Oral administration of Qing-Shu-Yi-Qi-Tang reduce lung cancer-induced cachexia in mice

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This study is to investigate anti-inflammatory and anti-cachectic effect of Qing-Shu-Yi-Qi-Tang (QSYQT) in RAW 264.7 cells and Lewis lung cancer cells bearing mice. We examined the effect of QSYQT on LPS-induced inflammatory activity in a murine macrophage cell line, Raw 264.7. We evaluated cachetic parameters, such as weight loss, food intake and tumor size in tumor-bearing and non-tumor-bearing mice treated with QSYQT decoction or a normal diet. Cytokine production in cell culture and in cachetic mice was quantified by ELISA. NF-kB expression was measured using real-time polymerase chain reaction (PCR). QSYQT significantly reduced the IL-1β, IL-6 and TNF-α production in LPS-stimulated RAW 264.7 cells. Treatment of QSYQT prevented weight loss in tumor-bearing mice without affecting food intake or tumor growth. Furthermore, the level of proinflammatory cytokines, IL-1β, IL-6 and TNF-α in sera were significantly reduced in tumor-bearing mice treated with QSYQT. NF-kB expression in spleen of LLC-derived mice was decreased in the presence of QSYQT. Our results revealed that QSYQT exerts an anti-cachectic effect on LLC-induced cachectic mice. The effect is conclusively associated with modulation of IL-6 production through NF-kB.

Key words: Chinese traditional medicine, cachexia, anti-inflammation, IL-6, NF-kB, palliative medicine.

INTRODUCTION

Cachexia is a syndrome characterized by progressive loss in weight, anorexia, fatigue and aberrant immune function (Esper and Harb, 2005). The disorder occurs in up to 80% of patients with advanced cancer and accounts for up to 20% death. Cancer cachexia is associated with the responsiveness to chemotherapy and correlated with survival time of cancer patients, leading to poor quality of life (Brown, 2002). The disorder is commonly seen in subjects with gastrointestinal, lung and prostate cancers, in contrast to haematological and breast malignancies. The exact nature of mechanism underlying pathogenesis of cachectic multifactorial complication in cancer patients remains sketchy. However, it is evident that persistent inflammatory response of the host in association with cytokines and catabolic factors produced by the tumor plays a critical role in the pathogenesis. Current therapeutic interventions in cancer cachexia are of limited benefit (Gagnon and Bruera, 1998). Drugs that modulate either immune responses or production of tumor-associated catabolic molecules represent promising candidates for anti-cachectic therapy. Herbal medicines serving as one arm of medication in Asian societies represent attractive remedy for their immuno-modulating activity in the aspect of palliative supplement (Yap et al., 2010; Yang et al.
Qing-Shu-Yi-Qi-Tang (QSYQT), a formula of Chinese medicine, has been traditionally used for treating fever and pulmonary disorders for centuries. The major ingredients of QSYQT have been individually investigated for their immunopharmacological action. Astragalus membranaceus has been reported to effectively reduce inflammatory responses through inhibition of NF-κB-mediated transcription (Lee et al., 2005; Ryu et al., 2008). In a clinical trial, Astragalus radix has been demonstrated to improve cancer-associated anoxia in patients with advanced cancer (Lee, 2010). Atractylodes macrocephala koidz, an ingredient of QSYQT, has been demonstrated to have anti-inflammatory effects using in vitro model (Dong et al., 2008; Li et al., 2007). The use of Panax ginseng C. A. Mey inhibits the expression of inflammation cytokines, including IL-6, IL-8 and TNF-α in lipopolysaccharide (LPS)-stimulated lymphocytes (Lee et al., 2008; Kim et al., 2007). Immuno-modulatory activity of Rhizoma Cimicifugae has been demonstrated in vitro and in vivo (Pan et al., 2009; Lin et al., 2006).

The present study was aimed to explore the effect of Chinese herbal decoction QSYQT on lung cancer induced cachexia. The anticachectic activity of QSYQT was investigated using a cachectic model established in mouse with Lewis lung carcinoma cells (Matthys et al., 1991). The physiological parameters, including body weight, food intake and tumor size were evaluated as well as immunological factors, such as cytokine production.

MATERIALS AND METHODS

Decoction preparation

The decoction used in this study was prepared by Chuang Song Zong Pharmaceutical Co. Ltd. (Ligang, Taiwan) as lyophilized powder of water extract from Astragalus membranaceus, Panax ginseng C. A. Mey, Atractylodes chinensis (DC.) Koidz, Cimicifuga foetida, L. A. macrocephala Koidz, Alisma orientale (Sam.) Juzep, Citrus reticulata Blanco, Massa Medicata Fermentata. Briefly, the aforementioned materials were mixed and decocted three times with boiling distilled water for 1 h. The decoction was filtered, collected, concentrated and lyophilized. The powder was dissolved in distilled water and administered in a volume of 10 ml/kg.

Cell culture

Vero and Lewis lung carcinoma (LLC) cell lines were obtained from Biosource Collection and Research Center (BCRC, Hsin-Chu, Taiwan) and maintained in Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% fetal calf serum (Gibco, USA), penicillin (100 U/ml), streptomycin sulfate (100 µg/ml) and 2.0 mM/ml glutamine. Cells were incubated at 37°C in a humidified 5% atmosphere of 95% air and 5% CO2. Cells were co-treated with LPS (1 µg/ml) and various concentrations of QSYQT for 24 h.

MTT assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described (Mantovani and Madeddu, 2009). Briefly, cells were seeded at a density of 4 x 10^4 cells/well in a 24-well plate and cultured with serum-free Dulbecco's Modified Eagle's Medium (DMEM) for 16 h. Then, the cells were treated with serial concentrations of 6-gingerol (0, 5, 10 and 15 µg/ml) for 24 or 48 h. Treatment at each concentration was performed in triplicate. After treatments, the medium was aspirated and cells were washed with phosphate buffered saline (PBS). Cells were subsequently incubated with MTT solution (5 mg/ml) for 4 h. The supernatant was removed, and formazan was solubilized in isopropanol and measured spectrophotometrically at 563 nm. The percentage of viable cells was estimated in comparison with untreated cells.

Evaluation of cytokines production

Concentrations of IL-6, IL-1β and TNF-α in culture supernatants or circulation of mice of each group were determined by the ELISA method following the instructions of the manufacturer (R&D Systems, Minneapolis, MN, USA).

RNA extraction and real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from harvested cells with the RNaseasy Midi Kit (Qiagen) and treated with RNase-free DNase (Qiagen) according to the manufacturer's instructions. The SuperScript II (Invitrogen) reagent set was used to convert 2 µg RNA to cDNA. All real-time PCR assays contained 10 mM Tris (pH 8.3), 50 mM KCl, 1 U of Taq polymerase (Sigma-Aldrich), 200 ng/µL bovine serum albumin (MBI Fermentas), 3 mM MgCl2, 0.3 mM dNTPs (Sigma-Aldrich), 1:100,000 SYBR Green I (Molecular Probes) and 400 nM primer polymerase chain reaction (PCR) primer each in 20 µl. The reverse transcription and real-time PCR primers were designed using Primer3. Real-time PCR was performed in a LightCycler (Roche Diagnostics) starting with 3 min of preincubation at 95°C, followed by 50 amplification cycles. The threshold cycle (Ct) was determined by the use of the maximum-second-derivative function of the LightCycler software. Formation of expected PCR product was confirmed by agarose gel electrophoresis (2%) and melting curve analysis.

Animal model and experimental design

Male C57BL/6 mice of 6 to 8 weeks old from National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and were housed as previously described. Animals were injected subcutaneously into upper flank of mice with Lewis lung carcinoma (LLC) cells (1 x 10^6 tumor cells/animal) using a 27-gauge needle. Animals were orally administered with either distilled water or decoctions once everyday after cell injection until the animals were sacrificed. Food intake and body weight were measured every 2 days after cell injection. Tumor volume was measured every 4 days throughout the experiment, and tumor volume was calculated as (Height x Width^2)/2.
**Statistical analysis**

The data are expressed as mean ± S.D. Student's t-test or Dunnett t-test was used to compare the differences between treated groups and control groups, and differences were considered significant at P < 0.05.

**RESULTS**

**Effect of Qing-Shu-Yi-Qi-Tang (QSYQT) on cell viability and cytokine production in vitro**

Cytotoxic effect of QSYQT was examined using Vero and LLC cell lines after treatment with serial concentrations of QSYQT. As shown in Figure 1, there was no significant change in cell viability following the treatment at concentrations up to effective dose of 2.5%, as assessed by MTT assay. We further investigated the effect of QSYQT on the production of proinflammatory cytokine in LPS-stimulated RAW 264.7 macrophages. Concentrations of TNF-α, IL-6, and IL-1β in culture medium of activated macrophages in presence of QSYQT were measured by ELISA. The results showed that concentrations of LPS-induced proinflammatory mediators were concentration-dependent and they decreased in the presence of QSYQT (Figure 2).

**Anti-cachectic activity of QSYQT in LLC cancer cachexia model**

According to the findings in cell-based model, we performed in vivo study using LLC cancer cachexia model to evaluate anticachectic effect of QSYQT at final concentration of 2.5%. The effects of 2.5% QSYQT treatment on body weight, tumor size and food intake in C57BL/6 mice bearing LLC are shown in Figure 3. QSYQT significantly prevented weight loss in tumor-bearing mice without changing tumor growth or food intake (P < 0.05). No difference in body weight and food intake was observed between control mice with or without QSYQT treatment.

**Effect of QSYQT on inflammatory mediators in vivo**

To determine whether QSYQT modulates the level of inflammatory mediators in vivo including IL-1β, IL-6 and TNF-α, LLC-induced cachectic mice were orally administered with QSYQT at a concentration of 2.5% (v/v). The cytokines concentrations were measured using ELISA. High levels of IL-1β, IL-6 and TNF-α were detected in the sera of tumor-bearing mice. Treatment of QSYQT resulted in a significant decrease in concentration
serum IL-1β, IL-6 and TNF-α of LLC-derived cachectic mice as compared to that of untreated tumor bearing animals (Figure 4).

As production of IL-6 is regulated by several transcription factors including NF-kB, we thereafter determine the effect of QSYQT on NF-kB expression. As shown in Figure 5, expression of NF-kB in mouse spleen was significantly inhibited in the presence of QSYQT at a concentration of 2.5% (v/v).

**DISCUSSION**

Patients with advanced malignancies, such as lung cancer, frequently develop cachexia that significantly affects clinical course, quality of life and survival of the patients (Esper and Harb, 2005; Gagnon and Bruera, 1998; Mantovani and Madeddu, 2009). Therapeutic strategies against cachexia have been developed and examined in human trial and tumor-bearing animal models based on the clinical symptoms (Brown, 2002; Gagnon and Bruera, 1998). The treatments include appetite stimulants, non-steroid anti-inflammatory drug, anabolic and anti-catabolic drugs (Madeddu and Mantovani, 2009). However, in clinical trials, most of the anti-cachectic therapies fail to meet expectation in terms of weight gain, nutritional status and quality of life (Topkan et al., 2007). Currently, much research is focused on exploring the mechanism of cachexia development in cancer setting. It is quite clear that cancer cachexia is associated with a chronic systemic inflammatory response and proinflammatory cytokines are linked to all pathways that induce cachexia. Manipulation of cytokine production is currently of significant interest for managing cancer cachexia. Extracts of a variety of herbs have been shown to exert immunopharmacological activity. Since most of the medicinal herbs have low toxicity and minor side-effects, herbal formula is considered as a palliative supplement for cancer patients with worsening physical condition. In the present study, the data revealed that QSYQT, a traditional Chinese medicine formula, can reduce productions of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. The finding is consistent with previous researches in which the same herbal ingredients were examined. The results in vitro prompted us to examine the anti-cachectic effect of QSYQT on Lewis lung carcinoma, a cachectic tumor that induced significant weight loss and remarkable change in biochemical factors (Bennani-Baiti and Walsh, 2011; Llovera et al., 1998). Using this model, we demonstrated that oral administration of QSYQT resulted in attenuation of cachexia symptoms. However, anorexia, other feature of cachexia was not improved and tumor growth of Lewis lung cancer cell was not inhibited in the presence of...
Figure 3. Effect of QSYQT on (a) body weight, (b) food intake and (c) tumor growth in mice bearing Lewis lung carcinoma cells. Values represent mean body weights, tumor volumes and food intakes for 6 mice in each group. Bars indicate SD. (a) QSYQT treatment significantly attenuated weight loss in tumor-bearing mice as compared to controls. (b, c) There were no statistical differences in tumor growth or food intake between the 2 experimental groups.
QSYQT. In cancer patients, cachexia development is attributed to the production of a combination of cytokines and other cachectic factors by both the host and the tumor. The continuing presence of tumor, resulted in a chronic inflammatory state, characterized by the production of T helper 1 (Th1) cytokines, such as IL-1β,
IL-6 and TNF-α, and the induction of an acute phase response (Penna et al., 2010; Argiles et al., 2009). Although, the presence of these cytokines is not capable of generating the whole syndrome of cachexia, systemic administration of such cytokines does induce some features of anorexia and wasting in animals (Moldawer et al., 1987; Langstein and Norton, 1991; Negri et al., 2001; Murray et al., 1997; Costelli et al., 1993). Additionally, some cachectic symptoms in animal models can be attenuated by monoclonal antibody therapy to IL-1β, IL-6 and TNF-α (Matthys et al., 1991; Madeddu and Mantovani, 2009; Carbo et al., 1994; Strassmann et al., 1992). Our result shows that, in consistency, improvement in cachectic symptoms by QSYQT was associated with a decrease in serum levels of IL-1β, IL-6 and TNF-α. This suggests that profound inflammation plays a critical role in cachexia development and represents a target for managing cachexia. Elevated level of IL-6 and TNF-α in cancer cachexia has been postulated to be as result of up-regulated expression of NF-κB (Paule et al., 2007; Moore-Carrasco et al., 2007; Zhog et al., 2003). Our result shows that reduced production of IL-6 and TNF-α after oral administration of QSYQT was associated with lower NF-κB expression in splenocytes as compared to that of tumor-bearing ones without treatment. In consistency with previous studies, it is suggested that QSYQT modulate inflammation response through NF-κB pathway.

Conclusion

Conclusively, the present results indicate that QSYQT exerted a significant anticachectic activity by modulating circulating levels of cytokine in LLC-induced cachectic model. Further studies are needed to elucidate the mechanism of this effect, and clinical trials will be necessary to establish this activity in humans.

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