Ginsenoside-Ro enhances cell proliferation and modulates Th1/Th2 cytokines production in murine splenocytes

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Abstract: Aim To study the effects of ginsenoside-Ro on cell proliferation and cytokine production in murine splenocytes. **Methods** The effect of ginsenoside-Ro on murine splenocytes proliferation was studied using [^{3}H]thymidine incorporation assay. Effects of ginsenoside-Ro on the production of cytokines interleukin-2 (IL-2), interferon-γ (IFN-γ) and interleukin-4 (IL-4) from murine splenocytes were detected by ELISA method. Effects of ginsenoside-Ro on mRNA level of Th1 cytokine IFN-γ and Th2 cytokine IL-4 were evaluated by reverse transcription polymerase chain reaction (RT-PCR) analysis. **Results** Ginsenoside-Ro showed no mitogenic effect on unstimulated murine splenocytes. It enhanced the proliferation of Con A-induced murine splenocytes and the production of IL-2 at concentrations of 1 - 10 μmol·L^{-1}. Moreover, ginsenoside-Ro increased the production and expression of Th2 cytokine IL-4 and decreased the production and expression of Th1 cytokine IFN-γ in Con A-induced murine splenocytes at concentrations of 2 - 10 μmol·L^{-1}. **Conclusion** Ginsenoside-Ro showed immunomodulatory effects by regulating the production and expression of Th1/Th2 cytokines in murine splenocytes.

Key words: ginsenoside-Ro; IL-2; IL-4; IFN-γ; splenocytes
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人参皂苷-Ro促进小鼠脾细胞增殖及调节小鼠脾细胞Th1/Th2细胞因子的产生

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摘要: 目的　研究人参皂苷-Ro对小鼠脾细胞增殖及细胞因子产生的影响。方法　[^{3}H]TdR参入法检测人参皂苷-Ro对小鼠脾淋巴细胞增殖的影响;酶联免疫吸附法检测人参皂苷-Ro对小鼠脾淋巴细胞产生细胞因子白介素-2、干扰素-γ和白介素-4的影响;逆转录聚合酶链式反应分析法研究人参皂苷-Ro对小鼠脾淋巴细胞中干扰素-γ、白介素-4 mRNA表达的影响。结果　人参皂苷-Ro在1 - 10 μmol·L^{-1}显著促进Con A诱导的小鼠脾淋巴细胞增殖及小鼠脾淋巴细胞白介素-2的产生;在2 - 10 μmol·L^{-1}促进Con A诱导的小鼠脾淋巴细胞产生和表达Th2细胞因子白介素-4, 而降低Con A诱导的小鼠脾淋巴细胞产生和表达Th1细胞因子干扰素-γ。结论　人参皂苷-Ro通过调节脾细胞内Th1型和Th2型细胞因子的转录和表达发挥免疫调节作用。

关键词: 人参皂苷-Ro; 白介素-2; 白介素-4; 干扰素-γ; 脾细胞

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The term ginseng means "the essence of man" in Chinese and *Panax ginseng* has been used as revitalizing agent by Chinese medical practitioners for

3 000 years[1]. The herb is still utilized in many Asian countries for a variety of conditions. In addition, there is evidence that the herb can stimulate cellular immune function. Thus, extracts of ginseng have augmented murine lymphocyte proliferation[2] and nature killer (NK) cell function[3] *in vitro*. One double-blinded, placebo-controlled study in normal human volunteers revealed an increase in neutrophil function, CD4 cell count and NK-function in individuals taking ginseng compared to those given placebo[4]. See DM *et al*[5] also reported that *Panax ginseng* enhanced cellular immune function of peripheral blood mononuclear cell (PBMC) both from normal individuals and patients with depressed cellular immunity.

Most pharmacological actions of ginseng are attributed to ginsenosides. To date, it has been reported that many kinds of ginsenosides play an important anti-inflammatory and immunomodulatory role by affecting cytokine production and lymphocyte proliferation[6-8]. Ginsenoside-Ro, an oleanane-type saponin has been well known for its anti-inflammation, and anti-platelet action[9,10]. However, the immunomodulatory activity of ginsenoside-Ro has not been reported till now.

The present study was conducted to investigate the immunomodulatory effect of ginsenoside-Ro on murine splenocytes and explore its related mechanisms of action.

Materials and methods

Reagents [^{3}H] Thymidine ([^{3}H] TdR) was purchased from Chinese Atomic Nucleus Research Institute. Concanavalin A (Con A) was purchased from Sigma. TRIzol reagent and M-MLV reverse transcriptase were from GIBCOBRL. Taq DNA polymerase was from TaKaRa. ELISA kits for murine recombined IL-2, IFN-γ, and IL-4 were from R&D systems.

Test compound Ginsenoside-Ro was isolated from roots of *P. ginseng* as described previously[11], the purity of which was more than 95% by HPLC analysis. Ginsenoside-Ro was prepared in stock solution 0.1 mol• L^{-1} with DMSO and stored at -20 ℃. Before using, the stock solution was diluted to appropriate concentrations in RPMI 1640.

Animals Male BALB/c mice, (17 ±1) g, 6-7 weeks old, were from the Experimental Animal Center, Chinese Academy of Medical Sciences and Peking Union Medical College (SPF, certificate No SCXK 11-00-0006). All animals were housed in groups under 12 h regime (lights on from 7:00 to 19:00) at (23 ±2) ℃ prior to the experiments, and were given standard laboratory chow and tap water *ad libitum*.

Preparation of murine splenocytes Mice were sacrificed by cervical dislocation, and spleens were removed aseptically. Spleens were placed in cold Hanks solution and teased apart with a pair of forceps and a needle. A single cell suspension from the teased tissue was obtained by passing it through a 200-mesh and hemolysed by the buffer solution containing 1 mmol• L^{-1} Tris-HCl and 1% NH_4Cl (pH 7.2). Cells were washed twice with RPMI 1640 medium and subsequently suspended in complete RPMI 1640 culture medium. Cell viability was determined by Trypan blue dye exclusion.

[^{3}H] Thymidine incorporation assay To determine the effect of ginsenoside-Ro on the proliferation of splenocytes, 2×10^6 cells• mL^{-1} splenocytes treated by different concentrations of ginsenoside-Ro with T lymphocyte mitogen Con A (1 μg• mL^{-1}) were cultured in flat bottom 96 well plates in a total volume of 200 μL/well. After incubation for 3 d at 37 ℃ in 95% humidity and 5% CO_2, cultures were pulsed with 3.7×10^4 μBq of [^{3}H]-thymidine/well, and maintained for an additional 18 h period prior to harvest. Incorporated [^{3}H]TdR was measured in a Beckman liquid scintillation beta counter. Results were expressed as the mean min^{-1} of [^{3}H] TdR incorporated in triplicate cultures. Each experiment was repeated at least five times.

Cytokine determination Splenocytes (2×10^6 cells• mL^{-1}) were treated with ginsenoside-Ro in presence of Con A (1 μg• mL^{-1}) for 48 h or 72 h, and cell supernatants were collected and levels of IL-2 (48 h), IFN-γ (72 h) and IL-4 (48 h) were measured by ELISA kits.

RT-PCR for cytokine gene expression The total RNA was extracted from 5×10^6 splenocytes stimulated by different concentrations of ginsenoside-Ro with Con A (1 μg• mL^{-1}) for 10 h. Cultured splenocytes were washed and the RNA was extracted with the TRIzol reagent according to the recommendation of the manufacturer. First strain cDNA was synthesized from equal amount of total RNA with M-MLV reverse transcriptase and random hexamer. Genes were amplified by PCR using sense and anti-sense primers of IFN-γ and IL-4, as described

before with some modifications[12]. IFN-γ and IL-4 PCR primers were as follows. IFN-γ: sense 5′-CGTCTTGG TTTTGCAGCTC-3′, anti-sense 5′-ACTCCTTTTCCTCT TCCTTA-3′; IL-4: sense 5′-ACGGCACAGAGCTATT GATG-3′, anti-sense 5′-ATGGTGGCCAGTACTACGA-3′; GAPDH: sense 5′-CATCACCATCTTCCAGGAGC G-3′; anti-sense 5′-GAGGGGCCATCCACAGTCTTC-3′. PCR annealing temperature IFN-γ: 53 ℃; IL-4: 65 ℃; GAPDH: 58 ℃. Semi-quantitative RT-PCR was performed using GAPDH as an internal control to normalize gene expression for the PCR templates. The PCR products were studied on a 1% agarose gel and the amplified bands were visualized after staining with ethidium bromide. The size of the amplified fragments was determined by comparison with a standard DNA marker.

Statistical analysis All values expressed as $\bar{x} \pm s$ were obtained from at least 3 separate observations performed in triplicate. Statistical analysis was carried out using one-way ANOVA, followed by multiple comparisons by Dunnett′s test using SPSS 11.5 for windows. A value of $P < 0.05$ was considered statistically significant.

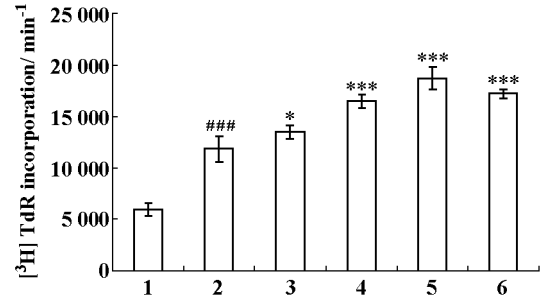
Results

1 Enhancement of Con A-induced cell proliferation by ginsenoside-Ro in murine splenocytes

Ginsenoside-Ro had no effect on unstimulated murine splenocytes (data not shown) but significantly increased ($P < 0.05$ or $P < 0.001$ *vs* control) Con A-induced murine splenocytes at concentrations of 1 - 10 μmol·L^{-1} with the maximal increase at 5 μmol·L^{-1} ($P < 0.05$ *vs* Con A group). As shown in Figure 1.

2 Effects of ginsenoside-Ro on cytokines production in murine splenocytes

To address the enhancement mechanism of ginsenoside-Ro on lymphocyte proliferation, we studied whether ginsenoside-Ro affects IL-2 production stimulated by mitogens, as IL-2 is the most potent cytokine to trigger lymphocyte proliferation. The data showed that ginsenoside-Ro increased the production of IL-2 in Con A-induced murine splenocytes at concentrations of 1 - 10 μmol·L^{-1} with the maximal increase at 5 μmol·L^{-1}. The production of IL-4 and IFN-γ, important cytokines of Th1/Th2 cell differentiation process were also evaluated. As shown in Table 1, ginsenoside-Ro increased the production of IL-4, while decreased the production of IFN-γ.



1: Untreated cells control; 2: Con A (1 μg·mL^{-1}); 3: Ginsenoside-Ro (1 μmol·L^{-1}) + Con A (1 μg·mL^{-1}); 4: Ginsenoside-Ro (2 μmol·L^{-1}) + Con A (1 μg·mL^{-1}); 5: Ginsenoside-Ro (5 μmol·L^{-1}) + Con A (1 μg·mL^{-1}); 6: Ginsenoside-Ro (10 μmol·L^{-1}) + Con A (1 μg·mL^{-1})

Figure 1　Effect of ginsenoside-Ro on the proliferation of murine splenocytes induced by Con A. Murine splenocytes were treated with different concentrations of ginsenoside-Ro in the presence of Con A (1 μg·mL^{-1}) for 72 h, and [^{3}H] TdR assay was performed to examine the proliferation. $n = 5$, $\bar{x} \pm s$. $^{###}P < 0.001$ *vs* control; $^{*}P < 0.05$, $^{***}P < 0.001$ *vs* Con A group

Table 1　Effects of ginsenoside-Ro on the production of IL-2, IL-4, and IFN-γ from murine splenocytes induced by Con A

Group / μmol·L^{-1}	IL-2 / pg·mL^{-1}	IL-4 / pg·mL^{-1}	IFN-γ / pg·mL^{-1}
Control	27 ± 6	55 ± 10	< 15
Con A 1 μg·mL^{-1}	1 613 ± 37$^{###}$	599 ± 20$^{###}$	1 064 ± 39$^{###}$
+ Ro 1	1 894 ± 21*	684 ± 54	998 ± 12
+ Ro 2	2 018 ± 105*	762 ± 66***	895 ± 9**
+ Ro 5	2 465 ± 223***	844 ± 38***	713 ± 37***
+ Ro 10	2 446 ± 204***	804 ± 54***	698 ± 43**

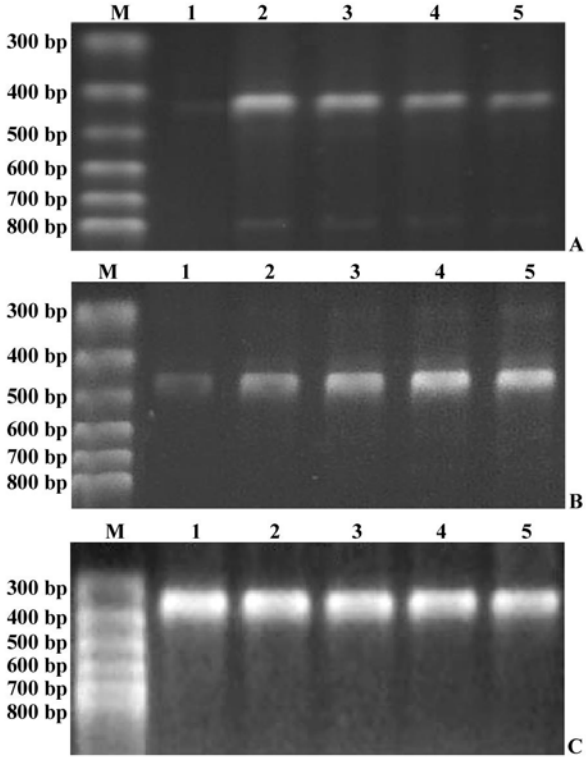
$n = 3$, $\bar{x} \pm s$. $^{###}P < 0.001$ *vs* control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* Con A alone

3 Effects of ginsenoside-Ro on the expression of IL-4 and IFN-γ mRNA in murine splenocytes

To determine if effects of ginsenoside-Ro on Th1 and Th2 cytokines are transcriptionally regulated, mRNA level of Th1-specific cytokine IFN-γ and Th2-specific cytokine IL-4 were measured using RT-PCR. The results were shown in Figure 2. Ginsenoside-Ro at concentrations of 2 - 10 μmol·L^{-1} increased the expression level of IL-4 and decreased that of IFN-γ in Con A-induced murine splenocytes.

Discussion

Lymphocytes are important immune cells and play a pivotal role in immune responses. These cells are able to produce many kinds of cytokines after



Lane M: DNA marker; Lane 1: Untreated cell control; Lane 2: Con A (1 μg• mL^{-1}); Lane 3: Ginsenoside-Ro (2 μmol• L^{-1}) + Con A (1 μg• mL^{-1}); Lane 4: Ginsenoside-Ro (5 μmol• L^{-1}) + Con A (1 μg• mL^{-1}); Lane 5: Ginsenoside-Ro (10 μmol• L^{-1}) + Con A (1 μg• mL^{-1})

Figure 2 mRNA levels of IFN-γ and IL-4 in murine splenocytes treated with different concentrations of ginsenoside-Ro in presence of Con A (1 μg• mL^{-1}). Murine splenocytes were incubated with ginsenoside-Ro at the concentrations of 2 - 10 μmol• L^{-1} in the presence of Con A (1 μg• mL^{-1}) for 10 h. Total RNA was extracted with TRIzol reagent and first cDNA was synthesized from equal amount of total RNA with M-MLV reverse transcriptase and random primers. mRNA levels of IFN-γ (A), IL-4 (B) and GAPDH (C) were detected by PCR using specific primers. The amplified cDNA were resolved on 1% (*w/v*) agarose gel and visualized by ethidium bromide

differentiation and activation. As the first step for T cell activation, lymphocytes should be proliferated by various signals such as bacterial products, including lipopolysaccharide or cytokines such as IL-2[8]. The immune response can be broadly categorized into cellular- or humoral-mediated response. The two types of immune responses are separately regulated by cytokines that control two general subsets of helper cells known as Th1 and Th2. IFN-γ is evaluated as representative Th1 cytokines mainly secreted from Th1 cells, while IL-4 is as key Th2 cytokines mainly secreted from Th2 cells[13]. Thus, cytokines such as IFN-γ, IL-2, and IL-4 are closely related to immune reaction.

The imbalance of Th1 /Th2 type responses plays an important role in the development and perpetuation of a number of immune disorders such as allergies, systemic lupus erythematous (SLE) and rheumatoid arthritis[14], and compounds that modulate expression of key cytokines that regulate immune responses would have clinical utility in treating patients with characteristically decreased cell-mediated immune responses or patients with chronic inflammatory and autoimmune diseases.

The present study demonstrated that ginsenoside-Ro not only enhanced murine splenocytes proliferation, but also increased IL-2 and IL-4 production and decreased IFN-γ production by regulation of cytokines gene expression in murine splenocytes, which may be responsible, at least partly, for the immunomodulatory effect of ginsenoside-Ro. These results suggest ginsenoside-Ro might be a desirable agent for the correction of Th1 dominant pathological disorders.

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