Anticachectic effects of *Coptidis rhizoma*, an anti-inflammatory herb, on esophageal cancer cells that produce interleukin 6

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Abstract

Herbs as alternative cancer therapies have attracted a great deal of recent attention due to their low toxicity and costs. In this study, the antitumor activity and anticachectic effect of *Coptidis rhizoma*, an anti-inflammatory herb, were investigated in nude mice carrying a human esophageal cancer cell line YES-2, which constitutively secretes interleukin-6 (IL-6) and induces cachexia when injected into these mice. In this study, in vivo growth of YES-2 cells was not affected by an oral supplement containing the extract powder of *C. rhizoma* at a final concentration of 1% (CR supplement). However, in comparison with normal diet, CR supplement significantly attenuated weight loss of tumor-bearing mice without a change in food or water intake. Tumor IL-6 levels were significantly lower in mice treated with CR supplement than in control mice (*P* < 0.001). Serum IL-6 was detectable in four (50%) of eight control mice; IL-6 was not detected in mice treated with CR supplement. We also confirmed that berberine (8–32 μM), a major component of *C. rhizoma*, dose-dependently inhibited secretion of IL-6 by YES-2 cells in vitro. Moreover, reverse transcription-PCR assay showed that treatment of YES-2 cells with berberine (8–32 μM) for 24 h reduced IL-6 mRNA expression. Our results suggest that *C. rhizoma* may have an anticachectic effect on esophageal cancer and an effect is associated with the ability of berberine to down-regulate tumor IL-6 production. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Coptidis rhizoma*; Berberine; Kampo medicine; Esophageal cancer; Cachexia; Interleukin 6

1. Introduction

Patients with esophageal cancer have a poor prognosis because of the rapid spread and of the cancer associated malnutrition due to dysphagia and cachexia [1], although significant progress has been made in surgical treatment and adjuvant chemotherapy for esophageal cancer [2]. Cancer cachexia is a paraneoplastic syndrome that worsens the quality of life of patients with a variety of malignant tumors [1,3,4]. Numerous studies have suggested that circulating...
IL-6 secreted from tumor cells plays an important role in cancer-induced cachexia [5±7]. These findings are supported by our clinical data that IL-6 might be related to the nutritional status of patients with esophageal cancer [1]. Wang et al. [8] recently reported an inverse association between serum IL-6 levels and prognoses in esophageal cancer patients. Thus, IL-6 is likely to play a key role in cancer-induced cachexia, suggesting that down-regulation of tumor IL-6 levels may improve cachexia or malnutrition in patients with esophageal cancer.

Our recent study showed that *Coptidis rhizoma*, a natural herb containing high levels of berberine, had direct antitumor effects on human esophageal cancer cells in vitro [9]. *C. rhizoma* or *Berberis vulgaris* root has long been used in European and Far Eastern countries for treating inflammatory diseases such as gastroenteritis [10,11], suggesting that it might prevent the inflammatory response caused by cancer progression. Indeed, Yasukawa et al. [12] showed that berberine inhibits 12-O-tetradecanoylphorbol-13-acetate-induced inflammation in mice. Fukuda et al. [13] reported that berberine inhibits the activity of activator protein 1 (AP-1), which transactivates inflammatory cytokines such as IL-6 [14]. Therefore, we postulated that *C. rhizoma* containing high levels of berberine could have both antitumor and anticachectic effects on esophageal cancers. Thus, we examined both antitumor activity and anticachectic effect of oral administration of *C. rhizoma* in nude mice carrying YES-2 cells, a IL-6-producing esophageal cancer cell line [15]. To our knowledge, this is the first study that evaluates the anticachectic effect of a natural herb on human esophageal cancer xenografts.

2. Materials and methods

2.1. Cell line

YES-2 cells, a human esophageal squamous cell carcinoma cell line [2,15], were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 5% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin G and 100 μg/ml streptomycin. This cell line constitutively secretes interleukin 6 (IL-6) but not IL-1β or tumor necrosis factor-α [15].

2.2. Agents

The extract powder of *C. rhizoma* [9] was donated from Tsumura & Co. (Tokyo, Japan) and mixed in standard mouse diet at a final concentration of 1% using a modification of previously described procedure [11]. Berberine was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Berberine has molecular weight of 371.8 and it is reported to be present in the extract powder of *C. rhizoma* at a concentration of approximately 20% [16]. Berberine was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO was kept at less than 0.1% to avoid its inhibitory effects on the proliferation of YES-2 cells.

2.3. In vivo studies

Six-week-old male BALB/c nu/nu mice were purchased from Japan SCL (Hamamatsu, Japan). YES-2 cells were injected with a 27-gauge needle subcutaneously into the right lower abdominal quadrant. The total number of tumor cells injected per animal was $5 \times 10^5$. Tumor volumes were measured using a slide caliper and determined by the following formula [17]: $a \times b^2 ÷ 2$, where $a$ is the larger and $b$ is the smaller of the two dimensions. In a *C. rhizoma* supplement (CR supplement) group ($n = 8$), a standard mouse diet containing the extract powder of *C. rhizoma* at a final concentration of 1% was fed throughout the experiment from 7 days before the injection of YES-2 cells. In a normal diet group ($n = 8$), the standard mouse diet was fed throughout the experiment. Tumor volume and body weight were measured once or twice per week after cell injection. All mice were killed by cervical dislocation 4 weeks after cell injection, and both tumor and serum samples were collected and stored at $-80^\circ$C until use. To evaluate whether mouse cachexia was caused by YES-2 cells, the body weights of intact mice in the CR supplement group ($n = 8$) and normal diet group ($n = 8$) were measured once or twice per week. All animal experiments were conducted in accordance with the guidelines of Animal Care and Use Committee of Yamaguchi University School of Medicine.

The concentrations of IL-6 in sera and tumor tissues were measured by enzyme-linked immunosorbent assay (ELISA) using the Quantikine Human IL-6
ELISA kit (R&D Systems Inc., Minneapolis, MN). The minimal detectable concentration of IL-6 was 3.12 pg/ml. Using murine colon 26 adenocarcinoma cells, we confirmed that the ELISA kit used in this study did not react with mouse IL-6 (data not shown). For measurement of tumor IL-6, tumor tissues were thawed, weighed quickly, placed in 2 ml phosphate-buffered saline (PBS), and homogenized for 30 s in a tissue homogenizer. The homogenates were then centrifuged twice at 4°C at 10 000 × g, and aliquots of the supernatants were prepared for the IL-6 assay as described previously [1].

2.4. In vitro studies

To examine whether IL-6 production in YES-2 cells is affected by berberine, ELISA and reverse transcription (RT)-PCR were performed as described previously [1,15]. The tissue or plasma concentration of berberine after oral administration of C. rhizoma remains unknown. Our preliminary study showed that 24-h treatment with berberine at concentrations of more than 40 μM significantly inhibited the proliferation of YES-2 cells. Therefore, in the present study, we used a dose of 4–32 μM to avoid inhibitory effects of berberine on the proliferation of YES-2 cells. Briefly, 1 × 10⁵ YES-2 cells per well were inoculated into 6-well plates with DMEM containing 5% FCS. After 24-h incubation, the medium was changed to DMEM with 5% FCS plus 0–12 μg/ml berberine. The culture media and cells were collected after 24-h incubation with or without berberine at each concentration. The concentrations of IL-6 in culture media were measured by ELISA as described above. For analysis of IL-6 mRNA expression, total cellular RNA from YES-2 cells was extracted with TRIzol (Gibco, Bethesda, MD), and 4 μl of total RNA (1 μg) was reverse-transcribed in 16 μl RT-mix [18] containing 200 units of Maloney-murine leukaemia virus reverse transcriptase (Gibco) at 42°C for 1 h. Subsequently, 4 μl of cDNA solution (equivalent to the cDNA from 0.2 μg of initial RNA) was amplified in 96 μl PCR-mix containing 10 × PCR buffer, 2.5 units of Taq DNA polymerase (Gibco), 0.2 mM dNTPs, 1.5 mM MgCl₂, and 25 pmol each primer for the IL-6 or β-actin genes. PCR amplification was performed for 35 cycles for IL-6 and 25 cycles for β-actin. Each cycle consisted of 94°C for 1 min (denaturation), 58°C for 45 s (annealing) and 72°C for 2 min (elongation). The primers used in this study were as follows: IL-6, 5’-GAACCTTCTCTCCACAGCG-3’ (sense) and 5’-GATCCAGATTGGAAGCATCC-3’ (antisense); and β-actin, 5’-CCAGAGCAAGAGAGGTAT-3’ (sense) and 5’-CTGTGTTGTGTAAGGTGTA-3’ (antisense). The expected sizes were 316 and 436 bp for IL-6 and β-actin genes. Finally, 10 μl of PCR products and a molecular weight marker (1 kb Plus DNA Ladder, Gibco) were separated by electrophoresis on a 1% agarose gels and visualized under ultraviolet light after ethidium bromide staining.

2.5. Statistical analysis

Body weights and the data for IL-6 levels in culture media were analyzed by analysis of variance (ANOVA), and where appropriate, Fisher’s PLSD test or Scheffe’s adjustment for comparison was used. Tumor volumes were compared using a one-way ANOVA with repeated measures. Tumor IL-6 levels in mice treated with or without CR supplement were compared using Student’s t-test. P < 0.05 was accepted as statistically significant.

3. Results

3.1. In vivo effects of oral administration of C. rhizoma on tumor growth and tumor-induced cachexia

YES-2 cells induced cachexia when injected into nude mice, as shown in Fig. 1A. The CR supplement significantly attenuated weight loss of tumor-bearing mice when compared with normal diet (Fig. 1A). No differences in food and water intake were observed between the two groups (data not shown). No difference in body weight was observed between intact mice treated with CR supplement and those treated with normal diet (Fig. 1A). In comparison with normal diet, CR supplement did not significantly inhibit in vivo growth of YES-2 cells (Fig. 1B). Histological examination revealed no remarkable change in tumor cell morphology or mononuclear cell infiltration into tumor tissues (data not shown). Tumor IL-6 levels were 783 ± 127 and 1649 ± 139 pg/g tissue in mice given CR supplement and those given normal diet, respectively. Thus, tumor IL-6
levels were significantly lower in mice given CR supplement than in those given normal diet ($P < 0.001$, Student’s $t$-test) (Fig. 2). Serum IL-6 was detectable in four of eight mice (50%) given a normal diet. Serum IL-6 levels in these four mice ranged from 3.7 to 12.2 pg/ml. In contrast, IL-6 was not detected in any of the mice given the CR supplement.

### 3.2. Down-regulation of IL-6 production in YES-2 cells in vitro by berberine

Berberine is a major component of *C. rhizoma* and has both antitumor and anti-inflammatory activities [9,11,13]. Therefore, we examined the effects of berberine on IL-6 production in YES-2 cells in vitro. As shown in Fig. 3A, berberine dose-dependently inhibited IL-6 secretion from YES-2 cells into culture medium at concentrations of 8–32 μM. Cell viability was unchanged after 24 h of exposure to berberine at concentrations of 4–16 μM; however, viability was reduced to approximately 85% control levels after 24-h exposure to 32 μM berberine. RT-PCR showed that treatment of YES-2 cells with more than 8 μM berberine for 24 h reduced in IL-6 mRNA expression (Fig. 3B).
4. Discussion

Many studies have demonstrated the anticachectic effects of agents such as indomethacin, melatonin, fish oil, dexamethasone, medroxyprogesterone acetate and eicosapentanoic acid [7,19–23]. However, there have been no reports regarding the anticachectic effects of natural herbs. The present study suggests that *C. rhizoma*, an anti-inflammatory herb, has an anticachectic effect on human esophageal cancer xenografts in nude mice.

Many molecules have been investigated as mediators of tissue wasting in cancer-induced cachexia [4,24]. Of these factors, numerous reports have suggested that circulating IL-6 secreted from tumor cells plays an important role in cancer-induced cachexia [1,5–7]. These reports have suggested that the anticachectic effect of *C. rhizoma* might be associated with tumor IL-6 production. In the present study, the CR supplement significantly prevented weight loss and decreased tumor IL-6 levels. Moreover, we confirmed that both IL-6 secretion and IL-6 mRNA expression in esophageal cancer cells are down-regulated in vitro by berberine, a major component of *C. rhizoma*. This is consistent with a previously reported finding that berberine directly inhibits the activity of AP-1, which is responsible for transactivation of IL-6 [13]. In the present study, serum IL-6 was not detected in 50% of the mice treated with normal diet. These mice showed dramatic weight loss. This suggests that other factors responsible for this cachexia are present, although we did not evaluate mouse IL-6 levels in this study. Tisdale suggested that IL-6 could be a marker of the process rather than the actual mediator of cancer cachexia, because direct administration of this cytokine to experimental animals fails to induce cachexia [24]. Recent studies have identified novel cachectic factors including neurotrophin-1/B cell-stimulating factor-3, lipid-mobilizing factor and proteolysis-inducing factor [25–28]. Further studies are needed to evaluate the effects of *C. rhizoma* on expression of these factors.

Berberine is a benzodioxolo–benzoquinolizine alkaloid present in the extract powder of *C. rhizoma* at a concentration of approximately 20% [16] and has direct antitumor activity on some malignant tumors including esophageal cancer [9,29–31]. We postulated that *C. rhizoma* might have an antitumor effect in vivo. In the present study, in vivo growth of YES-2 cells was not affected by a diet containing 1% *C. rhizoma*, although tumor IL-6 levels in these mice decreased significantly. Consistent with the finding, our in vitro study demonstrated that berberine inhib-
ited IL-6 secretion from YES-2 cells at the concentrations which did not inhibit the cell proliferation. In the present study, the tissue or plasma concentration of berberine after oral administration of *C. rhizoma* remains unknown. However, these results support the possibility that berberine levels in tumor tissues could inhibit tumor IL-6 production but not tumor growth. Studies of pharmacokinetics of berberine or unknown components of *C. rhizoma* are required to gain further insight into our results and to elucidate the mechanism by which *C. rhizoma* might prevent cancer-induced cachexia.

We conclude that *C. rhizoma* might have an antica- chic effect on human esophageal cancer and that berberine, the major component, might prevent cancer-induced cachexia. In this study, we investigated histologically organs including alimentary tracts and liver in mice after oral administration of *C. rhizoma*. We confirmed that there is no tissue injury in mice treated with *C. rhizoma* (data not shown). These results suggest that the treatment with *C. rhizoma* show less toxicity. Some herbs have recently attracted a great deal of attention as alternative therapies from the viewpoint of both reduced toxicity and cost. Several clinical trials with herbs and other natural products are under way in Europe and the United States [32,33]. Indeed, DiPaola et al. [34] demonstrated the clinical efficacy of an estrogenic herbal combination on prostate cancer. Thus, the use of herbs may be an effective strategy in the treatment of cancer.

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References


