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Immunostimulatory Lipid Nanoparticles from Herbal Medicine

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Abstract

Reproducibility is an important issue in biological characterization of drug candidates and natural products. It is not uncommon to encounter cases in which supposedly the same sample exhibits very different biological activities. During our characterization of macrophage-stimulatory lipids from herbal medicine, it was found that the potency of these lipids could vary substantially from experiment to experiment. Further analysis of this reproducibility issue led to the discovery of solvent-dependent nanoparticle formation by these lipids. While larger nanoparticles (approximately 100 nm) of these lipids showed modest macrophage-stimulatory activity, smaller nanoparticles (<10 nm) of the same lipids exhibited substantially higher potency. Thus, the study revealed an unexpected link between nanoparticle formation and macrophage-stimulatory activity of plant lipids. Although nanoparticles have been extensively studied in the context of vehicles for drug delivery, our finding indicates that drugs themselves can form nanoassemblies, and their biological properties may be altered by the way they assemble.

Keywords

atomic force microscopy; dynamic light scattering; Juzen-taiho-to; macrophages; macrophages; nanoparticles; β -sitosteryl β -D-glucoside

Reproducibility is an important issue when biological properties of organic molecules are studied. This is particularly an important issue in the studies of herbal natural products. When reproducibility becomes an issue, the problem is usually attributed to batch-to-batch variability of chemical constituents in herbs, which is a well-recognized problem in the study of herbal medicines (1,2). However, what if the same batch of an herbal extract exhibits different biological activities? There may be a number of possible reasons, such as decomposition of active constituents, loss of volatile ingredients, and even poor experimental skills or could there be a sound scientific reason behind such an observation?

We encountered such a problem during our study on an Oriental herbal formulation called Juzen-taiho-to (JTT; Shi-Quan-Da-Bu-Tang in Chinese). JTT is an immunostimulatory herbal formulation used widely in East Asia (3,4). It is clinically used for patients with

Conflict of Interest

The authors declare no conflict of interest.

cancer to improve their immunologic functions (3–6). JTT is known to stimulate macrophages (MΦ), which, in turn, activate the adaptive immune system (7). To understand the potent immunostimulatory activity of JTT, our group has been examining chemical constituents in JTT (8). In the course of the study, we purified a lipid mixture with a MΦ-stimulatory activity. That was when we encountered the reproducibility issue: The same batch of this lipid mixture exhibited very different potency from experiment to experiment. Further studies revealed that the potency difference could be attributed to the difference in molecular assembly, which changed dramatically by simple solvent treatments.

Methods and Materials

Materials

All solvents for purification were in the HPLC grade and were purchased from Fisher Scientific. The Juzen-taiho-to (JTT) formulation was purchased from Honso Pharmaceutical Co. (Nagoya, Japan). Unless specified otherwise, all other chemicals and reagents were obtained through Fisher Scientific (Pittsburgh, PA, USA) and used without further purification.

Purification of the MΦ-stimulatory lipid mixture from JTT

The lipid mixture was purified using a protocol described elsewhere (8). Briefly, purification of MΦ-stimulatory factors was guided by a qRT-PCR assay of intercellular adhesion molecule 1 (ICAM-1) using a human monocytic leukemia cell line (THP-1); ICAM-1 is a well-known biomarker of MΦ-stimulation (9,10). JTT (dried powder, 400 g) was extracted with 3 L of chloroform/methanol (2:1). The extract was dried by rotary evaporator. The dried material was partitioned between *n*-butanol and water. The 1-butanol layer, which contained the activity, was dried and subjected to an open silica gel column (silica gel = 100 g; column i.d. 3.5 cm) using step-gradient of 1%, 10%, 20%, and 30% methanol in dichloromethane (480 mL each). The 20% methanol in dichloromethane exhibited the most potent activity. This fraction was dried and then reconstituted in 50% methanol in water (500 μL) and loaded onto a solid-phase extraction C₈ column (Alltech SPE C-8). The loaded sample was eluted with 50% MeOH_{aq} (4 mL), 75% MeOH_{aq} (4 mL), and 100% MeOH (8 mL). The '100% MeOH' fraction, which showed the activity, was subjected to further purification using reversed-phase HPLC. The reversed-phase HPLC was carried on an Agilent 1100 series (Santa Clara, CA, USA) using a semi-preparative C₁₈ column (Alltech®) with isocratic elution using 100% methanol. HPLC eluent was monitored by an Agilent 1100 photodiode array detector and an evaporative light scattering detector (ELSD) model 800 (Alltech®). UV signals were collected at 220, 254 and 280 nm. ELSD analysis was performed with nitrogen in a drift temperature of 43 °C and nebulizer pressure of 4.4 bar. The lipid mixture described in this study was the largest ELSD peak at 13 min, which contained β-sitosterol β-D-glucoside (BSSG) as the major constituent and small amounts of inseparable glycolipids (8). The purified material was free of endotoxin contamination as determined by Limulus amoebocyte lysate test (Lonza, Allendale, NJ, USA).

ICAM-1 qRT-PCR assay

THP-1 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and $1 \times$ penicillin and streptomycin, that is, 100 units/mL and 100 $\mu\text{g/mL}$, respectively, 0.005 mM β -mercaptoethanol and maintained in a 37 °C humidified 5% CO₂ incubator. On the day before each qRT-PCR experiment, THP-1 cells were plated on a 6-well plate (200 000 cells/well). After 24 h, cells were treated with the lipid samples (final concentration, 5 $\mu\text{g/mL}$), JTT (100 $\mu\text{g/mL}$; positive control), and DMSO (vehicle control) and incubated for 4 h in a 37 °C humidified 5% CO₂ incubator. Total RNA was purified by RNeasy mini kit (Qiagen, Valencia, CA, USA). RNA samples were quantified using UV absorbance at 260 nm. Samples with the 260/280 nm ratio of 1.8 or higher were used for the subsequent study. The first-strand cDNA samples were synthesized with Gibco BRL Superscript Choice system and oligo (dT)_{12–18} primer. TaqMan® Gene Expression Assays (Life Technologies, Grand Island, NY, USA) were carried out on Applied Biosystems 7500 Real-Time PCR system using preoptimized assays for ICAM-1 (Life Technologies Assay Id: Hs00277001_m1) and GAPDH endogenous control (Life Technologies Catalog Number: 4352934E). The C_T method was employed to quantify the ICAM1 gene induction. The raw data were first normalized by the endogenous control (GAPDH) for individual samples. Then, the relative quantification (RQ) values, that is the fold-change ratios, were obtained by comparing the normalized data against the DMSO vehicle control. Minimum triplicate experiments were carried out for each experimental condition.

Atomic force microscope

Samples were spotted onto a freshly cleaved mica substrate. After overnight exposure, they were washed with DMSO and ultrapure water and dried with nitrogen. Then, the samples were imaged with an atomic force microscope (AFM) (MFP-3D Stand Alone Atomic Force Microscope from Asylum Research) working in alternating contact mode. Silicon tips with 10 nm radius of curvature, and typical spring constant and the resonant frequency values of 40 nN/nm and 325 kHz, respectively, were employed. The resulting images were processed with WSxM software (Nanotec Electronica S.L., Madrid, Spain).

Dynamic light scattering

Dynamic light scattering (DLS) measurements were recorded using the PD2000DLS (PDDLS/Cool Batch 90T from Precision Detectors) instrument in batch mode at 25 °C and 12- μL glass microcuvettes to determine the size distribution of nanoparticles in DMSO. The sample concentration was 100 $\mu\text{g/mL}$ for both '90% MeOH' and '100% MeOH' samples.

Results and Discussion

The lipid mixture with potent M Φ -stimulatory activity was purified from JTT using a protocol described elsewhere (8). This mixture contained β -sitosteryl β -D-glucoside (BSSG) as the major constituent and minute quantities of inseparable glycolipids (8). Further attempts to separate individual glycolipids resulted in the loss of activity. Therefore, we decided to characterize this sample as a mixture. During the characterization, we first noticed that the M Φ stimulatory potency of this sample could depend on the solvent system at the final stage of purification. The activity seemed high when methanol (100% MeOH)

was used as the HPLC eluent, whereas it seemed weaker when 90% MeOH aq. was used. Yet, this initial observation was too qualitative. We therefore conducted a more systematic study to see whether the difference in the solvent systems (100% MeOH and 90% MeOH aq) was responsible for the seemingly different biological activity. To this end, two samples were prepared using the protocol outlined in Scheme 1. From a methanol solution of the lipid mixture (1 mg/mL), two aliquots (100 μ L each) were taken into vials and dried. One was then dissolved in 90% MeOH aq., dried, and redissolved in DMSO ('90%' sample). The other was dissolved in 100% MeOH, dried, and redissolved in DMSO ('100%' sample). These samples were subjected to a qRT-PCR assay of ICAM-1 using THP-1 cells, which was used to measure the M Φ -stimulatory activity.

The qRT-PCR assay showed distinct difference between the two samples (Figure 1). Compared to the DMSO vehicle control, approximately 25-fold induction of ICAM-1 was observed when THP-1 cells were treated with the '100%' sample. On the other hand, induction of ICAM-1 by the '90%' sample was only about 3-fold. Thus, simple solvent treatments could make a large difference in the potency of this lipid mixture. The next obvious question was the origin of this potency difference. As the two samples have the same amount of the lipid mixture, the potency difference could not be attributed to variability in lipid quantities. We therefore postulated that the difference might arise from some difference in molecular assembly. To test this hypothesis, the two samples were analyzed by atomic force microscope (AFM) and dynamic light scattering (DLS).

The difference between the two samples was striking. The '90%' sample showed predominantly large (approximately 100 nm) aggregates, as evidenced by AFM and DLS (Figure 2A). On the other hand, the '100%' sample showed much smaller (<10 nm) nanoparticles according to AFM and DLS (Figure 2B). Thus, these observations indicated that the small lipid nanoparticles were responsible for the M Φ -stimulatory activity. Solvent-dependent formation of nanomaterials has been widely studied in the context of drug delivery vehicle over the last decade (11–16). In addition, it has been shown that aggregation of organic molecules could cause promiscuous inhibition of enzymes (17,18). Yet, the possibility of nanoparticle formation, let alone the biological properties of nanoparticles, is hardly considered in the studies of herbal natural products.

In retrospect, it is not surprising that a mixture of BSSG and glycolipids forms molecular assemblies in a solvent-dependent manner. When the lipid mixture in 90% MeOH aq. is dried, methanol evaporates first and water is left behind in the vial (Figure 3). As a result, the polarity of the solution increases as the sample dries. On the other hand, when the 100% MeOH solution is dried, the solvent polarity remains the same until the end. It is likely that the polar environment encourages the aggregation of hydrophobic lipids and results in the formation of large nanoaggregates. On the other hand, a less polar environment leads to the formation of smaller nanoparticles. The difference in size can cause differences in the cellular uptake and metabolism, which, in turn, can result in the potency difference. Further studies are underway to understand the molecular mechanism of M Φ stimulation by lipid nanoparticles from JTT.

Conclusion

In conclusion, the current study revealed that molecular assembly could substantially affect the potency of the MΦ stimulatory lipid mixture. This observation may not be limited to herbal natural products. Rather, the current finding points to a possibility that similar nanoassemblies are playing important roles in the biological activities of many organic molecules and drugs.

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Figure 1.

The MΦ-stimulatory activity of '90%' and '100%' samples by qRT-PCR of ICAM-1. Cells were treated with the sample for 4 h and subjected to RNA isolation and qRT-PCR analysis. DMSO (vehicle control), JTT (100 μ g/mL: positive control), '90%' sample (5 μ g/mL), and '100%' sample (5 μ g/mL).

Figure 2. Characterization of '90%' and '100%' samples by AFM and DLS. (A) The '90%' sample showed mostly large BSSG aggregates. (B) The '100%' solution mainly composed of small nanoparticles. The insets in the AFM images are the height profiles of representative particles. The lower DLS panels in (A) and (B) show the particle distributions, which were consistent with the AFM results.

Figure 3.

Possible solvent effect on lipid aggregation. When the sample solution in 90% methanol aq. is dried, methanol is evaporated first, leaving a concentrated solution in water. Because of the poor solubility of BSSG in water, large aggregates form as the '90%' sample is dried.

Scheme 1.

Preparation of two samples of the MΦ-stimulatory lipid mixture. The two samples had the same amount of the lipid mixture but underwent different solvent treatments. One was dissolved in 90% MeOH aq., dried, and redissolved in DMSO ('90%' sample). The other was dissolved in 100% MeOH, dried, and redissolved in DMSO ('100%' sample).