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Ying Liu, Qian Wu, Peng Li, Weijie Liu, Yongri Jin, Xuwen Li, Xiaolei Shi*

Langerhans cell-like dendritic cells treated with ginsenoside Rh₂ regulate the differentiation of Th1 and Th2 cells *in vivo*

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Abstract: Ginsenoside Rh₂ is one of the rare ginsenosides extracted from *Panax ginseng* C. A. Mey. The anti-allergic activity of ginsenoside Rh₂ has been documented in some literature. In this work, an anti-allergic mechanism of ginsenoside Rh₂ was investigated by focusing on the differentiation of T cells through Langerhans cells (LCs). Langerhans cell-like dendritic cells (LDCs) were generated *in vitro* and were used as substitute for LCs. *In vivo*, the mRNA expression for IFN- γ and CXCR3 of T cells was increased after being injected with ginsenoside Rh₂-treated LDCs thereby increasing the concentration of IFN- γ in the culture supernatants of CD3⁺/CD28⁺ T lymphocytes. However, *in vitro*, the expression of mRNA for CD40 and CD80 on ginsenoside Rh₂-treated LDCs was up-regulated significantly and the endocytic activity of LDCs was down-regulated slightly. These findings indicate that T cells differentiation could be regulated by ginsenoside Rh₂ through LDCs *in vivo* by altering the antigen presenting capacity, maturation and phagocytosis of LDCs.

Keywords: anti-allergic activity; ginsenoside Rh₂; Langerhans cells; T cells differentiation.

1 Introduction

Ginseng (*Panax ginseng* C. A. Mey.) is widely used in Asia in daily routine. Ginsenoside Rh₂ is one of the rare ginsenosides extracted from ginseng [1]. It is also known as a main metabolite of ginsenosides by bacteria from human intestine [2]. Ginsenoside Rh₂ has been widely concerned for its excellent pharmacological activities such as anti-

tumor, anti-oxidant and anti-allergic effects [3-5]. Anti-allergic effects of ginsenoside Rh₂ were well documented in literature in the past. Park et al. reported that ginsenoside Rh₂ has potent inhibitory activity on β -hexosaminidase release from RBL-2H3 cells and in the passive cutaneous anaphylaxis reaction in mice, it also inhibited nitric oxide and prostaglandin E₂ in lipopolysaccharide-stimulated RAW 264.7 cells [5]. In addition, ginsenoside Rh₂ has been reported to have anti-allergic activity *in vivo* by Li et al., the mechanism focused on the regulation of p38 MAPK/NF- κ B signaling in a murine model of asthma [6]. According to the pathogenesis of allergic diseases, the differentiation of Th1 or Th2 cells affects allergic reactions directly. However, the exploit effects of ginsenoside Rh₂ on T cell response has not been reported previously.

Dendritic cells (DCs) play a critical role in immunoregulation as antigen presenting cells (APCs). It has been proved that Langerhans cells (LCs) is a subgroup of DCs as it participates in various allergic diseases such as pathogen infection, contact hypersensitivity and atopic dermatitis, exerting either pro-inflammatory or regulatory functions [7-9]. LCs could constantly survey external environment and deliver signals and antigens to lymphoid organs. Once infected, LCs would capture the invading pathogens, get activated and migrate to drain the lymph nodes, finally present the antigen to T cells to elicit immune response [10]. Thus, LCs could work as APCs, and allergic inflammation might be relevant with regulation of LCs function. Furthermore, peptide-major histocompatibility complex displayed on the surface of LCs could trigger the T cell receptor and results in T cells proliferation and differentiation. It has been proved that LCs plays a major role in Th1 and Th2 differentiation in a report [11]. Langerhans cell-like dendritic cells (LDCs) are usually generated *in vitro* and are used as substitute for LCs.

Considering that ginsenoside Rh₂ has potent anti-allergic activity, while its regulation on T cells and LDCs were seldom reported. This is why, the effects of ginsenoside Rh₂ on T cells differentiation through LDCs *in vivo* were investigated in the current work.

*Corresponding author: Xiaolei Shi, College of Food Science and Engineering, Jilin University, Changchun 130062, P. R. China, E-mail: xlshi@jlu.edu.cn

Ying Liu, Qian Wu, Peng Li, Weijie Liu, Yongri Jin, Xuwen Li: College of Chemistry, Jilin University, Changchun 130012, P. R. China

2 Materials and methods

2.1 Chemicals and reagents

RPMI-10 medium was made up of RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum, 25 mmol/L HEPES (Sigma, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Solarbio, China). Recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF), recombinant murine IL-4 and recombinant human TGF-β1 were purchased from PeproTech (USA). Anti-mouse I-A^d monoclonal antibody was obtained from Cedarlane (Canada). Anti-mouse IgG antibody was obtained from Sigma Co. (St. Louis, MO, USA). Dynabeads® mRNA DIRECT™ Micro Kit was purchased from Life Technologies (Norway). PrimeScript™ 1st strand cDNA Synthesis Kit and Ex Taq® DNA polymerase were purchased from Takara (Japan). SYBR Green I was obtained from BioTeke (China). Primers were synthesized by Invitrogen (China). Anti-mouse CD4-FITC and CD183-APC antibody were obtained from BD (USA) and anti-mouse CD194-PE antibody was obtained from Biolegend (USA). Dynabeads® mouse T-Activator CD3/CD28 was obtained from Invitrogen Dynal AS (Oslo, Norway). Enzyme-linked immunosorbent assay (ELISA) Kits for IFN-γ and IL-4 were purchased from eBioscience (USA). Other reagents were analytically pure.

2.2 Sample preparation

The roots of *Panax ginseng* C. A. Mey. were obtained from Jingyu County, Jilin Province. Powdered, dried roots of ginseng (150 g) were extracted with 2500 mL 50% (v/v) acetic acid at 80°C under reflux for 1 h. Sodium hydroxide was used to regulate the pH value of the solution to 7.0, and ethyl acetate was used to extract the ginsenosides from the solution. After vacuum evaporation, the extract was subjected to silica gel column using EtOAc-EtOH-H₂O (v/v/v, 10:2:1) as eluent, five pooled fractions (A, B, C, D and E) were obtained. Fraction D was subjected to silica gel column eluted with EtOAc-EtOH-H₂O (v/v/v, 15:1:1) and then ODS column eluted with MeOH-H₂O (v/v, 8:1) to afford ginsenoside Rh₂ (85mg).

The purity of ginsenoside Rh₂ was evaluated by an Accchrom S3000 HPLC instrument coupled with a UV detector and Agilent SB C18 column (250×4.6 mm, 5 µm). Acetonitrile and water (57:43) were used as eluents. The flow rate was 0.8 mL/min, the temperature of the column was 30°C and the detection wavelength was 203nm. The

Figure 1: The chemical structure of ginsenoside Rh₂.

purity of ginsenoside Rh₂ was more than 98%, calculated by normalization method.

The structures of ginsenoside Rh₂ were elucidated by nuclear magnetic resonance based on data reported in the literature [1]. ¹H-NMR [Pyridine-*d*₅ (Brucker, Swiss)] and ¹³C-NMR [Pyridine-*d*₅ (Brucker, Swiss)] were measured on a Bruker Avance-600 spectrometer, using TMS as international standard. The structure of ginsenoside Rh₂ was shown in Figure 1.

2.3 Mice

Female BALB/c mice aged 6 to 8 weeks were obtained from Changchun Institute of Biological Products Co, Ltd. (Certificate No.: SCXK (Ji) 2011-0003, Changchun, China). Mice were kept in plastic cages in a clean environment, allowed free access to a standard laboratory diet and water. The room temperature was controlled at 24 ± 2°C. All experiments were under the direction of Animal Welfare and Research Ethics Committee of Jilin University.

2.4 LDCs generation *in vitro*

The generation of murine LDCs *in vitro* was modified according to the report [12]. Bone marrow cells were obtained from the spinal cavity of mice and cultured in RPMI-10 medium at 37°C with 5% CO₂, treated with GM-CSF (20 ng/mL), IL-4 (100 ng/mL) and TGF-β1 (10 ng/mL). The medium was supplemented 3 d later. 4 d later, the supernatant of the cells were removed. The cells were suspended in RPMI-10 medium and treated with anti-mouse I-A^d antibody. After shaken on ice for 1 h, the cells were purified by anti-mouse IgG antibody. Cells obtained

were used as LDCs accordingly. LDCs (2×10^5 cells/mL) were treated with OVA (30 $\mu\text{g/mL}$) and ginsenoside Rh₂ (50 $\mu\text{mol/L}$) for 18 h.

2.5 T cells differentiation *in vivo*

18 h later, OVA and ginsenoside Rh₂ were removed from the LDCs, and LDCs (5×10^4 cells/mouse) were injected into both footpads of mice. 2 d later, OVA (30 μg) was injected into both footpads of mice as a booster. 5 d later, lymph nodes were dissected from the leg bends of mice and grinded to obtain lymph node cells.

2.6 Detection of cytokines and chemokine receptors on T cells by RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to detect mRNA expression in T lymph node cells. An mRNA extract kit was used to extract mRNA from lymph node cells. A cDNA synthesis kit was used to synthesize cDNA from the mRNA. PCR amplification of the cDNA products (2 μL) was performed in 15 μL of reaction mixture using an Ex Taq kit according to the manufacturer's protocol. The primers used were listed below, according to the report [12]: IFN- γ , forward 5'-GCT ACA CAC TGC ATC TTG GCT TTG-3' and reverse 5'-CAC TCG GAT GAG CTC ATT GAA TGC-3'; IL-4, forward 5'-AGT TGT CAT CCT GCT CTT CTT TCT C-3' and reverse 5'-CGA GTA ATC CAT TTG CAT GAT GCT C-3'; CXCR3, forward 5'-ATC AGC GCC TCA ATG CCA C-3' and reverse 5'-TGG CTT TCT CGA CCA CAG TT-3'; CCR4, forward 5'-TCT ACA GCG GCA TCT TCT TCA T-3' and reverse 5'-CAG TAC GTG TGG TTG TGC TCT G-3'. Thermal conditions for denaturation, annealing, and extension were as follow: 94°C for 4 min; 94°C for 45 s, 61°C for 45 s and 72°C for 2 min (35 cycles); 72°C for 7 min. The PCR products were mixed with SYBR Green I for 10 min and resolved in 2% agarose gels. The β -actin (forward 5'-GTG GGC CGC TCT AGG CAC CAA-3' and reverse 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3') was detected as an internal control. Values were normalized to β -actin as relative expression levels.

2.7 Detection of Th1/Th2 cytokines and chemokine receptors by FCM and ELISA

Lymph node cells were selected by anti-mouse CD4-FITC antibody. Receptors related to Th1/Th2 differentiation on

CD4⁺ T cells were detected by flow cytometric (FCM), using anti-mouse CD183-APC (CXCR3) and anti-mouse CD194-PE (CCR4) antibody. Lymphocytes were washed with ice-cold PBS 3 times. FCM analysis was performed using FACS Calibur and Cell Quest software (BD Biosciences) immediately.

Lymph node cells (2×10^5 cells/well) were incubated in a 96-well plate, treated with mouse T-Activator CD3/CD28. The culture supernatants of CD3⁺/CD28⁺ lymphocytes were collected after incubation for 48 h at 37 °C in a humidified atmosphere with 5% CO₂, and the concentrations of IFN- γ and IL-4 were determined with the ELISA kits.

2.8 Detection of surface molecules on LDCs by RT-PCR and FCM

An mRNA extract kit was used to extract mRNA from lymphocytes. A cDNA synthesis kit was used to synthesize cDNA from the mRNA. The primers used were listed as follows according to the report [13]: CD40, forward 5'-CCT GTA AGG AAG GAC AAC AC-3' and reverse 5'-ATC ACG ACA GGA ATG ACC AG-3'; CD80, forward 5'-GAA GAC CGA ATC TAC TGG CA-3' and reverse 5'-GGA AGC AAA GCA GGT AAT CC-3'; CD86, forward 5'-AGC CTG AGT GAG CTG GTA GT-3' and reverse 5'-CCT GTT ACA TTC TGA GCC AG-3'. The PCR reactions and data analysis were performed as the method above.

2.9 Phagocytosis assay of LDCs

Endocytic activity of LDCs was assessed using FITC-OVA. LDCs were cultured with 1 mg/mL of FITC-OVA for 30 min at 4°C. LDCs were washed with ice-cold PBS 3 times. FCM analysis was performed using FACS Calibur and Cell Quest software (BD Biosciences) immediately.

2.10 Statistical analysis

Values are expressed as mean $\pm$ SD. Statistical evaluation of the data was determined by one-way ANOVA. PASW Statistics 18 software was used in the evaluation. $P < 0.05$ was considered to be statistically significant and $P < 0.01$ was considered to be statistically very significant.

Figure 2: Effects of ginsenoside Rh₂ on mRNA expression for Th1/Th2 cytokines and chemokine receptors in T lymph node cells. Bands were quantified by densitometric analysis relative to β-actin. (Mean ± SD, N=3), *P<0.05 vs. control; ** P<0.01 vs. control.

3 Results

3.1 Th1 and Th2 regulation by LDCs treated with ginsenoside Rh₂

The investigation of Th1/Th2 differentiation mainly focuses on cytokines and chemokine receptors expression on T cells. As the results of RT-PCR shown in Figure2, the mRNA expression of IFN-γ and CXCR3 were increased by ginsenoside Rh₂-treated LDCs significantly. There was nosignificant change in the mRNA expression of IL-4 and CCR4.

FCM was applied to analyze the expression of CXCR3 and CCR4 on CD4⁺ T cells after the harvest of T lymph node cells. As the results shown in Figure3, ginsenoside Rh₂-treated LDCs could increase the expression of CXCR3 and suppress the expression of CCR4 on CD4⁺ T cells, but statistically no significant differences were shown (seen)).

Much more, the cytokines in supernatants of CD3⁺/CD28⁺ T lymphocytes were detected by ELISA. As the results shown in Figure4, the production of IFN-γ was changed from 5667.5 ± 187.5 pg/mL to 11870.6 ± 703.1 pg/mL and production of IL-4 was changed from 270.0 ± 14.0 pg/mL to 178.5 ± 25.5 pg/mL. Th1-prone immune response for increasing IFN-γ production was induced by ginsenoside Rh₂-treated LDCs significantly, while Th2-prone immune response for decreasing IL-4 production was affected slightly although not significantly.

According to the results above, ginsenoside Rh₂-treated LDCs could enhance the differentiation of Th1

cells significantly through LDCs *in vivo* and then affect the balance of Th1/Th2. Since ginsenoside Rh₂ was removed before the injection, it would be appropriate to assume that ginsenoside Rh₂ affected the functions of LDCs by acting on LDCs.

3.2 Effects of ginsenoside Rh₂ on LDCs functions

Aimed at clarifying the functions of LDCs after being treated by ginsenoside Rh₂, surface molecules and endocytic activity of LDCs were investigated. The mRNA expression for CD40, CD80 and CD86 on LDCs were investigated by RT-PCR. As it is shown in Figure5, ginsenoside Rh₂ has up-regulated the expression of CD40 and CD80 mRNA on LDCs, while the expression of CD86 mRNA was not affected drastically.

In the presence and absence of ginsenoside Rh₂, the endocytic activity of LDCs was detected by FCM. The results were shown in Figure6. The average fluorescence intensity of engulfed FITC-OVA in LDCs treated without ginsenoside Rh₂ was 10.22±1.34%. However, LDCs cultured with ginsenoside Rh₂ showed lower average fluorescence intensity as 9.01 ± 1.43%. The results indicate that endocytic activity of LDCs was suppressed by treatment of ginsenoside Rh₂.

Figure 3: Effects of ginsenoside Rh₂ on CXCR3 and CCR4 among CD4⁺ T cells. (Mean $\pm$ SD, N=3), *P<0.05 vs. control; ** P<0.01 vs. control.

4 Discussion

Ginsenoside Rh₂ has been proved to prevent allergic airway diseases. However, studies in the past have been stuck in the apparent phenomenon, such as release of inflammatory substances and organ damage [5-6]. As an important part of immune response, investigation related to the effects of ginsenoside Rh₂ on T cells regulation was necessary. In this work, we tried to clarify whether the anti-allergic effects of ginsenoside Rh₂ were realized by

regulation of Th1 and Th2 cells differentiation through LDCs.

Most allergic diseases like bronchial asthma, allergic rhinitis and atopic dermatitis are thought to be related with the Th1/Th2 immune imbalance [14-16]. As we all know, levels of IFN- γ and IL-4 are usually used to reflect the Th1/Th2 balance in allergy [17-19]. CXCR3 is a typical chemokine receptor expressed on Th1 cells while CCR4 is a typical chemokine receptor expressed on Th2 cells [20-23]. Thus, the levels of these cytokines and chemokine receptors could indicate the differentiation of T cells. According to the results of Th1 and Th2 regulation by ginsenoside Rh₂-treated LDCs, the mRNA expression for IFN- γ and CXCR3 was significantly increased. Although the decrease of mRNA expression for IL-4 and CCR4 did not reach the statistical significance and the Th1/Th2 immune balance was affected without a doubt. The results of FCM based on CD4⁺ T cells were consistent with the results above. Further-more, ginsenoside Rh₂-treated LDCs also increased the production of IFN- γ significantly and decreased the production of IL-4 slightly in the culture supernatants of CD3⁺/CD28⁺ T lymphocytes. By combining the experimental results of RT-PCR, FCM and ELISA assays, we can draw a conclusion that ginsenoside Rh₂-treated LDCs could induce the differentiation of Th1 cells and regulate the Th1/Th2 immune balance toward a Th1-prone immune response.

DCs have a strong stimulating effect on T lymphocytes, and even a small number of DCs and very low levels of antigen can also induce an immune response to T lymphocytes, thereby initiating cellular immune response [24-25]. The signals from DCs cause T cells to differentiate

Figure 4: Effects of ginsenoside Rh₂ on IFN- γ and IL-4 in the culture supernatants of T lymph node cells. (Mean $\pm$ SD, N=3), *P<0.05 vs. control; **P<0.01 vs. control.

Figure 5: Effects of ginsenoside Rh₂ on mRNA expression for surface molecules CD40, CD80 and CD86 on LDCs. LDCs were pulsed with OVA for 18h in the absence or presence of ginsenoside Rh₂. Bands in A were quantified by densitometric analysis relative to β -actin. (Mean $\pm$ SD, N=3), *P<0.05 vs. control; **P<0.01 vs. control.

Figure 6: Effects of ginsenoside Rh₂ on endocytic activity of LDCs. Percentages of OVA positive cells among LDCs reflect the antigen uptake of LDCs. (Mean $\pm$ SD, N=3), *P<0.05 vs. control; **P<0.01 vs. control.

into different subtypes [26]. To clarify the mechanism further, cell surface molecules on LDCs and endocytic activity of LDCs were investigated. In the present work, LDCs were generated *in vitro* and used as the substitution of LCs. According to a literature report, LDCs obtained expressed Langerin and E-cadherin, which were thought to be typical LC surface markers [12]. Co-stimulatory molecules CD40, CD80 and CD86 are typical molecules on LCs, which are related to the endocytic ability of LCs [27-28]. The results of RT-PCR showed that ginsenoside

Rh₂ increased the expression of mRNA for co-stimulatory molecules CD40 and CD80. Although the expression of CD40, CD80 and CD86 seemed a little weak, they might augment upon maturation after injected *in vivo*. CD40 was reported to enhance Th1 differentiation by promoting IL-12 production from APCs [29]. Therefore, an increase of CD40 expression would explain why ginsenoside Rh₂-treated LDCs enhanced Th1 cell's development. In fact, some Notch ligands related to Th2 cell differentiation were investigated simultaneously. However, the expression was

very weak, so the data were not shown here. The expression of CD80 is usually associated with the maturation of DCs, which in turn affects the antigen uptake of DCs [30]. In this work, there was a significant increase in expression of CD80, and the uptake of FITC-OVA was decreased with a little relatively. The results of endocytic activity did not show significant differences due to the limited sample size. The results indicated that ginsenoside Rh₂ acted on LDCs and changed the functions of LDCs.

In conclusion, the present work indicates that Th1 and Th2 cells differentiation could be regulated by ginsenoside Rh₂ through LDCs *in vivo*. The regulation was achieved by changing the antigen presenting capacity, maturation and phagocytosis functions of LDCs *in vitro*. This work draws a new medicinal action of ginsenoside Rh₂ in allergic diseases.

Conflict of interest: Authors declare no conflict of interest.

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