radix. After two weeks feeding with the diet containing the above agents, the mice uteri were resected and cut in half longitudinally. One half was quickly frozen in the liquid nitrogen for the following experiments, while the other specimen was submitted to a pathological examination.

Reverse transcription-polymerase chain reaction (RT-PCR).

Total-RNA was isolated from frozen tissue specimens by the 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, Gaithersburg, MO) in 20 μM Tris-HCl buffer (pH 8.4) with 50 μM KCl, 2.5 μM MgCl₂, 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. RT reaction was heated at 94°C for 5 min to inactivate MMLV-RTase. For COX-1 (450 bp), extension of 35 cycles was performed consisting of 1 min at 94°C for denaturation, 1 min at 57°C for annealing, and for COX-2 (583 bp) of PCR consisting of 1 min at 72°C 15 sec at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for extension. PCR (30 or 25 cycles), consisting of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1.5 min at 72°C for extension, for TNF-α (369 bp) and IL-1α (401 bp). They were carried out in reverse transcribed cDNAs with 0.1 mM specific primers described below, using the IWAKI thermal sequencer TSR-300 (IWAKI Glass, Tokyo) with Vent DNA polymerase (New England Biolabs, Beverley, MA) in 20 μM Tris-HCl buffer (pH 8.8) with 10 μM KCl, 10 μM (NH₄)₂SO₄, 2 μM MgSO₄, 0.1% Triton X-100, and 0.15 μM deoxynucleotide phosphates. PCR (20 cycles) for geralddehyde-3-phosphate dehydrogenase (GAPDH, a house-keeping gene) mRNA (252 bp) as an internal standard was performed at the same time.

The following oligodeoxynucleotides were synthesized as specific primers in PCR according to the published information [cDNA for COX-1/2 (21), IL-1α (22) TNF-α (23) and GAPDH (24)] as shown in Table I.

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ranged from 0 to 12 (26). The immunohistochemical findings were analyzed by two independent investigators counting >200 cells and discordant results were reviewed jointly. The staining intensity for IL-1α and TNF-α protein was assigned as: (+), positive; (+/-), minimally or randomly positive; (-), negative (27).

Experimental protocol for long-term effects of GL. The protocol was: A total number of 125 female ICR mice, 12 weeks of age, underwent a 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 MNU exposure, the animals were divided into 6 groups. Group 1 (35 mice) was given the diet with 0.0625% GL and 5ppm E2. Group 2 (30 mice) was given the 5 ppm E2-containing diet alone. Group 3 (30 mice) was given the diet with 0.0625% GL. Group 4 (30 mice) was treated with the basal diet only as a control. At 30 weeks after the MNU exposure, all animals were sacrificed and autopsied. All major organs, especially the reproductive organs, were grossly inspected. Any lesions in the uterus, ovaries, vagina suspected of being neoplastic and hyperplastic were cut in half. The tissue specimens were submitted to a histopathological examination. The tissue specimens were then cut in 3-μm slices and then were stained with hematoxylin and eosin.

Histology of the uterine lesion. According to the WHO criteria (28), uterine endometrial lesions were divided into 4 types of lesions: a) endometrial hyperplasia, simple; b) endometrial hyperplasia, complex; c) atypical endometrial hyperplasia; d) adenocarcinoma.

Statistical analysis. A statistical analysis was done according to either the χ² test or Student’s t-test.

Results

Short-term experiment. The expression of COX-1/2 mRNAs is shown in Fig. 1. The level of COX-1 mRNA expression did not change after any treatment, however, the COX-2 mRNA expression which had been overexpressed in the E2 group significantly decreased after either the Gl radix or GL treatment (P<0.001, P<0.05, respectively). The results of the protein expression are summarized in Table II. The immunohistochemical score significantly decreased after treatment with either Gl radix or GL (P<0.05). The representative expression pattern of COX-2 protein is indicated in Fig. 2A and B.
protein expression decreased after the E2 plus Gl radix treatment (Fig. 2A) in comparison to that of the E2 group (Fig. 2B).

The expression of IL-1α and TNF-α mRNAs is shown in Fig. 3. Gl radix or GL exposure significantly decreased the level of IL-1α mRNA induced by the E2 treatment (P<0.001). Gl radix or GL also decreased the expression of TNF-α mRNA generated by E2 treatment (P<0.05). The results of the immunohistochemical analysis are summarized in Table III.

**Discussion**

We previously demonstrated that Gl radix has an inhibitory effect on E2-related endometrial carcinogenesis in mice (6). In the present study, the exposure to GL decreased the uterine weight in the long-term experiment, thus suggesting that GL has some anti-estrogenic effects at the dose used in this experiment. In the long-term experiment, the incidences of
adenocarcinoma and atypical hyperplasia of the group treated with E2 and Gl radix were significantly lower than those of the group treated with E2 alone (6). A similar tendency was confirmed by GL treatment. Since atypical hyperplasia is considered to be a direct precursor of endometrial adenocarcinoma (29), the decreased expression of atypical hyperplasia was suggested to support the chemopreventive effects of these agents in the endometrial carcinogenesis.

It has been reported that Gl radix contains not only GL but also isoflavones such as liquiritin, licoricone, licoflavone and formononetin (30). Isoflavones are known to act as chemopreventive agents for carcinomas including endometrial carcinoma (16). As summarized in Fig. 4, the incidence of adenocarcinoma in the Gl radix plus E2 group was lower than that in the GL plus E2 group (11.8 and 19.4%). The effectiveness for the preventive effect of Gl radix may therefore be related to the presence of other isoflavones in Gl radix.

Clinical studies have shown that the COX-2 expression is associated with the estrous cycle in the uterus (31). In the present study, the COX-2 mRNA expression significantly increased after E2 stimulation, and COX-2 mRNA was overexpressed in adenocarcinoma and atypical endometrial hyperplasia in the mouse uterus, thus suggesting the overexpression of COX-2 to be related to E2-induced endometrial carcinogenesis in mice. It is therefore suggested that the suppression of the overexpression of COX-2 is an effective strategy for endometrial cancer prevention.

Table III. Immunohistochemical expression of IL-1α and TNF-α in the ovarectomized mouse uteri.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>IL-1α</th>
<th></th>
<th>TNF-α</th>
<th></th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Glandular cells</td>
<td>Stromal cells</td>
<td>Glandular cells</td>
<td>Stromal cells</td>
</tr>
<tr>
<td>1</td>
<td>E2 + Gl radix</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>2</td>
<td>E2 + GL</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>3</td>
<td>E2 alone</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Gl radix alone</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>5</td>
<td>GL alone</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

(++) strongly positive; (+), positive; (±), minimally or randomly positive.

Table IV. Mean body weight and mean left uterine corpora in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Initial number of animals</th>
<th>Effective number of body weight (g)</th>
<th>Wet weight of uterine corpora (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>animals</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>MNU + E2 + Gl</td>
<td>35</td>
<td>32</td>
<td>44.3±6.2*</td>
</tr>
<tr>
<td>2</td>
<td>MNU + E2 alone</td>
<td>30</td>
<td>25</td>
<td>43.1±3.0</td>
</tr>
<tr>
<td>3</td>
<td>MNU + GL alone</td>
<td>30</td>
<td>27</td>
<td>36.9±4.1</td>
</tr>
<tr>
<td>4</td>
<td>MNU alone</td>
<td>30</td>
<td>27</td>
<td>48.0±6.0</td>
</tr>
</tbody>
</table>

*Mean ± SD. *Significantly different from the corresponding group (P<0.05).

In this study, GL and Gl radix suppressed expression of COX-2 as well as the internal cytokines, IL-1α and TNF-α expression in the mouse uterine corpora. Meanwhile, it is known that TNF-α and IL-1α also play a significant role in both human and rodent carcinogenesis (16,32). TNF-α has also been reported to stimulate tumor promotion and the progression of initiated cells and premalignant cells in mice (33). Therefore, the inhibition of TNF-α and IL-1α mRNA expression is considered to contribute to cancer prevention (16,32,33).

In summary, we report herein that Gl radix is an efficient preventive agent for endometrial carcinogenesis, and that GL acts as an important constituent in Gl radix, for the suppression of the carcinogenesis related to the suppression of COX-2, TNF-α and IL-1α expression.

Acknowledgements

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References