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Metabolism of ginsenoside Rg_1 by intestinal bacteria Ⅱ. Immunological activity of ginsenoside Rg_1 and Rh_1[1]

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KEY WORDS ginseng; saponins; cytokines; radioimmunoassay; reverse transcriptase polymerase chain reaction

ABSTRACT

AIM: To compare the effect of ginsenoside Rg_1 and its metabolite Rh_1 on proinflammatory cytokines and their mRNA expression by THP-1 cells. **METHODS**: Human peripheral blood mononuclear cells (PBMC) were incubated with Rg_1 and Rh_1 at concentrations of 0.1, 1, 10, and 100 mg/L, and the cell proliferation was measured 24 h after incubation. Radioimmunoassay (RIA) was used to detect the production of proinflammatory cytokines, TNFα, IL-1α, and IL-8. TNFα mRNA level was detected by reverse transcription polymerase chain reaction (RT-PCR) after administration of Rg_1 and Rh_1. **RESULTS**: Rg_1 and Rh_1 (at concentration of 0.1, 1, 10, 100 mg/L) had no effect on PBMC proliferation. Rh_1 1 mg/L could upregulate the productions of TNF (and IL-8 induced by lipopolysaccharides (LPS) 10 mg/L plus phorbol myristate acetate (PMA) 200 nmol/L, however, Rg_1 showed an inhibitory effect on TNFα production induced by LPS 100 mg/L. Rg_1 1 mg/L and Rh_1 100 mg/L enhanced the production of IL-1α level in THP-1 cells in the presence of LPS 10 mg/L. RT-PCR revealed that Rh_1 stimulated TNFα mRNA expression in suitable stimulatory conditions. **CONCLUSION**: Rg_1 and Rh_1 have different effects on the production of cytokines produced THP-1 cells stimulated by LPS and PMA.

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INTRODUCTION

Ginsenoside Rg_1 was converted into Rh_1 and 20 (S) protopanaxtriol (Ppt) by intestinal bacteria, and Rh_1 was detected in serum and urine of both rat and human after oral adiministration of Rg_1[1]. The pharmacological activity of Rg_1 possesses immune modulation and anti-aging activities[2-4]. In order to reveal the genuine active component on oral administration of Rg_1, comparison between Rg_1 and Rh_1 was carried out regarding the immunopharmacological aspects.

MATERIALS AND METHODS

Drugs Ginsenoside Rg_1, ginsenoside Rh_1 were purchased from Department of Natural Medical Chemistry of Norman Bethune University of Medical Sciences, China.

Cell lines Human myelomonocytic leukemic cell line, THP-1 (Monocyte, human, TIB202, ACTT, USA); Human peripheral blood mononuclear cells (PBMC).

Reagents Human recombinant cytokines (IL-1α, TNFα, IL-8) were kind gifts from Tsukuba Research Institute, Banyu Pharmaceutical Company (Tsukuba, Japan); All the rabbit polyclonal antibodies for above cytokines were prepared in our laboratory; ^{125}I was purchased from China Institute of Atomic Energy; new born calf serum (NCS) was purchased from Dalian Biological Reagents Factory (Dalian, China); lipopolysaccharides (LPS, *E coli* serotype 0111:B4) and phorbol myristate acetate (PMA) were purchased from Sigma (USA). RPMI-1640 medium, trypan blue and TRIZOL reagent were products of Gibco (NY USA); Primers for TNFα and β-actin cDNA amplification were purchased from *Life* Tech Oriental Company, Japan; diethyl pyrocarbonate

(DEPC), Taq DNA polymerase and RNase were purchased from Sigma. RPMI-1640 medium contained 10 % heat-inactived newborn bovine serum; 1 % *L*-glutamine 2 mmol/L; 1 % HEPES 10 mmol/L, penicillin 100 kU/L, and streptomycin 100 mg/L (pH 7.4).

RIA buffer Contained phosphate buffer 500 mL 0.5 mol/L 10 mL, BSA 10 % 12.5 mL, sodium azide 2 % 1.5 mL, NaCl 0.15 mol/L 465 mL.

PEG PEG (polyethylene glycol, MW:8000) solution contained PEG 6 % and goat anti-rabbit IgG 2 % in 10 mmol/L phosphate buffer.

THP-1 cell culture and determination of optimal cell density and concentration of LPS and PMA The cells were counted by hemocytometer and adjusted to cell densities ranging from 0.08×10^9 to 1.25×10^9 cells/L in RPMI-1640 medium containing 10 % NCS, then seeded in 96-well culture plates (180 μL/well), different doses of LPS (10 μL/well) and various concentrations of PMA (10 μL/well) were added into the THP-1 cell culture and then incubated at 37 ℃, 4 % CO_2, for 20 h. Production of IL-1α, TNFα, and IL-8 was quantitated using RIA. It was proved that the production of these three cytokines was maximal at 0.32×10^9 cells/L, therefore, this cell density was used in this study. The highest amount of IL-1α and TNFα were produced by LPS 10 mg/L and PMA 200 or 400 nmol/L, while IL-8 production reached maximal stage in the presence of LPS 10 mg/L and PMA 200 nmol/L.

Separation and incubation of human PBMC

1 Separation Heparin 0.1 mL was taken into a 50 mL injection syringe. The blood was obtained from three healthy adult volunteers, 40 mL/person. PBMC were separated by Ficoll-Hypaque density gradient solution, then washed 3 times with 0.9 % sodium chloride.

2 Incubation PBMC were suspended in RPMI-1640 medium containing human AB serum 2 % and adjusted to 5×10^9 cells/L, then seeded into 96-well culture plate (90 μL/well). The cell suspensions were disturbed and non-adherent cells were discarded 1 h after incubation. The adherent cells were washed with saline 3 times, and RPMI-1640 medium containing human AB serum 2 % and ginsenoside samples (Rg_1 and Rh_1, 10 μL/well) were added, followed by a 40-h incubation.

3 Measurement of cell number The adherent cells were detached by pipetting, stained by trypan blue, then counted under microscopic, magnified 100 times.

Effect of Rg_1 and Rh_1 on secretion of cytokines from THP-1 cells THP-1 cells were treated by different doses of Rg_1 and Rh_1 (10 μL/well). After incubation for 1 h, LPS 10 or 100 mg/L and PMA 200 nmol/L were added to the cell culture, then the cell incubation was continued for 20 h. The cells were collected into Eppendorf tubes after lysis by three freeze and thaw cycles, centrifuged at 1 200 × *g* for 10 min, then the concentration of IL-1α, IL-8, and TNFα were by quantitated RIA.

Cytokine quantitation by RIA Cytokine quantitation was performed according to previous reports[5,6].

Reverse transcription polymerase chain reaction (RT-PCR) THP-1 cell suspensions (0.32×10^9 cells/L) were added to the 6-well culture plate (6 mL/well) and cultured as previously described. The cells were collected after incubation for 0, 1, 3, 5, and 8 h, centrifuged at 500 × *g* for 10 min, then the supernatants were discarded. Total RNA was extracted by using TRIZOL (1 mL/tube). According to our previous results, the optimal incubation time for the highest production of TNFα mRNA was observed at 3 h. Therefore, various doses of Rg_1 and Rh_1 were added and the cells were cultured for 3 h to determine the effects of the compounds on expression of mRNA for TNFα by RT-PCR method[7].

1 Primers for TNFα TNFα primers were synthesized as below:

Primer 1: 5′ ATGAGCACTGAAAGCATGATC

Primer 2: 3′ TCACAGGGCAATGATCCCAAAGTAGACCTGCCC

2 Reaction conditions:

24 cycles: 94 ℃ 45 s, 60 ℃ 45 s, 72℃ 90 s

30 cycles: 94 ℃ 45 s, 60 ℃ 45 s, 72℃ 90 s

RESULTS

Effect of Rg_1 and Rh_1 on proliferation of human PBMC Rg_1 and Rh_1 had no obvious stimulatory or inhibitory effect on proliferation of human PBMC from three volunteers (data not shown).

Effect of Rg_1 and Rh_1 on the production of cytokines from THP-1 cell Under the stimulation of LPS 10 or 100 mg/L and PMA 200 nmol/L, Rg_1 had no obvious effect on production of TNFα, on the other hand, Rh_1 enhanced the production of TNFα stimulated by LPS 100 mg/L and PMA 200 nmol/L in a dose-dependent manner. However, Rh_1 1 mg/L induced 6.5 times higher production of TNFα than control in the presence of LPS 10 mg/L and PMA 200 nmol/L (Tab 1).

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Tab 1. Effect of Rg_1 and Rh_1 on TNFα production by THP-1 cells. $n = 4$ wells. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* the same concentration of Rg_1.

	TNFα production (μg/L)		
	+ LPS 100 (mg/L) + PMA 200 (nmol/L)	+ LPS 10 (mg/L) + PMA 200 (nmol/L)	Absence of stimulant
Control	107 ± 58	17 ± 4	0.29 ± 0.13
Rg_1 100 (mg/L)	30 ± 5^c	7.6 ± 1.7	0.52 ± 0.21
10	26 ± 15^c	7 ± 11	0.5 ± 0.3
1	10 ± 8^c	11 ± 7	0.8 ± 0.3
Rh_1 100 (mg/L)	290 ± 21^{cf}	19 ± 4	0.9 ± 0.5
10	151 ± 10^f	23 ± 19	1.8 ± 0.5
1	140 ± 95	110 ± 39^{cf}	8.5 ± 1.7

Tab 3. Effect of Rg_1 and Rh_1 on IL-8 production by THP-1 cells. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* the same concentration of Rg_1. $n = 4$ wells.

	IL-8α production (μg/L)		
	+ LPS 100 (mg/L) + PMA 200 (nmol/L)	+ LPS 10 (mg/L) + PMA 200 (nmol/L)	Absence of stimulant
Control	29 ± 16	19 ± 11	< 4
Rg_1 100 (mg/L)	28 ± 28	< 4	< 4
10	22 ± 22	9.5 ± 2.3	7 ± 4
1	22 ± 9	41 ± 39	< 4
Rh_1 100 (mg/L)	23 ± 21	9 ± 5	< 4
10	25 ± 20	10 ± 5	< 4
1	16 ± 5	710 ± 156^{cf}	< 4

Rg_1 and Rh_1 had no obvious effect on the production of IL-1α at higher doses of LPS 100 mg/L and PMA 200 nmol/L, but Rg_1 1 mg/L and Rh_1 100 mg/L increased the production of IL-1α significantly at lower doses of LPS 10 mg/L and PMA 200 nmol/L (Tab 2).

Tab 2. Effect of Rg_1 and Rh_1 on IL-1α production by THP-1 cells. $n = 4$ wells. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* the same concentration of Rg_1.

	IL-1α production (μg/L)		
	+ LPS 100 (mg/L) + PMA 200 (nmol/L)	+ LPS 10 (mg/L) + PMA 200 (nmol/L)	Absence of stimulant
Control	3.6 ± 1.2	2.3 ± 0.5	0.26 ± 0.14
Rg_1 100 (mg/L)	3.33 ± 0.12	1.4 ± 0.3	0.5 ± 0.3
10	6 ± 3	2.1 ± 0.5	0.188 ± 0.017
1	2.9 ± 0.5	8 ± 3^c	0.4 ± 0.3
Rh_1 100 (mg/L)	2.5 ± 0.7	8.5 ± 2.0^{cf}	0.21 ± 0.08
10	3.3 ± 1.6	4.6 ± 2.5	0.4 ± 0.03
1	4.8 ± 2.6	2.5 ± 1.5	0.4 ± 0.4

Rh_1 1 mg/L induced remarkably higher production of IL-8 than the control at LPS 10 mg/L and PMA 200 nmol/L (Tab 3).

Effect of Rg_1 and Rh_1 on the mRNA expression for TNFα by THP-1 cells Under the coherence of β-actin, effects of Rg_1 and Rh_1 on the production of TNFα mRNA produced by THP-1 cells were compared. After incubation for 0, 1, 3, 5, and 8 h, the highest production of TNFα mRNA was detected at 3 h. According to the result of RIA, the effects of Rg_1 and Rh_1 on the TNFα mRNA expression were compared, and several groups which had obvious influence on cytokine production from control were selected. Two concentrations of Rg_1 (100 and 1 mg/L) and Rh_1 (100 mg/L) were selected under the stimulation of LPS (100 mg/L) plus PMA (200 nmol/L). Rh_1 (1 mg/L) was also selected under the stimulation of LPS (10 mg/L) plus PMA (200 nmol/L) (Fig 1).

At 24 cycles of RT-PCR, there was no obvious DNA band on lane C or lane H, and on lane I a light DNA band appeared. At 30 cycles, DNA bands appeared in lane C and lane H, in lane I, cDNA amplification had reached a plateau stage. Therefore, we could compare the effect of Rg_1 and Rh_1 on original amount of cDNA at 24 cycles, where original mRNA amount for the cytokine was reflected.

According to the results of RIA, when the production of TNFα protein was inhibited by Rg_1 (Tab 1 LPS 100 mg/L), the production of TNFα mRNA (Fig 1 Lane A, B) was almost the same as that of control (Lane J). Rh_1 100 mg/L improved the production of TNFα protein under the stimulation of LPS 100 mg/L (Tab 1), but the amount of mRNA was reduced (Lane C). Rh_1 1 mg/L increased both TNFα protein production and its mRNA expression (Lane D).

From results mentioned above, we could find that there was no direct relation between mRNA amount and protein production at higher concentration of LPS 100 mg/L. On the other hand, there was a close relationship at low dose of LPS 10 mg/L. The ginsenoside sample control did not induce obvious expression of mRNA (lane E-H).

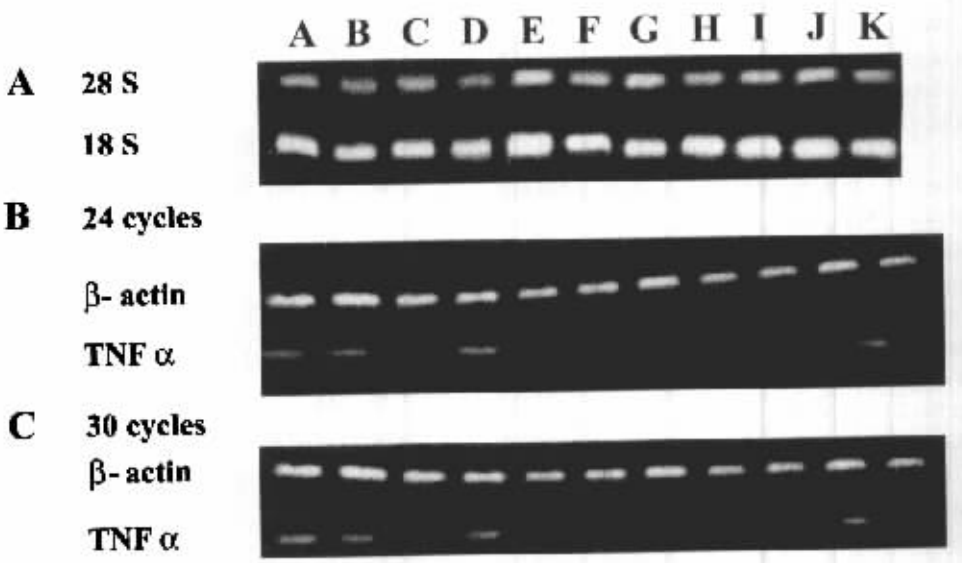

Fig 1. Effect of Rg_1 and Rh_1 on TNFα mRNA expression of THP-1 cells by semi-quantitative RT-PCR. Lane A: Rg_1 100 mg/L + LPS 100 mg/L + PMA 200 nmol/L. Lane B: Rg_1 1 mg/L + LPS 100 mg/L + PMA 200 nmol/L. Lane C: Rh_1 100 mg/L + LPS 100 mg/L + PMA 200 nmol/L. Lane D: Rh_1 1 mg/L + LPS 10 mg/L + PMA 200 nmol/L. Lane E: Rg_1 100 mg/L. Lane F: Rg_1 1 mg/L. Lane G: Rh_1 100 mg/L. Lane H: Rh_1 1 mg/L. Lane I: LPS 10 mg/L + PMA 200 nmol/L. Lane J: LPS 100 mg/L + PMA 200 nmol/L. Lane K: cell control

DISCUSSION

It has been reported that Rg_1 can enhance the immune function and anti-aging activity[2-4]. Proved by our previous study, Rg_1 was metabolized by rat and human intestinal bacteria. Among the metabolites, Rh_1 was detected in the blood and urine of humans[1].

THP-1 cell line has a kind of human myelomonocytic leukemic cell origin and it has the similar reaction as the human macrophage to a variety of stimulation[6,9], therefore, we observed the effect of ginsenosides on cytokine production by THP-1 cells. It was found that Rg_1 and Rh_1 had no toxic effect on PBMC culture at concentrations of 0.1, 1, 10, or 100 mg/L.

It has been reported that some drugs, especially traditional Chinese medicine (TCM), have bi-directional regulation on cytokine production. Low dose of TCM can enhance the release of some cytokines, while at higher dose they reduce the production.

Protein kinase C (PKC) and P38 protein kinase, mitogen-activated protein kinases (MAPK), play a pivotal role in a variety of cellular signal transduction pathways, especially, of induction of proinflammatory (TNFα and IL-1) cytokine production. The mechanism of the bi-directional regulation of Rh_1 on the production of the proinflammatory cytokines might be attributed to the activities of these two kinases[10-12]. The relationship between them remains to be elucidated.

In this study, it was proved that Rh_1 1 mg/L could stimulate THP-1 cell to produce TNFα on the stimulation with low dose LPS. However, Rh_1 at high concentration 100 mg/L could inhibit the proliferation of three tumor cell lines (data not shown). Therefore, whether there is a direct relationship between TNF production and reduced tumor cell proliferation waits further studies. According to our results, Rh_1 1 mg/L can increase the production of IL-8 induced by LPS 10 mg/L. However, Rg_1 1, 10, and 100 mg/L and Rh_1 10, 100 mg/L have no significant effect on the production of IL-8.

The results revealed that the prodrug Rg_1 and metabolite Rh_1 absorbed into serum showed different immuno-pharmacological activities *in vitro*, attributed to their different chemical structures. Rh_1 at suitable concentration can induce production of some proinflammatory cytokines on stimulation while Rg_1 shows no significant effect. Whether the results can be consistent with *in vivo* research still need to be further studied.

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REFERENCE

1 Wang Y, Wang BX, Liu TH, Wang W. Studies on metabolism of ginsenoside Rg_1 by intestinal bacteria (I). The absorbed components in serum after intestinal bacteria metabolism of ginsenoside Rg_1. Acta Pharm Sin 2000; 35: in press.

2 Liu M, Zhang JT. Immunoregulatory effects of ginsenoside Rg_1 in aged rats. Acta Pharm Sin 1995; 30: 818 - 23.

3 Liu M, Zhang JT. Studies on the mechanism of immunoregulatory effects of Ginsenoside Rg_1 in aged rats. Acta Pharm Sin 1996; 31: 95 - 100.

4 Li JQ, Zhang XG, Zhang JT. Study on the anti-apoptotic mechanism of ginsenoside Rg_1 in cultured cortical neurons. Acta Pharm Sin 1997; 32: 406 - 10.

5 Van der Meer JWM, Enders S, Lonnemann G, Cannon JG, Ikejima T, Okusawa S, *et al*. Concentration of immunoreactive human tumor necrosis factor alpha produced by human mononuclear cells *in vitro*. J Leukocyte Biol 1988; 43: 216 - 23.

6 Endres S, Ghorbani R, Lonnemann Y, Van der Meer JWM, Dinarello CA. Measurement of immunoreactive interleukin-1 beta from human mononuclear cells: optimization of recovery, intrasubject consistency, and comparison with interleukin-1 alpha and tumor necrosis factor. Clin Immunol Immunopathol 1988; 49: 424 - 38.

7 Yabe T, Yamada H. Induction mechanism of nerve growth factor synthesis by Kami-nutan-to; Role of cyclic AMP and c-*fos* mRNA accumulation. Phytomedicine 1997; 4: 191 - 8.

8 Grey ST, Csizmadia V, Hancock WW. Differential effect of tumor necrosis factor-alpha on thrombomodulin gene expression by human monocytoid (THP-1) cell versus endothelial cells. Int J Hematol 1998; 67 : 53 - 62.

9 Baqui AA, Meiller TF, Chon JJ, Turng BF, Falkler WA Jr. Granulocyte-macrophage colony-stimulating factor amplification of interleukin-1 beta and tumor necrosis factor alpha production in THP-1 human monocytic cells stimulated with lipopolysaccharide of oral microorganisms. Clin Diagn Lab Immunol 1998; 5: 341 - 7.

10 Zeng N, Yang K, Shen YJ. Effect of Xue lian chong cao jing on the activity of NK cells and IL-2. Chin J Integrated Tradit West Med 1995; 2: 36 - 37.

11 Zhao KS, Ding LX, Kong HY, Tian SY, Jin BQ, Liu XS. Research on the enhancement of *Astragalus membranaceus* polysaccharide on TNF produced by human PBMC. Chin J Herbal Pharmacol Ther 1993; 13: 263 - 265.

12 Fu TH, Xie ZR, Deng BF. Effect of doxorubicin on IL-2 release and LAK activity of human fetal thymocytes. Chin Pharmacol Bull 1996; 12: 140 - 2.

人参皂苷 Rg_1 的肠内菌代谢Ⅱ. 人参皂苷 Rg_1 和 Rh_1 免疫活性[1]

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关键词 人参; 皂苷类; 细胞因子类; 放射免疫测定; 逆转录聚合酶链反应

目的: 比较人参皂苷 Rg_1 及其代谢产物 Rh_1 对细胞因子及其 mRNA 表达的影响. **方法**: 将 Rg_1 及 Rh_1 加入正常人外周血单核细胞(PBMC)培养 24 小时后, 计数细胞, 观察其对正常细胞增殖的影响. 用放射免疫法(RIA)观察 Rg_1 及 Rh_1 对人组织瘤细胞(THP-1)分泌与炎症有关的细胞因子(IL-1α, TNFα, IL-8)产生的影响, 用逆转录酶链式聚合反应(RT-PCR)方法, 检测 Rg_1 及 Rh_1 对 TNFα 的 mRNA 表达的影响. **结果**: Rg_1 及 Rh_1(0.1, 1, 10, 100 mg/L)对 PBMC 增殖无明显影响. 但在脂多糖 10 mg/L 和 PMA 200 nmol/L 存在下, Rh_1 的低浓度(1 mg/L)能促进 THP-1 细胞产生 TNFα 与 IL-8. 而 Rg_1 在 LPS 100 mg/L 时抑制 TNFα 的产生. Rg_1 1 mg/L 及 Rh_1 100 mg/L 均能促进 IL-1α 的产生. RT-PCR 实验结果表明, Rh_1 能显著促进 TNFα mRNA 的表达. **结论**: Rg_1 与 Rh_1 的免疫活性有所不同, 在有些方面甚至相反.

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