Journal of Ethnopharmacology 134 (2011) 141–146

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

The protective effect of schisandra lignans on stress-evoked hepatic metastases of P815 tumor cells in restraint mice

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ARTICLE INFO

Article history:
Received 13 July 2010
Received in revised form
22 November 2010
Accepted 27 November 2010
Available online 3 December 2010

Keywords:
Fructus schisandrae
Schisandra lignans
Hepatic metastases
Restraint stress
Immobilization
Immunomodulation

ABSTRACT

Aim of the study: The present study was conducted to investigate the effects of schisandra lignans extract (SLE) on stress-evoked hepatic metastases of mastocytoma P815 tumor cells, which was closely related with immune function.
Materials and methods: The high-performance liquid chromatography (HPLC) fingerprint of SLE was recorded and the percentage composition of schisandra lignans was determined as 82.63%. The contributions of the immunomodulatory properties of SLE to the protective effects on stress-induced hepatic metastases were studied.
Results: Our results found that restraint stress significantly promoted hepatic metastases of P815 tumor cells. However, oral administration of SLE (100 and 200 mg/kg/d, 14 d) significantly reduced the number of metastatic colonies in liver of restrained mice. SLE was further found to be significantly improving T lymphocyte proportions and increasing cytotoxic T lymphocyte (CTL) activity of immunized spleen cells in stressed mice.
Conclusion: These results indicated that the protective effects of SLE on hepatic metastases were related to its alleviation of the adverse effects of stressors for bio-homeostasis and immunoprotection. The obtained data provided evidences to elucidate the traditional use of *Fructus schisandrae* as a tonic or sedative.

1. Introduction

Fructus schisandrae, fruit of *Schisandra chinensis* (Turcz.) Baill, was regarded as a popular herb in traditional Chinese and Russian herbal medicine. In Traditional Chinese Medicine (TCM), it is a common ingredient in prescriptions such as Shenqi Wuweizi Pian included in Chinese Pharmacopoeia, and could also be used alone as a tonic or sedative (Deng et al., 2008). Modern pharmacological studies have demonstrated that the traditional use was related with its anti-stress and immune-protective activities (Panossian and Wikman, 2008). The major active compounds of *Fructus schisandrae* are schisandra lignans (Avula et al., 2005). Schisandra lignans provide hepatoprotection, anti-oxidation, anti-stressand anti-tumor activities (Schobert et al., 2008). The stress-protective effects of schisandra lignans, both *in vitro* and *in vivo*, have also been confirmed in recent studies (Chiu and Ko, 2004; Lee et al., 2007). Treatment with schisandra lignans significantly reduced serum corticosterone levels and prevented stress-induced reduction in spleen size and serum interleukin-2 levels (Lee et al., 2007). These results suggested that schisandra lignans could be used to treat stress disorders, in part, by its immunomodulatory effects.

Restraint or immobilization induces "non-specific" generalized physiological responses that are characteristics of stress, i.e. disorders of autonomic nervous system and hormones, suppressions of immune system, and damages of organ functions (Bodnar et al., 2004; Fuchikami et al., 2009). Our previous studies confirmed that immunomodulatory mechanism and oxidative stress in immunocytes play important roles in fighting against stress and natural products had protective effects (He et al., 2009b). Other researchers' studies demonstrated that restraint stress promoted tumor growth

Abbreviations: SLE, schisandra lignans extract; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; ORAC, oxygen radical absorbance capacity; *F. schisandrae*, *Fructus schisandrae*; NK, natural killer; CTL, cytotoxic T lymphocyte; TCM, Traditional Chinese Medicine; HPA, hypothalamic pituitary adrenal; FITC, fluorescein isothiocyanate; PE, phycoerythrin; DiO, 3′-dioctadecyloxacarbocyanine perchlorate; PI, propidium iodide; TBARS, thiobarbituric acid-reactive substances.

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doi:10.1016/j.jep.2010.11.070

and virus replication in animal by suppressing antibodies production and cytotoxicity of natural killer (NK) cell (Hunzeker et al., 2004; Kurihara et al., 2002). As a traditional tonic, *Fructus schisandrae* was reported containing compounds modulating human immune system (Yip et al., 2007). There have also been many reports about the antiproliferative effects of lignans isolated from *Fructus schinensis* on human tumor cells *in vitro* (Min et al., 2008). However, there have been no previous studies on the effect of these compounds on tumor cells migration induced by stress *in vivo*. In the present study, we investigated the effects of schisandra lignans extract (SLE) on stress-evoked hepatic metastases of P815 tumor cells in mice treated with restraint stress. Moreover, contributions of the immunomodulatory properties of SLE to its effect on stress-evoked hepatic metastases of P815 tumor cells were also investigated.

2. Materials and methods

2.1. Preparation of schisandra lignans extract

Fructus schisandrae was supplied by Liaoning Ludan Ltd. (Liaoning, China). A voucher specimen (2009WWZ0006) was maintained in Institute of Traditional Chinese Medicine & Natural Products, Jinan University, Guangzhou, China. Authentic standards of Schizandrol A, Schizandrol B, Schisantherin A, Deoxyschizandrin, Schizandrin B, Schisandrin C were purchased from Scholar Biotech Ltd. (China, Chengdu). Air-dried *Fructus schisandrae* (4.0 kg) was powdered and soaked in 15 volumes of distilled water overnight. The macerated crude drug was refluxed for 1.5 h for two times. Then the combined aqueous extracts were centrifuged at 3000 rpm for 15 min. The centrifuged supernatant was then concentrated under reduced pressure until its density was between 1.05 and 1.10. The concentrated aqueous extract was then subjected to chromatographic separation on D101 macroporous adsorption resin with water, 30%, 60%, 90% EtOH–H_2O to give four fractions. The 90% EtOH–H_2O fraction was concentrated under reduced pressure and then dried in vacuum to give yellowish brown oil as SLE. SLE was qualitatively analyzed employing HPLC–MS method. Total lignans was quantitated by measuring against Schizandrol A standard calibration curve. HPLC–MS analysis was carried out on an Agilent series 1100 HPLC system equipped with UV–vis detector and coupled online with a Bruker Esquire 2000 mass spectrometer (Bruker Co., Switzerland).

2.2. Chemicals and reagents

Mouse immunoglobulin G1-fluorescein-isothiocyanate (FITC) or phycoerythrin (PE), anti-CD3 (FITC), anti-CD4 (PE), and anti-CD8 (PE) were all purchased from Beckman (USA). Sodium fluorescein (FL), 2′,2′-azobis (2-amidinopropane)-dihydrochloride (AAPH), and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.3. Animals and tumor cell lines

Seven week-old female DBA/2 mice were purchased from Slac Laboratory Animal (Shanghai, China). DBA/2 mice were kept in a specific pathogen-free animal room at $23 \pm 1\,^{\circ}C$ with a 12-h dark–light cycle and were fed a normal diet and water. The animals were allowed to acclimatize to the environment for 1 w before the experiment. All mice cared and maintained in accordance with the guidelines set forth by the National Institutes of Health. The protocol was approved by the Institutional Laboratory Animal Care and Use Committee of Jinan University (No: 0033051). The mouse mastocytoma tumor cell line P815 was purchased from Sun Yat-sen University and maintained by propagation in DBA/2 mice ascites.

2.4. Stress procedure and hepatic metastases of P815

Hepatic metastasis was induced by i.v. injection of mouse cutaneous mastocytoma P815 cells according to the method of Kurihara et al. (2002). The experimental mice were divided into five groups: normal control, P815 control (P815 injection only), model control (stress + P815), and two SLE groups (stress + P815 + SLE 100 or 200 mg/kg). SLE groups received oral administration of SLE suspended in drinking water at a final concentration of 10.0 and 20.0 mg/ml, while the three control groups received water only. On the second day of administration, mice were physically restrained in a 50 ml polypropylene centrifuge tube with holes for 20 h (He et al., 2009a), and then placed in the home cage with food and water before the assay. Multiple liver metastases were induced by injection of 0.1 ml of PBS solution containing 1×10^4 viable mouse P815 cells into the tail vain of the DBA/2 mice on day 1 after stress loading. The normal control group mice were injected with blank PBS at the same volume. SLE was consecutively administered orally to the mice from 1 d before the restraint stress to 1 d before sacrifice for 14 d. All mice were sacrificed 12 d after inoculation of tumor cells. The livers were removed and fixed in Bouin's solution for 5 min, and then the metastatic tumor nodules on the liver surface were counted.

2.5. Splenocyte preparation

The spleens were collected and splenocytes were prepared by disrupting the spleen with a grinder in PBS (pH 7.4). The total splenocyte number was determined with a blood-cell counting chamber (Erma, Japan). After a 10 min centrifugation at 1500 rpm to separate debris, erythrocytes were lysed using ammonium chloride reagent (0.02 M Tris–0.13 M NH_4Cl, PH 7.2). The cells were washed twice with PBS and suspended in 1 ml of cold RPMI-1640 medium with 10% FBS. The viability of splenocytes was determined by trypan blue exclusion.

2.6. Determination of T lymphocyte subsets

Samples containing 1×10^6 splenocytes in RPMI-1640 medium were treated with selected monoclonal antibodies conjugated with FITC or PE (Beckman, USA). We used the following double-staining combinations: anti-CD3 (FITC)/anti-CD4 (PE), and anti-CD3 (FITC)/anti-CD8 (PE). Mouse IgG1-FITC and -PE were used as control staining. After 15 min incubation at room temperature in the dark, the cells were washed with PBS and resuspended in 0.5 ml of cold PBS and analyzed using a FACS Epics XL (Beckman, USA). Usually, 10,000 cells were scanned for each sample, and the results were expressed as the percentage of cells yielding a specific fluorescence in a gated lymphocyte region.

2.7. CTL activity assay

The experiments employed two fluorescent stains as previous reported (He et al., 2009b). CTL cell activity was detected with the freshly isolated splenic mononuclear cells. Target cells for detection of CTL cytotoxicity were P815 cell line stained with 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO, from Sigma). Mixtures of the stained P815 cells and the splenocytes were incubated at different ratios for 4 h. Then propidium iodide (PI, from Sigma) was added to the mixtures at a concentration of 5 μg/ml at room temperature for 15 min. Dio stains the intended tumor cell population homogeneously, and PI stains damaged