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Mung bean (*Vigna radiata* (L.)) coat extract modulates macrophage functions to enhance antigen presentation: A proteomic study

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ABSTRACT

The immunomodulatory effect of mung bean is mainly attributed to antioxidant properties of flavonoids; however, the precise machinery for biological effect on animal cells remains uncertain. To understand the physiological change produced by mung bean consumption, proteomic and metabolomic techniques were used. In vitro assay confirmed the importance of synergistic interaction among multiple flavonoids by *IL-6* expression. Proteomic analysis detected that the abundance of 190 proteins was changed in lipopolysaccharide-stimulated RAW264.7 cells by treatment with coat extract. Pathway mapping revealed that a range of proteins were regulated including an interferon-responsive antiviral enzyme (2′-5′-oligoadenylate synthetase), antigen processing factors (immunoglobulin heavy chain-binding protein and protein disulfide-isomerase), and proteins related to proteasomal degradation. Major histocompatibility complex pathway was activated. These results suggest that mung bean consumption enhances immune response toward a Th2-promoting polarization.

Biological significance: This study highlighted the immunomodulation of RAW264.7 cells in response to treatment with mung bean seed coat extract, using gel-free proteomic technique. The mechanism of immunomodulation by mung bean has not been described until today except for a report which identified HMGB1 suppression as a pathway underlying the protective effect against sepsis. This study suggested that the mung bean is involved in the regulation of antigen processing and presentation, and thus shifts immune response from acute febrile illness to specific/systemic and long-lasting immunity to protect the host.

1. Introduction

Chronic inflammation has become main focus in control of a wide variety of diseases such as atherosclerosis, cancer, diabetes, and neurodegenerative diseases [1,2,3]. Traditional medicine can be used to modulate chronic inflammatory processes and reduces pain with less adverse effects [4]. Plant-derived dietary compounds are of interest in the search for nutraceuticals that have health benefits. It is known that plants contain a diversity of bioactive compounds as a sequel of their secondary metabolism. Accumulating researches imply that biological effect of medicinal plants cannot be explained by a single component but synergistic and mutually potentiating interaction was postulated between different compounds [5]. Mixture of selected flavonoids often affords coordinated effect to enhance immune cell response [6], prevent osteoclastic cell growth [7], and strengthen antimicrobial effect against *Staphylococcus aureus* [8]. In general whole plant extracts are more effective than one of its components or fractions [9]. The striking aspect is that whole extracts are able to suppress the effect of toxic fraction derived from whole extract [10], suggesting complex interactions among phytochemicals. Mung bean (*Vigna radiata* (L.) R. Wilczek), a leguminous crop, has a wide range of biological activity. The content of a major flavonoid vitexin and other potentially bioactive compounds varies with cultivar and habitat [11,12]. It is of interest to evaluate biological activity of cultivars with different flavonoid composition.

Some studies implied positive involvement of mung bean in modulation of immune function [13]. Prescriptions in traditional Chinese medicine reported that mung bean can be used to alleviate heat stress or administered to patients with purulent skin [14]. Modern research showed that at the individual level, both aqueous and ethanolic extracts

Abbreviations: PS, lipopolysaccharide; PVPP, polyvinylpolypyrrolidone; PCR, polymerase chain reaction; LC, liquid chromatography; TOF, time of flight; MS, mass spectrometry; IL, interleukin; IFI44, interferon induced protein 44; COX2, cyclooxygenase 2; TLR, Toll-like receptor; PGE2, prostaglandin E2; Th, T helper; MHC, major histocompatibility complex.

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of mung bean coat can rescue mice from infectious diseases like lethal sepsis and suppress inflammation during pathogenesis of type 2 diabetic complications [13,15]. Experiments using cultured cell lines revealed that ethanolic extract of whole bean or seed coat can inhibit induction of various pro-inflammatory cytokines caused by stimulation with lipopolysaccharide (LPS) [13,16,17]. Antioxidative activities of two flavonoids, vitexin, and isovitexin, were assumed to be a rationale of biological activities of mung bean; however, it is now accepted that effect of crude extract of mung bean is far complex and pleiotropic than that of single compounds [13,18]. Treatment of RAW264.7 cells with crude extract can attenuate LPS-induced release of both HMGB1 and several cytokines; whereas, treatment with single compound can reproduce only HMGB1 suppression but cannot rescue cytokine production as much [13,18]. Therefore, comprehensive analysis is required to obtain perspective view of immune regulation by mung bean.

In the present study, synergistic interaction between major component vitexin and other constituents was assessed by in vitro assay using RAW264.7 macrophage cell line. Metabolomic profiling of flavonoids was combined with in vitro assay to determine the relationship between metabolite composition and biological activity of mung bean. Furthermore, gel-free proteomic analysis was applied to reveal pleiotropic biological effect of mung bean coat extract on LPS-stimulated RAW264.7 cells, describing multi-faceted function of mung bean.

2. Materials and methods

2.1. Plant materials and treatment

Mung beans (*Vigna radiata* (L.) R. Wilczek) from different habitats in Asian countries were purchased from local supermarkets in Tokyo and Yokohama, Japan. The places of production of the mung beans are as follows: two cultivars from China, one cultivar from Thailand, and one cultivar from Myanmar. Mung bean of Japanese native cultivar was a generous gift from Dr. K. Suzawa. These cultivars were referred as China 1, China 2, Thailand, Myanmar, and Japan in the present study. To prepare mung bean extract for cell culture assay, mung bean seeds (30 g) were soaked in milliQ water at room temperature for 16 h and the coat and flesh were separated. The coat or flesh was boiled in 100 mL of water for 10 min. The water-soluble fraction was centrifuged at 3100 ×*g* at 4 °C for 30 min, filtered through a 0.22 μm filter (Millipore, Billerica, MA, USA), and dried in a rotary evaporator for storage. To remove polyphenols and flavonoids from the extract, polyvinylpolypyrrolidone (PVPP; Sigma Aldrich, Saint Louis, MO, USA) was used as described by Fujimura et al. [19]. Briefly, the PVPP was swollen in milliQ water of 10 times weight for 30 min and centrifuged. The precipitated gel pellet was mixed with a one-half volume of mung bean extract for 15 min. After centrifugation, the supernatant was referred as PVPP-treated extract and dried in a rotary evaporator for storage.

2.2. Cell culture and stimulation

Murine macrophage-like RAW264.7 cells (RIKEN Institute of Physical and Chemical Research Cell Bank, Tsukuba, Ibaraki, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS; HyClone, GE Healthcare, Chicago, IL, United States). The cells were incubated in the presence of 5% CO_2 at 37 °C and subcultured in every three days. For treatment, coat and flesh extract, vitexin, or vitexin 2-O-rhamnoside (Sigma Aldrich) were dissolved in dimethyl sulfoxide (DMSO) with 15 cycles of 30 s sonication and 60 s intervals. The DMSO was used to dissolve carbohydrate contained in the flesh extract, which reduces solubility of the dried powder. RAW 264.7 cells were pre-incubated for 24 h with or without the extract (15 mg/L), vitexin, or vitexin 2-O-rhamnoside and were stimulated with LPS (400 ng/mL; Sigma Aldrich) for 4 h.

2.3. Reverse transcription polymerase chain reaction

Inhibitory effects of coat extract on the expression level of cytokines in LPS-stimulated RAW264.7 cells were determined by reverse transcription polymerase chain reaction (RT-PCR). For detection of cytokine production in RAW264.7 cells, total RNA was extracted with an ISOGEN II kit (Nippon Gene, Tokyo, Japan) from RAW264.7 cells stimulated with mung bean extract. First-strand cDNA was synthesized from 500 ng RNA with a Prime Script RT Master Mix (Takara, Shiga, Japan). EmeraldAmp MAX PCR Master Mix (Takara) was used for semi-quantitative PCR. The PCR condition was as follows: 22 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Primer sequences are as follows: *IL-6*, forward 5′-GTACTCCAGAAGACCAGAGG -3′, reverse 5′- TGCTGGTGACAACCACGGCC -3′; *β-actin*, forward 5′- GCCATGTACGTAGCCATCCAG -3′, reverse 5′-CATGAGGTAGTCTGTCAGGTC -3′. In addition, *IL-6* expression was monitored by quantitative real-time PCR (qPCR) using *GAPDH* gene as an internal control with a SYBR Premix Ex Taq II (Takara). Primer sequences are as follows: *IL-6*, forward 5′- TAGTCCTTCCTACCCCAATTTCC -3′ and reverse 5′- TTGGTCCTTAGCCACTCCTTC -3′; *GAPDH*, forward 5′- CCTGGAGAAACCTGCCAAGTATG -3′ and reverse 5′-AGAGTGGGAGTTGCTGTTGAAGTC -3′. Expression analysis of flavonoid biosynthesis enzymes in mung bean was performed using coat as well as root and hypocotyl of two day-old seedlings to assess enzyme activity. Two day-old seedling was used because RNA levels in coat were extremely low due to seed dehydration during maturation. Total RNA was extracted from a portion (100 mg) of mung bean coat and seedlings. The PCR conditions were as follows: 95 °C for 30 s, followed by 45 cycles of 95 °C for 10 s and 60 °C for 30 s. Gene expression was normalized using the *18S rRNA* gene as an internal control. Primer sequences are shown in Supplemental Table 1.

2.4. Metabolite extraction

Air-dried coat was crushed into fine powder and the samples (0.1 g) were extracted using 1 mL of 75% methanol by ultrasonication for 30 min. After centrifuged at 10,000 ×*g* for 20 min at 4 °C, the supernatant was collected and dried in a rotary evaporator for storage. For liquid chromatography (LC) coupled mass spectrometry (MS) analyses, extracts were dissolved in 200 μL of 75% methanol and were filtered through a 0.45 μm filter (Merck KGaA, Darmstadt, Germany). The standard compounds were provided by the Zhejiang Institute for Food and Drug Control (Hangzhou, China). For quantification, calibration curves were constructed using the standard solutions diluted in methanol at different concentrations; vitexin and isovitexin (0.1, 0.2, 0.4, 0.6, and 0.8 $mg \cdot mL^{-1}$), kaempferitrin (0.02, 0.04, 0.08, 0.12, and 0.24 $mg \cdot mL^{-1}$), isoorientin (0.02, 0.04, 0.06, 0.08, and 0.1 $mg \cdot mL^{-1}$), adenosine (0.0125, 0.0250, 0.0500, 0.0075, 0.0100, and 0.0200 $mg \cdot mL^{-1}$), tryptophan and uridine (0.025, 0.050, 0.100, 0.200, and 0.300 $mg \cdot mL^{-1}$). For HPLC analysis, 10 μL of the standard solutions and samples was used.

Analysis of metabolites was performed according to the method of Tang et al. with minor modifications [14]. HPLC analysis was performed on a Waters 2695 Alliance HPLC system (Waters, Milford, MA, USA) equipped with a photodiode array detector and an online degasser and auto sampler for solvent delivery. Compounds in samples were separated using reversed-phase HPLC. A C18 column (4.6 mm ID × 250 mm, Agilent, Santa Clara, CA, USA) was used with a flow rate of 1 $mL \cdot min^{-1}$ at 30 °C. Gradient elution was employed for qualitative and quantitative analyses using a mobile phase of 0.2% acetic acid and methanol: 0–10 min (2%–25% methanol), 11–15 min (25%–36% methanol), 16–35 min (36%–42% methanol), and 36–43 min (42%–100% methanol). Spectra were measured at wavelength of 260 nm, and peaks were compared with the retention time, ultraviolet spectra, and MS of the standards. The sample injection volume was 10 μL.

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