Enzyme Hydrolysates of Ginseng Marc Polysaccharides Promote the Phagocytic Activity of Macrophages Via Activation of TLR2 and Mer Tyrosine Kinase

Jeong Yeon Seo¹, Ji Won Choi¹, Jae Yeon Lee², Young Shik Park², and Yong Il Park^{1*}

¹Department of Biotechnology, The Catholic University of Korea, Bucheon 14662, Republic of Korea

²R&D Center for Life Science, Biotopia Co., Ltd., Chuncheon 24398, Republic of Korea

Received: January 3, 2018

Revised: March 13, 2018

Accepted: March 17, 2018

First published online
April 12, 2018

*Corresponding author

Phone: +82-2-2164-4512;

Fax: +82-2-2164-4846;

E-mail: yongil382@catholic.ac.kr

pISSN 1017-7825, eISSN 1738-8872

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Although ginseng marc is a by-product obtained during manufacturing of various commercial ginseng products and has been routinely discarded as a waste, it still contains considerable amounts of potential bioactive compounds, including saponins and polysaccharides. Previously, we reported that ginseng oligosaccharides derived from ginseng marc polysaccharides by enzymatic hydrolysis exert immunostimulatory activities in macrophages and these activated macrophages are in turn able to inhibit the growth of skin melanoma cells by inducing apoptosis. In the present study, a more detailed investigation of the immunostimulatory activity and underlying action mechanisms of an enzymatic hydrolysate (GEH) containing these oligosaccharides derived from ginseng marc polysaccharides was performed. The levels of proinflammatory cytokines and anti-inflammatory cytokines were measured in GEH-stimulated RAW264.7 macrophages using RT-PCR analysis and ELISA. The expression levels of Toll-like receptor 2 (TLR2) and TLR4, Dectin-1, and MerTK were measured by RT-PCR analysis or western blot analysis, and the phagocytic activities of GEH-challenged bone marrow-derived macrophages toward apoptotic Jurkat cells were assayed using fluorescence microscopy. GEH induced the production of both proinflammatory cytokines TNF- α and IL-6, and anti-inflammatory cytokine IL-10 in RAW 264.7 cells. The expression of the TLR2 and MerTK mRNAs was increased upon GEH treatment. Phagocytosis of apoptotic Jurkat cells was enhanced in GEH-treated macrophages. Based on the results, this enzymatic hydrolysate (GEH) containing oligosaccharides exerts immunostimulatory effects by maintaining the balance between M1 and M2 cytokines, facilitating macrophage activation and contributing to the efficient phagocytosis of apoptotic cells. Therefore, the GEH could be developed as value-added, health-beneficial food materials with immunostimulatory effects.

Keywords: Ginseng polysaccharides, enzymatic hydrolysate, ginseng oligosaccharides, immunostimulation, macrophages, Toll-like receptor 2

Introduction

Panax ginseng C. A. Meyer has been consumed over thousands of years as a medicinal herb to treat various medical symptoms, owing to its various health benefits, such as cardioprotection, vasorelaxation, stimulation of the central nervous system, anticarcinogenic effects, anti-allergic effects, and host resistance against infection [1].

Over the last several decades, various active compounds, including ginsenosides, polysaccharides, peptides, poly-acetylenic alcohols, and fatty acids, have been isolated and identified from *P. ginseng* and their pharmacological activities have been extensively investigated [1]. Since ginseng-derived functional foods are manufactured and usually sold in the form of water extracts, substantial amounts of ginseng marc are produced after the extraction

process, which are discarded as wastes or used as animal feeds. However, these wastes contain considerable amounts of bioactive materials, such as polysaccharides and saponins [2]. Acidic polysaccharides found in ginseng marc exhibit significant antitumor effects and immunostimulatory activities [2]. In addition, red ginseng marc containing polysaccharides was used as a potential inhibitor against thermal stress in lipopolysaccharide (LPS)-exposed laying hens [3]. However, it has been generally recognized that the innate structural variations and complexity, high molecular weights, and viscous nature of polysaccharides may limit their successful applications as health-beneficial food materials or therapeutic agents, suggesting that smaller-sized oligosaccharides would overcome these problems [4]. Indeed, significant efforts have focused on preparing bioactive oligosaccharides from marine polysaccharides, aiming for their pharmaceutical usage [4].

Macrophages are a typical group of immune cells in charge of host defence against bacterial infections. They play an essential role in phagocytosing invading microorganisms, such as viruses and bacteria, as well as dying self cells and cancer cells, and produce many cytokines and inflammatory mediators, such as nitric oxide (NO) and superoxide, when activated by various stimuli [5]. Macrophages connect the innate immunity and adaptive immunity by expressing high levels of class II major histocompatibility complex molecules to present antigens on their surface [6].

We previously reported that glucan-type ginseng oligosaccharides prepared from ginseng marc polysaccharides by enzymatic hydrolysis exert immunostimulatory activities in macrophages, and these activated macrophages are in turn capable of inhibiting the growth of skin melanoma cells by inducing apoptosis [7]. In the present study, in an effort to develop ginseng marc-derived oligosaccharides as potent value-added, health-beneficial materials, an enzymatic hydrolysate containing these oligosaccharides was prepared from ginseng marc polysaccharides and in-depth evaluations of the immunostimulatory activity and underlying action mechanisms were performed.

Materials and Methods

Materials

Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-ethylenediaminetetraacetic acid (EDTA), PBS, penicillin, and streptomycin were purchased from GIBCO-BRL (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased

from Sigma-Aldrich (USA). Carboxyfluorescein succinimidyl ester (CFSE) and rhodamine-labeled phalloidin were purchased from Invitrogen Life Technologies (USA). Rabbit polyclonal antibodies against phospho-mer tyrosine kinase (p-MerTK) and total MerTK were purchased from FabGennix (USA) and Santa Cruz Biotechnology (USA), respectively.

Preparation of Enzymatic Hydrolysate from the Ginseng Marc

Ginseng marc powder was purchased from Kotobuki Bussan Co., Ltd. (Japan). Three kilograms of ginseng marc powder was dissolved in 15 L of distilled water and mixed for 3 h with gentle stirring. The water-soluble fractions were collected by centrifugation at $14,534 \times g$ for 30 min and precipitated with 3 volumes of ethanol overnight at 4°C. The ethanol precipitate was freeze-dried, dissolved in distilled water (dH_2O) to achieve a 20% solution (w/v), and digested with 100 units of pectinase (Pectinex ultra pulp; Novozymes, Denmark) for 90 min at 40°C. The enzyme reaction was stopped by heating the solution at 100°C for 10 min. The supernatant was collected after centrifugation at $9,302 \times g$ for 30 min and dialyzed (molecular weight cutoff, 10 kDa) against dH_2O for 36 h at 4°C. After spray-drying, the obtained hydrolysate, with a yield of 12.5% in dry mass, was tentatively named GEH and used to investigate the immunostimulatory activity and molecular mechanism of action. The endotoxin level in purified GEH was checked by using a chromogenic limulus amoebocyte lysate endotoxin assay kit (Genscript, USA), revealing an endotoxin content of less than 0.5 EU/ml (endotoxin unit/ml).

Chemical Analysis

The total carbohydrate content was determined using the phenol-sulfuric acid method at 490 nm [8]. Total proteins and total lipids were quantified using the Bradford method and Bligh and Dyer method, respectively [9, 10]. The total polyphenol content of GEH was determined using the Folin-Ciocalteu phenol reagent [11].

Analysis of Molecular Mass and Monosaccharide Composition of GEH

The average molecular mass of the enzymatic hydrolysate (GEH) from ginseng marc was determined by size-exclusion HPLC (Waters Alliance HPLC 2695) with a Shodex OHpak column (SB-shodex 804 HQ; Showa Denko Co., Japan). Ten micrograms of 1% GEH (in dH_2O) was injected, eluted with dH_2O at a flow rate of 0.7 ml/min at 30°C, and detected with an ELSD detector (Altech, USA). Pullulans (Sigma-Aldrich, USA) were used as the molecular mass standards: 1,320, 10,000, 21,700, 48,900, 113,000, 200,000, 366,000, and 804,000 Da. The monosaccharide composition of GEH was analyzed by HPAEC-PAD (high-performance anion-exchange chromatography pulsed electrochemical detection). One milligram of GEH was dissolved in 1 ml of dH_2O , and trifluoroacetic acid (TFA) was added to obtain a 2 M final concentration and incubated for 4 h at 100°C. After the reaction, the mixture was

vacuum-dried using a Speed-Vac. The GEH was hydrolyzed with 2M TFA for 4 h at 100°C, and the vacuum-dried materials were dissolved in dH₂O. Monosaccharides in the TFA-hydrolyzed GEH were analyzed by HPAEC-PAD using a Dionex DX600 chromatography system (Dionex Co., USA) with a Carbo-Pac PA1 column (Dionex Co., USA).

Cell Culture Conditions

The RAW264.7 murine macrophage cell line (ATCC TIB71) and Jurkat T lymphocyte cell line (ATCC TIB152) were cultured in DMEM and RPMI supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml). The cells were maintained at 37°C in a humidified 5% CO₂ atmosphere. Unless otherwise stated, the cells were incubated with varying concentrations (100–500 µg/ml) of GEH for 24 h. LPS was used as a positive control to prime the macrophages. Bone marrow-derived macrophages (BMDMs) were isolated from the bone marrow of female BALB/c mice (Oriental Bio, Korea) and differentiated in DMEM containing 20% L929 fibroblast-conditioned medium, according to a previous report [12].

Effects of GEH on Cell Viability

The MTT assay was conducted to determine the effects of GEH on RAW264.7 cell viability. Cells were seeded on 96-well plates at a density of 1×10^4 cells/well, cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO₂, and treated with different concentrations of GEH (100–500 µg/ml). After 24 h of treatment, the MTT (1 mg/ml) solution was added to each well and incubated for 4 h at 37°C. Intracellular formazan crystals formed by viable cells was solubilized with DMSO and the absorbance was measured at 570 nm using a microplate reader (Molecular Devices, USA). The percentage viability was calculated using the following equation: (OD of GEH-treated macrophages)/(OD of control macrophages (untreated)) $\times$ 100

Nitric Oxide Assay

RAW264.7 cells were treated with various concentrations of GEH (100–500 µg/ml) or LPS (0.1 µg/ml) for 24 h. Nitrite concentrations in the supernatant were determined colorimetrically after a reaction with the Griess reagent [13]. Briefly, 100 µl of supernatant was mixed with 50 µl of 1% sulfanilic acid in 5% phosphoric acid and 50 µl of 0.1% naphthylethylene diamine dihydrochloride and incubated for 30 min at room temperature. The absorbance was measured at 550 nm using a microplate reader.

Quantitation of Cytokine Levels

For the measurement of cytokine production, cells were seeded in 6-well plates at a density of 5×10^5 cells/well. After 24 h of treatment with GEH, the culture supernatants were collected and TNF- α , IL-6, IFN- γ , and IL-10 concentrations were measured using TNF- α , IL-6, IFN- γ , and IL-10 Enzyme-Linked Immunosorbent Assay (ELISA) kits (R&D Systems, USA), according to the

manufacturer's instructions.

Phagocytosis Assay

BMDMs were plated at 5×10^4 cells/8-well chamber slide. After 24 h, the BMDMs were treated with different concentrations of GEH (100–500 µg/ml) and incubated for an additional 48 h. Jurkat T cells were labeled with CFSE, exposed to UV irradiation at 254 nm for 10 min to induce apoptosis, and cultured at 37°C for 2.5 h. Approximately 70% of cells were apoptotic, based on an evaluation of their nuclear morphology using light microscopy [14]. Apoptotic Jurkat T cells were resuspended in DMEM after pelleting by centrifugation, and cocultured with BMDMs at a 4:1 ratio (target to phagocytes). Phagocytosis was allowed for 90 min at 37°C. The cells were then washed 4 times with ice-cold PBS to remove the unengulfed apoptotic Jurkat cells and stained with rhodamine-labeled phalloidin according to the manufacturer's instructions. The phagocytosis of apoptotic Jurkat cells by BMDMs was visualized using fluorescence microscopy (Carl Zeiss, Germany, Magnification, $\times$ 200).

Reverse-Transcription-PCR (RT-PCR)

Total RNA was extracted from RAW264.7 cells using TRIzol reagent (Invitrogen Corp., USA), according to the manufacturer's instructions. cDNAs were synthesized from 1 µg of total RNA from each sample using a high-capacity cDNA reverse transcription kit (Applied Biosystems, USA) and were amplified with mouse-specific primers for TNF- α , IL-6, iNOS, IL-10, TLR2, TLR4, Dectin-1, MerTK, and β -actin. The primer sets and product sizes are listed in Table 1. Each PCR product was separated on 2% agarose gels, stained with ethidium bromide, and visualized by UV transillumination. All data were collected by densitometry and normalized to the internal control β -actin.

Western Blot Analysis

Western blot analyses were performed using lysates from RAW264.7 cells cultured in DMEM in the presence of GEH for the indicated times. The cell lysates were prepared using a cold lysis buffer (pH 7.4) containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, and 1 µg/ml of leupeptin. The cell lysates were centrifuged at 13,000 rpm for 10 min at 4°C. Protein concentrations were determined using the Bradford method. Equal amounts of protein were separated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with Tris-buffered saline (TBS, pH 7.4) containing 5% nonfat dry milk and incubated with primary anti-rabbit p-MerTK, t-MerTK antibodies, primary anti-mouse p-JNK, p-ERK, p-p38, SOCS3, t-JNK, t-ERK, and t-p38, and β -tubulin overnight at 4°C. After washing with TBS containing 0.1% Tween 20, the membranes were incubated with anti-rabbit IgG-HRP for 2 h at room temperature. Specific protein bands were visualized using the enhanced chemiluminescence

Fig. 1. Determination of the molecular mass of the enzyme hydrolysate (GEH) of ginseng marc.

(A) Size-exclusion HPLC analysis of GEH. (B) Estimation of the molecular mass using pullulans (Sigma) as standard size markers: (a) 804,000, (b) 366,000, (c) 200,000, (d) 113,000, (e) 48,900, (f) 21,700, (g) 10,000, and (h) 1,320 Da. $R^2 = 0.99$.

system (Amersham Pharmacia Biotech, USA). The intensities of the bands were quantified using the ImageJ program (National Institute of Health, USA). Protein bands were normalized to the corresponding loading controls.

Inhibition Assay against TLR2 or MerTK

Cells were pretreated with or without a neutralizing antibody against TLR2 or IgG1 (20 µg/ml each) or MerTK inhibitor UNC2205 (20 nM) for 2 h, followed by further incubation with or

without GEH (500 µg/ml) for 24 h. The culture supernatants were collected to analyze levels of TNF-α, IL-6, and IL-10 using ELISAs.

Statistical Analysis

Data were analyzed using SAS software (SAS Institute, Inc., USA). All values were expressed as the mean ± SEM, and measurements were performed in triplicate. Statistical differences among groups were evaluated using one-way analysis of variance followed by Dunnett's multiple range tests.

Table 1. Primer sequences and product sizes.

Primers	Forward/Reverse	Sequence	Product size (bp)
iNOS	Forward	5'-GGGAGTAGCCTGTGTGCACCTGGAA-3'	310
	Reverse	5'-CTGCAGCACTTGGATCAGGAACCTG-3'	
TNF-α	Forward	5'-CCTGAGTTCTGCAAGGGAG-3'	339
	Reverse	5'-GCAAAAGAGGAGGCAACAAG-3'	
IL-10	Forward	5'-CCAAGCCTTATCGGAAATGA-3'	162
	Reverse	5'-TTTTACAGGGGAGAAATCG-3'	
IL-6	Forward	5'-GTTCTCTGGGAAATCGTGGA-3'	397
	Reverse	5'-TGGTCTTGGTCCTTAGCCAC-3'	
TLR2	Forward	5'-AGCATCCGAATTGCATCACC-3'	175
	Reverse	5'-ACCCAGAGAAGCATCACATGA-3'	
TLR4	Forward	5'-TTGCTGCCAACATCATCCAG-3'	359
	Reverse	5'-GGTCCAAGTTGCCGTTTCTT-3'	
Dectin-1	Forward	5'-ACAGTACACCAGACACAGGG-3'	249
	Reverse	5'-TGGCCAGACAGCATAAGGAA-3'	
MerTK	Forward	5'-TCCTACCTCCTGTTGCGTTT-3'	356
	Reverse	5'-ATTCACACTCTCAGGCTGCT-3'	
β-Actin	Forward	5'-TGGAATCCTGTGGCATCCATGAAAC-3'	463
	Reverse	5'-TAAAACGCAGCTCAGTAACAGTCCG-3'	

Table 2. Chemical composition of the enzyme hydrolysate (GEH) of ginseng marc.

Source	Carbohydrate ^a (mass %)	Protein ^b (mass %)	Lipid ^c (mass %)	Polyphenol ^d (mass %)	Neutral sugars ^e (mol% of all neutral sugars)			
					Glc	Gal	Ara	Rha
GEH	79.79	1.3	0.26	0.56	82.4	7.4	7.1	3.0

^aTotal carbohydrate was determined by the phenol-sulfuric acid method at 490 nm [8].

^bTotal protein was quantified by the Bradford method [9].

^cTotal lipids content was determined by the Bligh and Dyer method [10].

^dTotal polyphenols was determined using the Folin-Ciocalteu phenol reagent [11].

^eNeutral sugar composition was analyzed using HPLC-PAD.

Results and Discussion

Chemical Composition of GEH and Molecular Weight Determination

The size-exclusion HPLC analysis showed that the enzyme hydrolysate (GEH) of the ginseng marc polysaccharides consists of two major fractions with estimated molecular sizes of 677,720 Da (42.28% relative amount) and 2,515 Da (57.72%), respectively (Figs. 1A and 1B). As shown in Table 2, chemical composition analyses showed that GEH consists mainly of carbohydrates (79.79%, in mass) and other minor chemicals, such as protein (1.3%, in mass), lipids (0.26%), and polyphenols (0.56%). The monosaccharide analysis showed that GEH mainly consists of glucose (82.4 mol%) as the major sugar and other sugars, such as galactose (7.4), arabinose (7.1), and rhamnose (3.0). These results suggested that the GEH has water-soluble arabinogalactoglucan-type polymers mainly consisting of glucose residues with other minor sugars.

Effects of GEH on the Viability of RAW264.7 Cells

As an initial study of the immunostimulatory activity of GEH in RAW 264.7 macrophage cells, the effects of GEH on the viability of RAW264.7 cells that had been cultured with GEH (100–500 µg/ml) for 24 h were assessed using MTT assay. As shown in Fig. 2A, GEH did not disturb the viability of RAW264.7 macrophages at concentrations up to 500 µg/ml, but rather enhanced cell proliferation in a dose-dependent manner, with 7.30%, 16.05%, and 20.72% increases, suggesting that the GEH is nontoxic toward RAW264.7 macrophages.

Effects of GEH on the Production of Immunomodulatory Molecules in RAW264.7 Cells

Whether GEH improves the immune functions in RAW264.7 cells was examined. The expression levels of iNOS, TNF- α , and IL-10 mRNAs in GEH-treated RAW264.7

cells were examined by RT-PCR at various time points. The expression of the iNOS mRNA was significantly increased in a time-dependent manner, and its expression reached a peak at 24 h of incubation with 500 µg/ml of GEH (Fig. 2B). The GEH (100, 250, and 500 µg/ml) induced dose-dependent increases in the expression of the iNOS mRNA by 7.20-, 7.81-, and 8.25-fold and the protein levels by 1.98-, 2.26-, and 2.47-fold, respectively, and the NO release was also dose-dependently increased by 1.75%, 11.2%, and 39.1%, respectively (Fig. 3). The expression of TNF- α and IL-10 mRNAs peaked at 4 h of incubation and was gradually decreased in a time-dependent manner (Figs. 2C and 2D). After 4 h of exposure, GEH dose-dependently increased the expression of the TNF- α , IL-6, and IL-10 mRNAs in RAW264.7 cells (Fig. 4). The expression of the TNF- α mRNA increased by 6.3%, 8.1%, 10.7% compared to GEH-untreated control cells (0 µg/ml). In addition, TNF- α production was dose-dependently and significantly increased in the supernatant of GEH-treated RAW264.7 cells by 8.9-, 13.9-, and 16.6-fold, consistent with the mRNA expression data shown in Fig. 4A (Fig. 5A). Similarly, the levels of the IL-6 mRNA and protein were dose-dependently increased by the GEH treatment (11.4-, 36.8-, and 47.2-fold increases in the mRNA levels; and 2.6-, 16.9-, and 120.2-fold increases in protein release by 100, 250, and 500 µg/ml GEH treatment; Figs. 4B and 5B). Similar results were obtained in GEH-treated BMDMs (data not shown).

Macrophages are the most functionally diverse cells of the hematopoietic system and are found in almost every tissue. In recent decades, the new concept of macrophage polarization, which classifies macrophages into M1 (classically activated) macrophages or M2 (alternatively activated) macrophages, was proposed by Mills *et al.* [15] and has received great attention from researchers. In addition to their classical role of killing the attacking microorganisms, macrophages are also involved in the induction and resolution of inflammation, as well as tissue

Fig. 2. Effects of GEH on the cell viability and the expression of iNOS, TNF- α , and IL-10 mRNAs in RAW264.7 macrophages. (A) Cells were cultured in the presence of GEH (100–500 $\mu\text{g/ml}$) or LPS (0.1 $\mu\text{g/ml}$) for 24 h, and the cell viability was determined by MTT assay. (B, C, D) The expression of iNOS (B), TNF- α (C), and IL-10 (D) mRNAs was measured using RT-PCR. Relative increases in the levels of each band compared with the loading control β -actin were quantified using densitometry and ImageJ software. Values are expressed as the mean $\pm$ SEM of three separate experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the GEH-untreated cells (0 $\mu\text{g/ml}$).

repair. The M1 subtype exhibits inflammatory functions and more effectively presents antigens to the adaptive immune system, whereas M2 macrophages show immunosuppressive and anti-inflammatory functions, contribute to tissue remodeling, and promote Th2-mediated immune responses [16].

Since TNF- α and IL-6 are regarded as typical pro-inflammatory cytokines secreted by M1 macrophage, the increase in the mRNA and protein levels of these cytokines,

shown in Figs. 4 and 5, indicate that they are hallmarks of classic macrophage activation and a possible excessive immune reaction. In addition, a report using an anti-TNF- α antibody-administered mouse model showed that the TNF- α -depleted mouse showed significant reduction in illness and delayed clearance of respiratory syncytial virus (RSV)-infected cells, suggesting that TNF- α is important for the induction of an immune response, but it exacerbates illness during the effector phase of RSV infection [17]. Therefore,

Fig. 3. Effects of GEH on nitric oxide (NO) levels in RAW264.7 macrophages.

Cells were cultured with or without GEH (0–500 μg/ml) or LPS (0.1 μg/ml) for 24 h. The expression of the iNOS mRNA (A) and protein (C) was measured using RT-PCR and western blot analysis, respectively. Relative increases in the levels of each band compared with the loading control β-actin or β-tubulin were quantified using densitometry and ImageJ software. (B) NO levels in the culture supernatants were analyzed using Griess reagent. Values are expressed as the mean ± SEM of three separate experiments. ** $p < 0.01$ and *** $p < 0.001$ compared with the GEH-untreated cells (0 μg/ml).

if these excessive proinflammatory cytokines TNF-α and IL-6 are continuously produced in the body and are not

controlled appropriately and promptly, chronic inflammation may eventually occur and the resolution of inflammation

Fig. 4. Effects of GEH on the expression of TNF-α, IL-6, and IL-10 mRNAs in RAW264.7 macrophages.

Cells were cultured with or without GEH (0–500 μg/ml) for 4 h. The expression of the TNF-α (A), IL-6 (B), and IL-10 (C) mRNAs was analyzed using RT-PCR. Relative increases in the levels of each band compared with the loading control β-actin were quantified using densitometry and ImageJ software. Values are expressed as the mean ± SEM of three separate experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the GEH-untreated cells (0 μg/ml).

Fig. 5. Effects of GEH on TNF- α , IL-6, IFN- γ , and IL-10 production in RAW264.7 macrophages.

Cells were cultured with or without GEH (100–500 $\mu\text{g/ml}$) or LPS (0.1 $\mu\text{g/ml}$) for 24 h. The culture supernatants were analyzed for levels of TNF- α (A), IL-6 (B), IFN- γ (C), and IL-10 (D) using ELISAs. Values are expressed as the mean $\pm$ SEM of three separate experiments. *** $p < 0.001$ compared with the GEH-untreated cells (0 $\mu\text{g/ml}$).

will be delayed or inhibited. Therefore, potential questions have been raised regarding whether the increase in GEH-elicited TNF- α expression at both the transcriptional and translational levels is a representative sign of immune stimulation or an excessive pro-inflammatory status in macrophages. Changes in the mRNA and protein levels of the M2-generated cytokine IL-10 were investigated in GEH-treated macrophage to address this question. As shown in Figs. 4C and 5D, the expression of the IL-10 mRNA was significantly and dose-dependently increased by 3.3-, 5.3-, and 5.8-fold following treatment with various concentrations of GEH (100, 250, and 500 $\mu\text{g/ml}$, respectively), and its protein level was considerably increased by 1.2-, 2.1-, and 3.0-fold, respectively, compared with GEH-untreated normal controls, suggesting that M1 cytokines, such as TNF- α , and M2 cytokines, such as IL-10, are both

produced in RAW264.7 cells in the presence of GEH. This indicates that the M1/M2 balance is not disturbed by the GEH treatment, and rather is maintained.

Effects of GEH on the Expression of Toll-Like Receptor mRNAs in RAW264.7 Cells

Next, the molecular target of GEH was investigated using RT-PCR to identify the molecular mechanism by which GEH exerts its immunostimulatory effects on macrophages. Owing to its large size, GEH is presumed to be unable to penetrate the cell, thus it probably transduces its signal through pattern recognition receptors expressed on the surface of macrophages. The mRNA levels of surface receptors, such as TLR2, TLR4, and Dectin-1, were determined in GEH-treated macrophages. As shown in Fig. 7A, the expression of the TLR2 mRNA was dose-

Fig. 6. Effects of GEH on the expression of TLR2 and MerTK mRNAs.

The expression of the TLR2 (A) and MerTK (B) mRNAs was analyzed using RT-PCR. Relative increases in the levels of each band compared with the loading control β -actin were quantified using densitometry and ImageJ software. Values are expressed as the mean $\pm$ SEM of three separate experiments. * $p < 0.05$ and *** $p < 0.001$ compared with the GEH-untreated cells (0 h).

dependently increased by 2.6-, 3.2-, and 4.8-fold in response to the GEH treatment (100, 250, and 500 $\mu\text{g/ml}$). The TLR2 mRNA levels peaked after 4 h of exposure to GEH, returned

to basal levels within 10 h, and decreased below the basal levels (Fig. 6A). Unlike TLR2, the mRNA levels of TLR4 and Dectin-1 were not altered by different concentrations

Fig. 7. Effects of GEH on the expression of TLR2, TLR4, and Dectin-1 mRNAs in RAW264.7 macrophages.

Cells were cultured with or without GEH (100–500 $\mu\text{g/ml}$) for 4 h. The expression of the TLR2 (A), TLR4 (B), and Dectin-1 (C) mRNAs was analyzed using RT-PCR. Relative increases in the levels of each band compared with the loading control β -actin were quantified using densitometry and ImageJ software. Values are expressed as the mean $\pm$ SEM of three separate experiments. ** $p < 0.01$ and *** $p < 0.001$ compared with the GEH-untreated cells (0 $\mu\text{g/ml}$).

of GEH (Figs. 7B and 7C). Dectin-1, a C-type lectin receptor on macrophages, monocytes, and dendritic cells, recognizes the fungal wall-derived β -glucan found at high levels in mushrooms and promotes the phagocytosis of live yeast and fungal-derived zymosan [18]. Thus, GEH may interact with TLR2, but not with TLR4 or Dectin-1, on the surface of macrophages, transduce signals inside the cell, and ultimately induce the production of immunostimulatory mediators, such as TNF- α , IL-6, NO, and IL-10.

GEH Enhances Phagocytotic Activities of Macrophages toward Apoptotic Cells

The human body efficiently removes more than 10^9 apoptotic cells everyday using the TAM receptors (Tyro3, Axl, and MerTK) on macrophages and dendritic cells [19]. If these cells are not removed rapidly, inflammation persists owing to the release of various inflammatory molecules from the debris of dying cells.

The phagocytic activity of macrophages toward apoptotic Jurkat cells was assayed using CFSE-labeled Jurkat cells to address this question. As shown in Fig. 8, phagocytosed apoptotic Jurkat cells, shown in green, gradually appeared inside the rhodamine-phalloidin-labeled BMDMs in response to the increasing concentrations of GEH, suggesting that GEH is definitely capable of activating macrophages to engulf apoptotic cells. β -Glucan has been shown to stimulate BMDMs to recognize phosphatidylserine, which is exposed on apoptotic cells, and thereby enhance the phagocytosis of apoptotic cells [20]. Similar to this report, our data demonstrated that GEH stimulates macrophages to remove the apoptotic cells.

Effects of GEH on MerTK in RAW264.7 Cells

The TAM receptor tyrosine kinase has been known to play a pivotal role in resolution of inflammation [21]. Mice deficient in MerTK/Axl/Tyro3 produce autoantibodies

Fig. 8. Effects of GEH on MerTK mRNA and protein levels in RAW264.7 macrophages.

(A) Cells were cultured with or without GEH (0–500 $\mu\text{g/ml}$) for 24 h and expression of the MerTK mRNA was measured using RT-PCR. (B) RAW 264.7 cells were challenged with or without GEH (100–500 $\mu\text{g/ml}$) for 48 h and apoptotic Jurkat cells were co-cultured with RAW264.7 cells at a 10:1 ratio for 15 min. After removing the unengulfed Jurkat cells by washing with ice-cold PBS, lysates were prepared by RIPA lysis buffer. The phospho-MerTK (p-MerTK) and total MerTK (t-MerTK) levels were monitored by Western blot analyses. Relative increases in the levels of each band compared to β -actin or t-MerTK were quantified using densitometry and ImageJ software. Values are expressed as the means $\pm$ SEM of three separate experiments. * $p < 0.05$ and ** $p < 0.01$ compared with the GEH-untreated cells (0 $\mu\text{g/ml}$).

Fig. 9. Effects of GEH on MerTK mRNA and protein levels in RAW264.7 macrophages.

(A) Cells were cultured with or without GEH (0–500 μg/ml) for 24 h and expression of the MerTK mRNA was measured using RT-PCR. (B) RAW264.7 cells were challenged with or without GEH (100–500 μg/ml) for 48 h and apoptotic Jurkat cells were cocultured with RAW264.7 cells at a 10:1 ratio for 15 min. After removing the unengulfed Jurkat cells by washing with ice-cold PBS, lysates were prepared with RIPA lysis buffer. The phospho-MerTK (p-MerTK) and total MerTK (t-MerTK) levels were monitored by western blot analyses. Relative increases in the levels of each band compared with β-actin or t-MerTK were quantified using densitometry and ImageJ software. Values are expressed as the mean ± SEM of three separate experiments. * $p < 0.05$ and ** $p < 0.01$ compared with the GEH-untreated cells (0 μg/ml).

that spontaneously lead to development of autoimmune diseases, including rheumatoid arthritis and lupus, and are associated with chronic inflammation [19]. Macrophages derived from MerTK-negative mice show an approximately 40–50% reduction in the phagocytosis of apoptotic thymocytes [22]. The effect of GEH on changes in MerTK expression at both the mRNA and protein levels were investigated using RT-PCR and western blot analyses. As shown in Fig. 9A, the expression of the MerTK mRNA was significantly increased by increasing concentrations of GEH (100–500 μg/ml). Similar to the increased mRNA expression, apoptotic Jurkat cells significantly induced MerTK phosphorylation in GEH-activated macrophages in a dose-dependent manner, with 1.14-, 2.43-, and 2.93-fold increases compared with the GEH-untreated normal control (0 μg/ml), suggesting that GEH is capable of eliciting MerTK activation to facilitate the subsequent clearance of apoptotic cells.

GEH Stimulates RAW264.7 Cells to Produce TNF-α, IL-6, and IL-10 via TLR2 or MerTK Activation

To confirm the roles of TLR2 and MerTK in activation of

macrophage function, cells were pretreated with or without the TLR2 neutralizing antibody or MerTK inhibitor, followed by further incubation with or without GEH (500 μg/ml) for 24 h. Pretreatment with the neutralizing antibody against TLR2 significantly alleviated cytokine production of TNF-α, IL-6, and IL-10 in GEH-treated RAW 264.6 cells (Figs. 10A–10C). In addition, the MerTK inhibitor (UNC2205) significantly reduced GEH-induced cytokine production of TNF-α, IL-6, and IL-10 compared with GEH-treated cells (Figs. 10D–10F). It has been reported that overexpression of MerTK in epithelial cancer cells drives the clearance of apoptotic cells [23]. In contrast, homozygous deletion of MerTK results in systemic autoimmunity from the failure to clear apoptotic cells by myeloid cells in peripheral tissues [24, 25]. In our study, GEH activated macrophage functions to produce TNF-α, IL-6, and IL-10, thereby enhancing phagocytosis of apoptotic cells as shown in Figs. 5 and 8. As shown in Fig. 10, inhibition assay using the TLR2 neutralizing antibody and MerTK inhibitor definitely showed that these cytokine productions in GEH-stimulated RAW264.7 cells are mediated by TLR2 and MerTK, even though TLR2 and MerTK did not

Fig. 10. Association of TLR2 or MerTK with the GEH-induced production of TNF- α , IL-6, and IL-10 in RAW264.7 cells.

Cells were pretreated with or without neutralizing antibody against TLR2 or IgG1 (20 µg/ml each; A, B, and C) or MerTK inhibitor, UNC2205 (20 nM; D, E, and F) for 2 h, followed by further incubation with or without GEH (500 µg/ml) for 24 h. The culture supernatants were analyzed for levels of TNF- α (A), IL-6 (B), and IL-10 (C) using ELISAs. Values are expressed as the mean $\pm$ SEM of three separate experiments. * p < 0.05, *** p < 0.001 compared with the GEH-untreated cells (None); * p < 0.05, ** p < 0.01 compared with the GEH-treated cells (Control).

completely inhibit cytokine production of TNF- α , IL-6, or IL-10.

Effects of GEH on the Expression of p38, ERK, JNK, and SOCS3 in RAW264.7 Cells

The downstream signaling of TLR2 and MerTK upon stimulation with GEH treatment was investigated by western blot analysis. The downstream signaling molecules of TLR2, including MAPK, were investigated. As shown in

Fig. 11, the expression levels of phosphorylated p38 (p-p38) and phosphorylated ERK (p-ERK) were dose-dependently increased upon GEH stimulation. Moreover, the expression of SOCS3 (suppressor of cytokine signaling), which is a well-known downstream signaling molecule of MerTK, was also dose-dependently enhanced in GEH-stimulated RAW264.7 cells.

Taken together, the results of the present study showed that the enzymatic hydrolysate (GEH) of ginseng marc

Fig. 11. Effects of GEH on the expression of p38, ERK, JNK, and SOCS3 in RAW264.7 cells.

Cells were treated with different concentrations of GEH for 1 h (p-p38, p-ERK, and p-JNK) or 24 h (SOCS3). An equal amount of cell lysates was subjected to western blot analysis. Relative increases in the levels of each band compared with each loading control, total p38, total ERK, total JNK, and β-tubulin, were quantified using densitometry and ImageJ software. Values are expressed as the mean ± SEM of three separate experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the GEH-untreated cells (0 μg/ml).

polysaccharides exert immunostimulatory effects by maintaining the balance between M1 and M2 cytokines, facilitating macrophage activation, and thereby enhancing phagocytic removal of apoptotic cells. Therefore, the GEH could be developed as value-added, health-beneficial food materials with potent immunostimulatory effects.

Acknowledgments

This study was supported by grants from the Leading Industry Development for Economic Region fund (R0004760), Ministry of Trade, Industry & Energy (MOTIE), Korea Institute for Advancement of Technology (KIAT), and

Gangwon Institute for Regional Program Evaluation (GWIRPE).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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