Oriental Medicinal Herb, *Periploca sepium*, Extract Inhibits Growth and IL-6 Production of Human Synovial Fibroblast-Like Cells

Takayoshi Tokiwa,*a Kuniaki Harada,b Toshiharu Matsumura,b and Takashi Tukiyamaa

*a* Kohno Clinical Medicine Research Institute; Shinagawa-ku, Tokyo 140–0001, Japan; and b Roman Industries Co. LTD.; Fuku'ura, Yokohama 236–0004, Japan. Received July 5, 2004; accepted July 28, 2004; published online July 29, 2004

*Periploca sepium* (PS) has traditionally been used in oriental medicine for treatment of rheumatoid arthritis (RA). We investigated the aqueous extract of PS (PSE) in its effects on human rheumatoid arthritis-derived fibroblast-like cells. In cell culture studies, PSE inhibited the growth and IL-6 production of the cells in dose-dependent manners. The extract of *Glycyrrhiza glabra* (GG), which has also been used to treat RA and chosen as a reference here, slightly inhibited the growth of RA cells. A study of PSE fractionation indicated that the active material inhibiting IL-6 production is filterable by ultrafiltration, suggesting that substances with low molecular weight might be involved in an inhibition of IL-6 production. These results support the view that PSE represents a rich source of growth inhibition and anti-IL 6 production.

Key words *Periploca sepium*; anti-IL 6 production; rheumatoid arthritis; synovial fibroblast

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by joint swelling, synovial membrane inflammation, and joint destruction. Hyperplasia of the synovium and damage to articular connective tissue are major morphologic features of RA, and represent an important determinant of disease progression.1) Although the exact cause of pathologic processes in this chronic inflammatory joint disease is not fully understood, abnormal cell proliferation and excessive production of cytokines such as IL-1β, IL-6, and TNF-α by synovial cells are important potential targets for therapeutic intervention.

Because of the chronic nature of the disease, considerable therapeutic attention has been payed on the use of medicinal herbs, among which Tripterygium Wilfordii Hook F (TWHF)2) and *Glycyrrhiza glabra* (GG)3,4) have particularly been investigated. Looking for other oriental medicinal herbs, we noted that *Periploca sepium* (PS) has traditionally been used in China to treat RA.3) Major past attention on this plant, however, has been in its anti-tumor effects,5) while little has been investigated in its effects on RA in cellular and molecular terms.

In the present study, we examined the effect of PSE on the growth and IL-6 production of synovial fibroblast-like cells derived from RA tissue (SF cells). As shown in Results and Discussion, PSE presents a rich source of low molecular principles which inhibit the growth and IL-6 production of SF cells in culture.

MATERIALS AND METHODS

Synovial Tissue Sources Patients with RA, as defined by the revised criteria of the American College of Rheumatology, were recruited for this study. All patients (stage III) were positive for rheumatoid factor. Approval was obtained from the Human Studies Committee of Kohno Clinical Medicine Research Institute, and individual informed consent was provided from each patient before inclusion in the study. Synovial tissue was obtained from the joints of 4 patients undergoing joint replacement or synovectomy, and thickened villous synovial tissue was recovered for the experiments.

Cell Culture Second and 3rd passage cultures of SF cells were used in the experiments. Tissue collected from swollen knee joints was cut into approximately 1-mm pieces and placed in polystyrene dishes. Explant cultures were performed from primary cultures of synovial tissue, and after overgrowth in a dish, the cells were trypsinized and used in the experiments. The culture medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum, and it also contained antibiotics, penicillin (100 U/ml) and streptomycin (100 μg/ml). All cells were cultured in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

Preparation of Oriental Medicinal Herb Extracts Oriental medicinal herbs, dried chips of PS and GG, were purchased from Uchida Wakanyaku Co., Ltd. (Tokyo, Japan), and the extracts (E) were prepared as follows: dried chips, 2 g each, were steeped overnight in 200 ml of distilled water at room temperature, and then boiled for 60 min. The infusions were filtered through filter paper to remove insoluble material, and after Millipore (0.2 μm) filtration, they were used in the experiments as crude extracts (PSE and GGE). The filtrate of PSE was further divided into two equal aliquots, and one of which was lyophilized and used as fraction A-1 (308 mg). The other was further filtered by ultrafiltration (<10 kDa). The filtrate and nonfiltrate obtained thus were concentrated under reduced pressure, lyophilized and used as fraction A-3 (269 mg) and A-2 (23 mg), respectively.

MTT Assay Cells were plated in 96-well plates at a density of 3×103 cells/well, and after 24 h of incubation, the culture medium was replaced with medium containing one of the oriental medicinal herb extracts or of the fractions (A-1, -2, -3). After 3-day incubation, 10 μl of stock MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide, Wako Co., Ltd., Tokyo, Japan) (5 mg/ml) was introduced into each well of the plate. The well plate was further maintained at 37°C for 4 h, the supernatant gently removed, and then 100 μl 100% dimethylsulfoxide added to each well. The absorbance was measured on a microplate reader at 570 nm. The cell density was estimated from the calibration curves using CHO-K1 cells.

Cell Viability Test Cells (104 cells/well) were cultured in a medium containing or not containing PSE in 96-well plates at 37°C for 3 d. The cells were harvested, and viable cell numbers counted using 0.1% trypan blue.

Immunofluorescence of IL-6 Cells were plated in 96-well
plates at a density of $3 \times 10^3$ cells/well. The cells were cultured with or without IL-1β (1 ng/ml) in the presence or absence of different concentrations of extracts or fractions for 3 d. The culture supernatants were collected and preserved at $-20^\circ$C till submitted to enzyme-linked immunosorbent assay (ELISA). IL-6 concentrations in culture supernatants were measured with ELISA kits. Each value represents the mean±S.D. of three experiments.

Effect of Oriental Medicinal Herb Extracts on Cell Growth When SF cells were exposed to PSE or GGE, as shown in Figs. 1 and 2, PSE showed a dose-dependent inhibitory effect, although slight inhibition was observed with GGE. The inhibition was also time-dependent (data not shown). IC$_{50}$ of PSE was between 0.02 and 0.2 mg/ml, and GGE showed a dose-dependent inhibitory effect, although slight inhibition was observed with PSE (mg/ml) vs. b).

Effect of PSE on Cell Viability It is important to clarify that the growth inhibition is not due to the nonspecific cytotoxicity of the extract. To this end, we estimated cell viability of PSE-treated RF cells by trypan blue dye exclusion test. We found that no significant difference in cell viability was observed for SF cells cultured in the presence or absence of 0.02 or 0.2 mg/ml of PSE, as shown in Table 1. These results suggest that PSE contains substances that inhibit cell growth but do not have a direct cytotoxic effect below the concentrations of at least 0.2 mg/ml.

Effect of PSE and Its Fractions on IL-6 Production We therefore studied the effect of the extracts on IL-6 production. To this end, we estimated cell growth toxicity of the extract. To this end, we estimated cell viability of any concentration of extracts or fractions for 3 d. The cells plated in 96-well plates were cultured with IL-1β (1 ng/ml) in the presence or absence of different concentrations of extracts or fractions for 3 d. The cells were stimulated with IL-1β (1 ng/ml) in the presence or absence of PSE for 3 d. IL-6 concentrations in culture supernatants were measured with ELISA kits. Each value represents the mean±S.D. of three experiments. b) $p<0.001$ vs. a), by Student's $t$-test; c) $p<0.01$, and d) $p<0.001$ vs. b).

Table 1. Effect of PSE on RA-Derived Synovial Fibroblast-Like Cells

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Dose (mg/ml)</th>
<th>No. of cells ($\times 10^3$/well)</th>
<th>IL-6 level (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>8</td>
<td>1237.5±1127.8</td>
</tr>
<tr>
<td>A1</td>
<td>0.2</td>
<td>2.5</td>
<td>15.0±7.1</td>
</tr>
<tr>
<td>A2</td>
<td>2</td>
<td>3.5</td>
<td>1.0±1.4</td>
</tr>
<tr>
<td>A3</td>
<td>2</td>
<td>7.0</td>
<td>509.8±509.5</td>
</tr>
<tr>
<td>A2</td>
<td>0.2</td>
<td>3.5</td>
<td>409.8±424.2</td>
</tr>
<tr>
<td>A3</td>
<td>2</td>
<td>3.5</td>
<td>18.5±2.1</td>
</tr>
<tr>
<td>A3</td>
<td>2</td>
<td>3.5</td>
<td>10.0±0.0</td>
</tr>
</tbody>
</table>

The cells plated in 96-well plates were cultured with IL-1β (1 ng/ml) in the presence or absence of fractions for 3 d. IL-6 concentrations in culture supernatants were measured with ELISA kits. Each value represents the mean±S.D. of three experiments.

The effects of PSE fractions on SF cells were collectively shown in Table 2. Fraction A-1 was shown to induce a strong inhibition of IL-6 production as well as cell growth at 0.2 or 2 mg/ml. Fraction A-3 behaved similarly to A-1. However, both cell growth and IL-6 production were only weakly inhibited by A-2 fraction. The recovery of A-2 fraction was below 10% of that of A-3 fraction as stated in Materials and Methods. IL-1β, IL-6, and TNF-α are well known proinflammatory cytokines involved in the pathogenesis of RA, and all three are increased in the synovial fluid and serum of RA patients. We therefore studied the effect of the extracts on IL-6 production by SF cells. The cells were stimulated with IL-1β 1 ng/ml in the presence of GGE or PSE for 3 d, and culture supernatants were subsequently recovered to assess IL-6 production. As shown in Figs. 2 and 3, SF cells produced a large quantity of IL-6 in response to IL-1β stimulation; whereas exposure to GGE or PSE significantly suppressed IL-6 production.

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which showed inhibition in cell growth and IL-6 production. Glyderinine isolated from GGE has been found to exert a pronounced anti-inflammatory effect.4)

Anti-neoplastic active components have been isolated from PS.5) However, no reports have been concerned with anti-inflammatory effect of PS. These results support the view that PSE presents a rich source of low-molecular substances with cell growth inhibition and anti-IL-6 production, and thus of interests in the study of inflammation control, particularly in RA.

REFERENCES