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Stimulating effects on mouse splenocytes of glycoproteins from the herbal medicine *Atractylodes macrocephala* Koidz.

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Abstract

A traditional herbal medicine, *Atractylodes macrocephala* Koidz. (AMK), has long been used as a digestive and tonic. Recent investigations have suggested its potential ability in stimulating immune responses, although a scientific basis for this activity has not yet been elucidated. Based on previous results showing that the activity might be due to proteins, we purified protein samples from an original sample preparation of AMK and examined the stimulating ability of the protein samples on mouse splenocytes. The sample treatment markedly stimulated lymphocyte proliferation, antibody production, and cytokine secretion in mouse splenocytes. In particular, the samples showed the ability to induce the preferential stimulation of Th1 type, rather than Th2 type T lymphocytes. Stimulating activity of the samples was associated closely with glycoprotein(s) with molecular weights of around 30 kDa, especially with carbohydrate moiety rather than with protein residues of the glycoprotein(s). Our findings suggest that the glycoprotein(s) might play critical roles in modulating immune-response induction, and could potentially be used as medicinal and pharmacological agents.
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Keywords: *Atractylodes macrocephala* Koidz. (AMK); Traditional herbal medicine; Glycoproteins; Splenocytes; Immune stimulation

Introduction

Atractylodes macrocephala Koidz. (AMK) has been used widely as a digestive and tonic in traditional oriental medicines (Yim et al., 1988). Recently, there has been increasing interest in studying the medicinal and pharmacological roles of this drug, and numerous investigations have reported its biological effects (Yun et al., 1981; Park et al., 1999). Low molecular weight substances, including sesquiterpenoids and triterpenoids, were identified from AMK as inhibitors against Na^+–K^+-ATPase and neuromuscle connections (Kimura et al., 1995).

AMK, more importantly, has been used as one of the major components of traditional Korean medicines such as Soamsan and Boyangwhanotang, which have long been used to induce cancer remission and to prevent

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chronic illnesses (Ha and Lee, 1998; Kim and Lim, 1999). The action mechanism of the medicines is believed to be related to the modulation of immune response induction. This assumption has been proven by our previous reports that oral administration of the medicines markedly enhanced antigen-specific immune responses (Kim et al., 2002a, b). In addition, it was considered that AMK might play a pivotal role among the ingredients of the medicines in the immune modulation induced by the medicines, although a scientific basis for the role has not yet been elucidated. To this end, the present study focused on investigating whether AMK contains immune-stimulating activity, and elucidating the identity of the major ingredient(s) responsible for the activity.

Materials and methods

Chemicals, plastics, and mice

Unless otherwise specified, all chemicals and plastics used in this study were purchased from Sigma Chemical Co. (St. Louis, MO) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ), respectively. Antibodies and cytokine reagents were obtained from BD PharMingen Inc. (San Diego, CA). BALB/c mice (4–6-weeks old) were purchased from Orient Co. (Seoul, Korea).

Preparation of AMK samples

AMK was generously provided by Dr. W.-H. Woo (College of Oriental Medicine, Wonkwang University, Iksan, Korea) and authenticated by botanists at the Korea Institute of Oriental Medicine. A voucher specimen (# 2001-N3) has been deposited in the author's laboratory (Y.-S. Jang). Initially, air-dried AMK (100 g) was cut into small pieces, mixed with 1000 ml of distilled water in a screw-capped flask, and then shaken at 4 °C for 3 days to prepare a water extract of AMK. The extract was subsequently filtered through filter paper (Whatman no. 3) and centrifuged at 5000*g* for 10 min to remove debris. Collected supernatants were lyophilized to give an original sample preparation of 10.2 g, which was 10.2% of the initial amount. One gram of the sample was dissolved with 10 ml of 80% $(NH_4)_2SO_4$ and centrifuged to collect the protein precipitate. The precipitate was then dialyzed and lyophilized to yield 72 mg of the protein sample preparation. The samples were stored at −20 °C before use.

Cell culture and treatment

Spleen cell population was prepared using RPMI-1640 medium supplemented with antibiotics and 10% FBS (HyClone, Logan, UT). Two million spleen cells per milliliter were resuspended in either 500 or 100 μl media for spreading onto either 24- or 96-well flat-bottomed plates, respectively. Before the samples were added, the cultures were replaced with the medium supplemented only with 0.5% FBS. At varied times after the sample treatment, the cells were subjected to analyses for trypan blue staining, tritiated-thymidine uptake, and ELISA assays.

Lymphocyte proliferation and cytotoxicity assay

The level of lymphocyte proliferation was assessed through a tritiated-thymidine uptake assay as described previously (Kim et al., 2002b). Sample-mediated cytotoxicity was measured by trypan blue exclusion experiment (Hongo et al., 1986).

Measurement of immunoglobulin (Ig) and cytokine levels

Splenocytes maintained in 24-well tissue culture plates were exposed to the samples for 48 h. The amount of Ig subclasses and cytokines produced by the sample-stimulated splenocytes was determined by ELISA which was supported by the Bank for Cytokine Research (Chonbuk National University, Chonju, Korea) as described previously (Kim et al., 2002b).

SDS-page analysis

SDS-PAGE was performed using a Mini-Protean II cell (Bio-Rad, Hercules, CA) with 10–15% polyacrylamide gel. After electrophoresis, gels were stained either with Coomassie brilliant blue (CBB) to detect total proteins or with Schiff reagent to detect glycoproteins as described previously (Neville Jr and Glossmann, 1974.).

Characterization of active ingredients

The protein sample of AMK was treated with pronase E to degrade protein, or with $NaIO_4$ to degrade carbohydrate residues as described previously (Kim et al., 2002b). The protein sample (1 mg) was also incubated for 3 h at 37 °C in 1 ml of culture medium containing 1000 units of polymyxin B (PMB) to determine whether the protein sample contained lipopolysaccharide (LPS)-like compounds. In addition, 10 mg of protein sample were dissolved in 1 ml distilled water and the tube was soaked in a boiling water bath for 20 min. The ability of the treated samples to stimulate lymphocyte proliferation was measured by TdR uptake assay. Finally, we separated active compounds within the protein samples using HPLC

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