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Discovery of a sugar-based nanoparticle universally existing in boiling herbal water extracts and their immunostimulant effect

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ABSTRACT

Herbal medicine is mainly prepared from boiling herbal water extracts. Many epoch-making immunosuppressant drugs, such as glycyrrhizic acid (old example) and FTY720 (current example), were developed from herbal secondary metabolites in the boiling water extract by partition with organic solvents. However, few immunostimulants have been discovered by this method. Instead of the usual method, we aimed to find a novel immunostimulant component by two unique methods in the research of herbal medicine: ultracentrifugation and electron microscopy. The immunostimulant was not a secondary metabolite, as expected, but the structure was a nanoparticle formed by a polysaccharide. In addition, we clarified the immune effect of the nanoparticle. Intake of the nanoparticle by phagocytosis resulted in immunostimulant effects by increasing the genes and proteins of inflammatory cytokines in macrophage cells. The immunostimulant effects were inhibited by a phagocytosis inhibitor, cytochalasin D. To the best of our knowledge, this study is the first to describe the discovery of a nanoparticle in boiling herbal water extracts and its immunostimulant properties. This study will provide additional understanding of the efficacy of herbal medicine, in that the immunostimulant nanoparticle universally exists in boiling herbal water extracts. Thus, traditional herbal medicine may be an oldest known nanomedicine. Furthermore, this study suggests that the immunostimulant nanoparticle simply can be obtained from herbal medicine only by ultracentrifugation. We hope that this simple strategy will substantially contribute to drug development, including vaccine adjuvant, in the future.

1. Introduction

In traditional herbal medicine practiced worldwide (Chinese herbal medicine in China, Kampo medicine in Japan, Ayurveda in India, Unani in India, Central Asia, and North African countries, and so on) [1], the medicines are mainly prepared from boiling herbal water extracts [2]. The active components of these herbal medicines have been studied mainly in the form of secondary metabolites. Secondary metabolite products are usually obtained by partition with organic solvents [3]. As a result, a number of excellent modern medical drugs, particularly immunosuppressant drugs, are being developed from the secondary metabolites of these herbal medicines. As a current example, FTY 720 (fingolimod hydrochloride) is a novel immunosuppressant that is a functional antagonist of the first discovered S1P1 receptor, which was synthesized by structural transformation of the secondary metabolite of *Isaria sinclairii* parasitic on *Cordyceps*. FTY 720 is currently used as a drug for treating multiple sclerosis. Studies on secondary metabolites of herbal medicines are yielding tremendous benefits to humanity [4]. Herbal medicines are expected to be attractive candidates for components of new drugs [3].

There have been very few studies on the immunostimulant components of herbal medicines. We suggest that this is because it is hard to

Abbreviations: SEM, Scanning electron microscope; TEM, Transmission electron microscopy; *G. Radix*, *Glycyrrhizae Radix*

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find the immunostimulant components in boiling herbal water extracts by partition with organic solvents because these components are not secondary metabolites.

Using two unique methods in the research on herbal medicine, ultracentrifugation and electron microscopy, we discovered a novel unknown nanoparticle in herbal boiling water extract that had an immunostimulant effect. Here we report the discovery of the immunostimulant nanoparticle and describe its properties.

2. Materials and methods

2.1. Reagents and cells

Lipopolysaccharide (LPS; derived from *Escherichia coli* serotype 0111: B4, no. L-2630) and cytochalasin D were purchased from Sigma–Aldrich Japan (Tokyo, Japan). DiO, DAPI.RAW 264.7 cells (American Type Culture Collection, VA, USA), a murine macrophage cell line, were cultured in DEME medium (Thermo Fisher Scientific) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. All assays were performed when these cells covered 70% of the dish surface.

2.2. Isolation of the nanoparticle from boiling herbal water extracts

Glycyrrhizae Radix, Cinnamomi Cortex, Puerariae Radix, *Zingiberis Rhizoma, Paeoniae Radix, and Astragali Radix* were purchased from Tochimoto Tenkaido, Osaka, Japan. The boiling herbal water extracts was prepared by boiling a mixture (100 g) of the above herbal medicines gently in 500 mL of water for 50 min and then filtering the decoction. The herbal boiling water extract was centrifuged at 3000 × *g* for 5 min (KUBOTA 6800; KUBOTA, Tokyo, Japan), and the supernatant was collected and centrifuged at 20,000 × *g* for 20 min. The supernatant was collected and ultracentrifuged at 140,000 × *g* for 50 min twice, according to the preparation methods of exosome [5]. After removal of the supernatant, the clear transparent pellet was dispersed in distilled water and freeze-dried.

2.3. Transmission electron microscopy (TEM) and dynamic light scattering analysis

The nanoparticles were examined with a JEM-2000EX operated at 100 kV (JEOL Datum, Tokyo, Japan) at the Hanaichi UltraStructure Research Institute, Aichi, Japan. For negative staining with 2% (w/v) uranyl acetate (Cerac, USA), a 400-mesh grid with a carbon support film (10–20 nm in thickness) was used. The sizes of the suspended nanoparticles were confirmed by dynamic light scattering analysis using an FPAR-1000 particle analyzer (Otsuka Electronics, Osaka, Japan).

2.4. Elementary analysis and Raman spectroscopy

Elementary analysis was performed as described previously [6]. Elements of the freeze-dried nanoparticle were detected by Electron Probe Microanalyzer, EPMA (JEOL, Tokyo, Japan). Scanning electron microscope (SEM) observations were performed on the nanoparticles (Hitachi High-Technologies, TM3030, Tokyo, Japan). Raman spectroscopy was performed against the powder segment of the freeze-dried nanoparticles following the conditions of inVia Reflex Raman microscopy (Renishaw K.K, Tokyo, Japan), as follows: Laser: LD pumped green laser (532 nm), objective lens magnification × 50, irradiating laser beam diameter about 1.5 μm, irradiation laser power 1 mW or less, photometric Raman shift range 4000–150 cm^{-1}, wave number resolution about 6 cm^{-1}, number of integrations 10, data processing library search by spectral waveform comparison with database (Raman Library "RAMANDB," JEOL, Tokyo, Japan).

2.5. Analysis of sugar content

A sample of 0.3 g of the freeze-dried nanoparticles was added to 3 mL of 72% sulfuric acid at 30 °C for 1 h. This reaction solution was completely transferred to a pressure bottle while being mixed with 84 mL of purified water and then was thermally decomposed in an autoclave at 120 °C for 1 h. After thermal decomposition, the decomposed solution and the residue were separated by filtration. Quantitative analysis of monosaccharides in the decomposed solution was carried out by high-performance liquid chromatography (HPLC) (GL-7400 HPLC system, GL Science, Tokyo, Japan).

2.6. Confocal laser fluorescence microscope

The suspension of the nanoparticles was mixed with 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO; final concentration 0.001 mM) (Thermo Fisher Scientific, MA, US). To remove free DiO, the nanoparticles were precipitated with ultracentrifugation (139,000 × *g*, 70 min). The sediment containing the nanoparticles labeled with DiO was suspended with phosphate-buffered saline (PBS)(−). The DiO-labeled nanoparticles were added to the RAW 264.7 medium in the glass base dish, incubated for 2 h at 37 °C, and fixed with 4% paraformaldehyde for 10 min. After PBS washing, coverslips were inverted onto a slide with SlowFade Gold Antifade Reagent with DAPI (Life Technologies, CA, US). Finally, the cells were observed with a confocal laser scanning microscope (TCS-SP5, Leica Microsystems, Wetzlar, Germany).

2.7. Stimulation of RAW 264.7 cells by the nanoparticles in the presence of cytochalasin D

RAW 264.7 cells (5×10^5) cultured on 12-well plates were cultured for 24 h, and preincubated in the absence or presence of cytochalasin D (50 μmol/L) for 1 h at 37 °C. After preincubation, the nanoparticles (10 μg/mL) were incubated for 6 h and treated. The IL-6 levels in cell culture medium of these cells were determined with a mouse IL-6 ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Total RNA in the RAW 264.7 cells was extracted with a RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The cDNAs were amplified with FastStart Essential DNA Green Master (Roche, Pleasanton, CA, USA).

The forward/reverse transcription polymerase chain reaction (RT-PCR) primer pairs for mouse cDNAs were as follows: IL-6 (5′-GCTACC AAACTGGATATAATCAGGA-3′/5′-CCAGGTAGCTATGGTACTCCA GAA-3′); and β-actin (5′-CTAAGGCCAACCGTGAAAAG-3′/5′-ACCAGA GGCATACAGGGACA-3′). Quantitative RT-PCR (qRT-PCR) was performed was performed as described previously [7].

2.8. Statistical analysis

Statistically significant differences within each set of categorical data were determined by Tukey–Kramer HSD tests. Statistical analyzes were performed with JMP Pro software version 13 (SAS Institute Japan, Tokyo, Japan). $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Discovery and isolation of novel nanoparticles in herbal boiling water extract

Initially, we carefully observed various boiling herbal water extracts with naked eye. We were fortunately able to intuitively observe the presence of colloids in these extracts. Subsequently, we attempted to detect the presence of colloids. Because of limitations of space, the extract of *G. Radix* is shown in Fig. 1A as an example of one of the