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Research article

Ginsenoside Rg_3 promotes inflammation resolution through M2 macrophage polarization

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ABSTRACT

Background: Ginsenosides have been reported to have many health benefits, including anti-inflammatory effects, and the resolution of inflammation is now considered to be an active process driven by M2-type macrophages. In order to determine whether ginsenosides modulate macrophage phenotypes to reduce inflammation, 11 ginsenosides were studied with respect to macrophage polarization and the resolution of inflammation.

Methods: Mouse peritoneal macrophages were polarized into M1 or M2 phenotypes. Reverse transcription-polymerase chain reaction, Western blotting, and measurement of nitric oxide (NO) and prostaglandin E_2 levels were performed *in vitro* and in a zymosan-induced peritonitis C57BL/6 mouse model.

Results: Ginsenoside Rg_3 was identified as a proresolving ginseng compound based on the induction of M2 macrophage polarization. Ginsenoside Rg_3 not only induced the expression of *arginase-1* (a representative M2 marker gene), but also suppressed M1 marker genes, such as *inducible NO synthase*, and NO levels. The proresolving activity of ginsenoside Rg_3 was also observed *in vivo* in a zymosan-induced peritonitis model. Ginsenoside Rg_3 accelerated the resolution process when administered at peak inflammatory response into the peritoneal cavity.

Conclusion: These results suggest that ginsenoside Rg_3 induces the M2 polarization of macrophages and accelerates the resolution of inflammation. This finding opens a new avenue in ginseng pharmacology.

1. Introduction

Pharmacognostic studies on ginseng, the root of *Panax ginseng* Meyer, have determined that ginsenosides, triterpenoid saponins, are the bioactive ingredients of ginseng [1,2]. Ginsenosides are believed to mediate most of the pharmacological effects of ginseng, which include anticancer, anti-inflammatory, and antidiabetic activities [1,3,4]. Moreover, of the ginsenosides, ginsenoside Rg_3 is one of the most effective steroidal saponins in steamed ginseng [5]. Ginsenoside Rg_3 exhibits a wide range of therapeutic and pharmacological properties, which include anti-inflammatory, anticancer, antioxidant, and antiobesity effects [4,6–10].

The resolution of inflammation is now considered to be an active process orchestrated by proresolving mediators and their receptors [11,12]. M2 polarized macrophages are supposed to play key roles during the resolution of inflammation [13,14]. Although anti-inflammatory effects of ginseng and ginsenosides have been studied at the molecular level, the effects of ginsenosides on macrophage polarization and on the resolution of inflammation have not been studied. Therefore, we aimed: (1) to find whether any ginsenoside could induce M2 polarization of macrophages; (2) to confirm induction of M2 polarization and suppression of M1 polarization at molecular and cellular levels *in vitro*; and (3) to verify M2 polarization effect on inflammation resolution in a mouse peritonitis model *in vivo*.

First, we investigated the effects of ginsenosides on macrophage polarization, in order to determine which ginsenoside could accelerate the resolution of inflammation [15]. Of the 11 ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2, Rg_3, Rh_2, and Ro) tested, Rg_3 was found to inhibit M1 polarization and to induce M2 polarization in mouse peritoneal macrophages, and to promote the resolution of inflammation in a murine peritonitis model. The first report on

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ginsenoside Rg_3's effects on M2 polarization and resolution of inflammation would be the novelty of the present study.

2. Materials and methods

2.1. Materials

Ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2, Rg_3, Rh_2, and Ro) were purified in Kang-ju Choi's laboratory, the Korea Ginseng and Tobacco Research Institute (Daejeon, Republic of Korea), and the purities were more than 99.9%. They were dissolved in absolute methanol and stored at −20°C. All other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Animals

Eight- to ten-week-old male C57BL/6 (19–22 g) mice were purchased from Daehan Biolink (DBL; Seoul, Korea), housed in a laboratory animal facility at Pusan National University (Busan, Korea), and provided food and water *ad libitum*. The animal protocol used in this study was reviewed and approved beforehand by the Pusan National University-Institutional Animal Care Committee with respect to ethicality and scientific care.

2.3. Isolation and culture of mouse peritoneal macrophages

Mouse peritoneal macrophages were isolated from the peritoneal cavity of a 3% thioglycollate-treated C57BL/6 mouse 4 d after treatment and cultured at 37°C in a 5% CO_2 humidified incubator. Isolated macrophages were maintained in RPMI1640 containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mL penicillin, 50 μg/mL streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate for 18 h and then incubated in 0.5% fetal bovine serum-containing media for 24 h. RNA and protein samples were prepared after 5 h or 24 h of lipopolysaccharide (LPS) treatment (10 ng/mL or 100 ng/mL), respectively. Ginsenosides were added 1 h prior to adding LPS [15].

2.4. Reverse transcriptase-polymerase chain reaction

To determine the expressions of marker proteins of M1 or M2 polarization in macrophages by reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized with total RNA isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Synthesized cDNA products and primers for each gene were used for PCR, which was conducted using Promega Go-Taq DNA polymerase (Promega, Madison, WI, USA). Specific primers for tumor necrosis factor-alpha (TNF-α) (sense 5′-GAC CCT CAC ACT CAG ATC AT-3′, antisense 5′-TTG AAG AGA ACC TGG GAG TA-3′), transforming growth factor-beta 1 (TGF-β1) (sense 5′-TTGCTTCAGCTCCACAGAGA-3′, antisense 5′-TGGTTGTAGAGGGCAAGGAC-3′), Ym-1 (sense 5′-ACT TTG ATG GCC TCA ACC TG-3′, antisense 5′-AAT GAT TCC TGC TCC TGT GG-3′), and interleukin (IL)-10 (sense 5′-CCAAGCCTTATCGGAAATGA-3′, antisense 5′-TTTTCACAGGGGAGAAATCG-3′) were used to amplify gene fragments. PCR was performed over 30 amplification cycles (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s) in an Eppendorf Mastcycler gradient PCR machine (Eppendorf, Hamburg, Germany) [16]. Specific primers for arginase-1 (sense 5′-GTG AAG AAC CCA CGG TCT GT-3′, antisense 5′-CTG GTT GTC GGG GAG TGT T-3′), inducible nitric oxide synthase (iNOS) (sense 5′-ACC TAC CAC ACC CGA GAT GGC CAG-3′, antisense 5′-AGG ATG TCC TGA ACA TAG ACC TTG GG-3′), cyclooxygenase-2 (COX-2) (sense 5′-CCG TGG GGA ATG TAT GAG CA-3′, antisense 5′-CCA GGT CCT CGC TTA TGA TCT G-3′), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense 5′-TTCACCACCATGGAGAAGGC-3′, antisense 5′-GGCATGGACTGTGGTCATGA-3′) were used, and annealing was performed at 60°C. For IL-1β (sense 5′-GGAGAAGCTGTGGCAGCTA-3′, antisense 5′-GCTGATGTACCAGTTGGGGA-3′), annealing was undertaken at 57°C. Aliquots (7 μL) were electrophoresed in 1.2% agarose gels and stained with ethidium bromide [15].

2.5. Western blotting

Macrophages were harvested and resuspended in RIPA lysis buffer (GenDEPOT, Baker, TX, USA). Concentrations of proteins were determined using a BCA protein assay (ThermoScientific, Rockford, IL, USA). Proteins (30 μg) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose. Membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% skim milk, incubated with specific primary antibodies recognizing β-actin, COX-2, iNOS, and arginase-1, and then incubated HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA). Signals were developed using an enhanced chemiluminescence system (Pierce Biotechnology Inc., Rockford, IL) [16].

2.6. Nitrites measurement

NO production was estimated by measuring the amount of nitrite (a stable metabolite of NO) in medium using Griess reagent, as previously described [17]. Cells were pretreated with different concentrations of ginsenoside Rg_3 for 1 h and subsequently stimulated with LPS (100 ng/mL) for 24 h. Nitrite concentrations in medium were determined using the Griess Reagent System (Promega).

2.7. Prostaglandin E_2 production

Peritoneal macrophages were incubated with ginsenoside Rg_3 for 1 h and subsequently stimulated with LPS (100 ng/mL) for 24 h. Macrophage culture supernatants were harvested and immediately assayed using a prostaglandin E_2 (PGE_2) EIA kit (Cayman Chemical, Ann Arbor, MI) [18].

2.8. Induction of peritonitis and peritoneal cell counting

Peritonitis was induced by injecting 30 mg/kg of zymosan (Sigma) intraperitoneally (i.p.), and 12 h later, mice were treated i.p. with vehicle, 1 mg/kg, or 5 mg/kg ginsenoside Rg_3. Peritoneal washing was performed 24 h after zymosan treatment using 4 mL of ice-cold RPMI1640. Total cell numbers in peritoneal washings were calculated by counting after trypan blue staining. The cells obtained were washed with 0.1M phosphate buffer (pH 6.8), which was prepared by mixing 153 mL of 0.2M NaH_2PO_4 (Monobasic; AMRESCO, Solon, OH) and 147 mL of 0.2M Na_2HPO_4 (Dibasic; AMRESCO) and adding distilled water to a final volume 900 mL. The cells were attached to slides using a Cellspin (Hanil, Anyang, Korea), which was operated at 500 rpm for 5 min. Slides were then dried at room temperature for 30 min and fixed in methanol for 30 s. Cells were then stained with May–Grünwald solution (Sigma) and Giemsa solution (Fluka, Buchs, Switzerland) to identify individual cell types [19].

2.9. Statistics

Results are expressed as the means ± standard errors of the indicated numbers of determinations. The statistical significances of differences were determined by analysis of variance using Tukey's *post hoc*, and statistical significance was accepted for p

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