Effects of ginsenosides on regulatory T cell differentiation

Jisu Kim¹ · Hyeyoung Byeon¹ · Kyungtaek Im¹ · Hyeyoung Min¹

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Abstract Regulatory T cells (Treg cells) are a subpopulation of T cells defined as $CD4^{+}Foxp3^{+}CD25^{+}$. They mainly function as immunosuppressive T cells by down-regulating the induction and proliferation of effector T cells, but also modulate the immune system by maintaining self-tolerance and preventing autoimmune disease. In this study, the regulatory roles of ginsenosides, one of the active components in ginseng, *Panax ginseng* C. A. Meyer, in Treg cell differentiation were examined. The results demonstrated that ginsenoside Rd induced Treg differentiation by upregulating Foxp3 expression and increased the generation of TGF- β 1, IL-10 and IL-35. The data suggest that ginsenoside Rd may be a potential immunomodulating agent or supplement that can be applied for transplantation and autoimmune disorders.

Keywords Korean red ginseng · Ginsenoside · Regulatory T cell · Foxp3

Introduction

During T cell development, the early intrathymic precursors $CD4^{-}CD8^{-}$ double-negative T cells differentiate into $CD4^{+}CD8^{+}$ double-positive T cells, undergo positive and negative selection, and give rise to $CD4^{+}$ T helper cells (Th cells) or $CD8^{+}$ cytotoxic T lymphocytes (CTL) [1]. Th cells help other immune cells by secreting various cytokines, while CTL induces cell death of infected cells with

perforin and granzyme. Th cells have several subsets such as Th1, Th2, Th3, Th17, Th9, or Tfh (follicular helper T cells); among them, Th1 and Th2 cells have been widely studied [2].

Recently, $CD4^{+}CD25^{+}Foxp3^{+}$ T cells (Treg) were found as another subset of Th cells that negatively regulate other Th cells. Transforming growth factor (TGF)- β and IL-2 make naïve T cells differentiate into Treg cells by inducing a specific transcription factor, Foxp3 [3]. Although Treg cells mainly suppress other immune cells, they help to manage immune system with IL-10, IL-35, and TGF- β to prevent the occurrence of autoantigen production, autoimmunity, and graft-versus-host disease after transplantation [4–6]. Treg cells also reduce host immunity against pathogens to prevent tissue damage from vigorous immune disease [7]. Additionally, Treg cells repress tumor antigen-specific effector T cells, and thus are currently begin tested for treating cancer by depleting Treg [8].

Ginsenosides are one of the active components of Ginseng, *Panax ginseng* C. A. Meyer (Araliaceae), and can be classified into three groups: protopanaxadiol (Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2); protopanaxatriol (Re, Rf, Rg1, Rg2, Rh1); and oleanane (Ro) [9]. Ginsenosides can be metabolized by acid hydrolysis in the stomach, bacterial fermentation in the intestine, or heat-processing; such processes often result in the generation of pharmacologically active or readily absorbable forms such as compound K [20-O-D-glucopyranosyl-20(S)-protopanaxadiol]. Ginsenosides have been shown to possess anti-cancer, anti-atherosclerotic, anti-diabetic, and immunomodulatory effects [9].

Although numerous studies have reported the function of ginsenosides and compound K in various immune cells, their effects on Treg differentiation have not been well investigated, and the induction of Treg differentiation by

✉ Hyeyoung Min
hymin@cau.ac.kr

¹ College of Pharmacy, Chung-Ang University,
84 Heukseokro, Dongjakgu, Seoul 06974, Korea

ginsenoside Rp1 has been merely reported [10]. Therefore, the regulatory roles of various ginsenosides and compound K in Treg differentiation were examined in this study.

Materials and methods

Mouse

Three to six-week-old male C57BL/6 mice were purchased from Koatech (Pyungtaek, Korea). Foxp3-GFP C57BL/6 transgenic mice express green fluorescent protein (GFP) under the control of the mouse Foxp3 (forkhead box P3) promoter, and GFP is expressed in Foxp3⁺ Treg cells. The transgenic mice were used for convenience of experiments by skipping intracellular staining of Foxp3 for flow cytometry. The Foxp3^{GFP+} transgenic mice were kindly provided by Prof. K. W. Hwang (Chung-Ang University, Seoul, Korea).

Korean red ginseng extract and ginsenosides

Ginsenosides [Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, Rg1, Rg2(S), Rh1(S), Rh2(S), Rp1] and compound K were purchased from Ambo Institute (Daejeon, Korea) and dissolved in dimethyl sulfoxide at 20 mM.

CD4⁺ T cell preparation and cell culture

Spleens were harvested from mice, and single cell suspensions were prepared followed by red blood cell lysis. Cells were washed with RPMI1640 (Welgene, Daegu-si, Korea) and resuspended in MACS buffer. Anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added, and CD4⁺ T cells were positively separated using a magnetic MidiMACS Separator (Miltenyi Biotec). The purity of CD4⁺ T cells was greater than 95%. The purified CD4⁺ T cells were cultured in RPMI1640 containing fetal bovine serum (Welgene), penicillin/streptomycin (Gibco, Grand Island, NY, USA), MEM non-essential amino acids (Corning, Corning, NY, USA), L-glutamine (Corning), HEPES (Corning), sodium pyruvate (Corning), and 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). To further differentiate into Treg cells, CD4⁺ T cells were stimulated with plate-bound anti-mouse CD3e (1 µg/mL; clone 145-2C11, eBioscience, San Diego, CA, USA) and anti-mouse CD28 (1 µg/mL; clone 37.51, eBioscience) antibodies, and IL-2 (10 ng/mL, BD Biosciences, Franklin Lakes, NJ, USA) and TGF-β1 (2.5 ng/mL) were added to the cells. The cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere.

Cell viability assay

CD4⁺ T cells (4×10^5 cells) were seeded into a 96-well plate and cultured in Treg differentiation conditions together with ginsenosides. After 24 h, 10 µL of MTT solution (Sigma-Aldrich) and 100 µL of 0.04 N HCl-isopropanol were added to the culture 6 h apart. Absorbance was detected at 570 nm by a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Total RNA isolation and cDNA synthesis

CD4⁺ T cells were differentiated into Treg cells in the presence of ginsenosides for 3 days, and harvested for total RNA isolation and cDNA synthesis as described previously [11].

Quantitative real-time polymerase chain reaction (qRT-PCR)

cDNA, primers for each gene, and 2X SYBR green qPCR master mix (Enzynomics, Daejeon, Korea) were mixed to perform qRT-PCR using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Gene expression was normalized to the level of β-actin. Sequences of used primers are listed as follows: mouse Foxp3 (sense, 5'-GGC CCT TCT CCA GGA CAG A-3'; antisense, 5'-GCT GAT CAT GGC TGG GTT GT-3') [12], TGF-β (sense, 5'-CGC CAT CTA TGA GAA AAC C-3'; antisense, 5'-GTA ACG CCA GGA ATT GT-3') [13, 14], β-actin (sense, 5'-ACC CAC ACT GTG CCC ATC TAC-3'; antisense, 5'-GCC ATC TCC TGC TCG AAG TC-3') [15].

Enzyme-linked immunosorbent assay (ELISA) of TGF-β1

ELISA was performed to detect the secretion of TGF-β1 as previously described [16, 17]. Purified rat anti-mouse, human, pig TGF-β1 (clone A75-2, BD Biosciences), and biotinylated rat anti-mouse, human, pig TGF-β1 (clone A75-3, BD Biosciences) antibodies were used.

Flow cytometry

To detect Foxp3⁺ Treg cells secreting IL-10 and IL-35, 1×10^6 CD4⁺ T cells derived from Foxp3-GFP mice were differentiated into Treg cells in the presence of ginsenoside Rd for 3 days. The Treg cells were moved into 48-well plates at 6×10^5 cells/well, and cultured for an additional 2 days. Before harvest, cells were stimulated with cell stimulation cocktail (eBioscience) for 4 h. Harvested cells were fixed, permeabilized with Cytofix/Cytoperm™

Fixation/Permeabilization Kit (BD Biosciences), and blocked with anti-CD16/CD32 antibody (clone 93, eBioscience). Cells were then stained with PerCP Cy5.5-conjugated anti-IL-10 antibody (clone JES5-16E3, eBioscience) and PE-conjugated anti-IL-35 antibody (clone 27537, R&D Systems, Minneapolis, MN, USA). The stained cells were detected with a FACS Calibur (BD Biosciences) and analyzed using CellQuestPro software (BD Biosciences).

Statistics

All experiments were repeated at least 3 times, and data were expressed as the mean $\pm$ standard deviation. Significance of the data was determined by Student's *t* test and one-way ANOVA with Tukey's post hoc test. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

Results and discussion

Cytotoxic effects of ginsenosides on CD4⁺ T cells under Treg cell differentiation conditions

CD4⁺ T cells were treated with 100 μ M of ginsenosides and differentiated into Treg cells. Most ginsenosides did not affect cell viability, while Rh2(S), Rp1, and compound K showed cytotoxicity at 100 μ M (Fig. 1). Because of their cytotoxicity, Rh2(S), Rp1, and compound K were excluded for subsequent experiments.

Effects of ginsenosides on Treg cell differentiation

To examine the effects of ginsenosides on Treg differentiation, diverse ginsenosides [Rb1, Rb2, Rb3, Rc, Rd, Re,

Rf, Rg1, Rg2(S), Rh1(S)] were tested for Foxp3 expression. We observed that ginsenoside Rd (G-Rd) significantly upregulated the expression of Foxp3, and thus, G-Rd was used for further investigation. The data showed that the stimulatory effect of G-Rd on Treg differentiation was dose-dependent as determined by Foxp3 gene expression (Fig. 2B), which was confirmed by increased GFP expression in the experiments using Foxp3^{GFP+} transgenic mice (Fig. 2C).

Increase in the production of immunosuppressive cytokines by ginsenoside Rd treatment

To confirm the stimulatory effects of ginsenoside Rd on Treg differentiation, the production of immunosuppressive cytokines was measured. As shown in Fig. 3, G-Rd increased the production of TGF- β , and IL-35 significantly in a dose dependent manner, while the increase in IL-10 production was observed only at 100 μ M. Taken together, the increased production of immunosuppressive cytokines as well as upregulation of Foxp3⁺ Treg cells supports the regulatory function of G-Rd in Treg differentiation.

Wang et al. [16] reported that G-Rd had immunosuppressive effects on skin allograft rejection in rats. The exact mechanism of G-Rd in suppressing skin rejection remains unclear; however, based on our results, G-Rd suppressed allograft rejection possibly by inducing Treg cells. Although the effect of compound K on Treg differentiation could not be tested, Chen et al. [18] reported that compound K reduced adjuvant-induced arthritis by upregulating Treg cells and suppressing T cell activation in rats. As a final metabolite of protopanaxadiol-type ginsenoside by intestinal bacteria, ginsenosides with protopanaxadiol structures such as G-Rd are metabolized into compound K

Fig. 1 Cytotoxic effects of ginsenosides on Treg cells. Cell viability was observed by MTT assay under Treg differentiation condition, and expressed as the percent ratio of ginsenoside (100 μ M) treatment normalized over 0.5% DMSO control value

Fig. 2 Effects of ginsenosides on Treg differentiation. CD4⁺ T cells from C57BL/6 mice were differentiated into Treg cells in the presence of various ginsenosides, and Treg cell differentiation was determined based on Foxp3 expression. Effects of ginsenosides on Treg differentiation (A). Dose-dependent effects of ginsenoside Rd

(G-Rd) on Treg differentiation in C57BL/6 mice (B). Increase in Foxp3^{GFP+} Treg differentiation by G-Rd treatment (C, D). Flow cytometry plot shows the representative result of three experiments with similar results

Fig. 3 Changes in the production of inhibitory cytokines by G-Rd treatment. Expression levels of TGF-β (A), IL-10 (B), and IL-35 (C) were measured by ELISA for TGF-β and by flow cytometry for

IL-10 and IL-35 after G-Rd treatment. Flow cytometry plots show the representative results of three experiments with similar results

in vivo. Therefore, G-Rd, which upregulates Treg differentiation in vitro, may have the same immunosuppressive function as compound K in vivo.

In conclusion, we report the function of several ginsenosides in Treg differentiation including the induction of Treg differentiation by G-Rd. Given that rapamycin and alemtuzumab, which induce Treg cells to suppress immune responses [19], have been developed for the treatment of transplantation and multiple sclerosis, respectively, G-Rd may be a good drug candidate or supplement for transplantation and autoimmune disorders. Prospective studies will help to determine the detailed molecular mechanism of G-Rd for inducing Treg differentiation.

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