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Full Length Research Paper

Ginsenoside Rg1 decrease expression of TNF-α in rats with hyperlipidemia

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Proinflammatory cytokines, such as IL-6 and tumor necrosis factor-alpha (TNF-alpha), are suggested to have an important role in the process of hyperlipidemia. However, proinflammatory cytokines changes after using ginsenoside was little report. The aim of our study was to evaluate changes from tumor necrosis factor-alpha (TNF-α) of hyperlipidemia rats after using ginsenoside Rg1 treatment by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry technology. The results demonstrated that TNF-α was significantly down-regulated in Rg1 treatment group in comparison to the hyperlipidemia group on mRNA level ($P < 0.05$), and Rg1 group and control group had no significantly difference on mRNA level ($P < 0.05$). On protein level, TNF-α was also highly decreased in Rg1 treatment group in comparison to hyperlipidemia group. The present study provides improvement in understanding the molecular pathogenic mechanism of hyperlipidemia and development ginsenoside Rg1 as effective treatment drugs.

Key words: Hyperlipidemia, Ginsenoside Rg1, tumor necrosis factor-alpha (TNF-α), reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), immunohistochemistry.

INTRODUCTION

Hyperlipidemia is commonly diagnosed each year, which is a strong correlation between hypercholesterolemic and coronary heart disease (Kota et al., 2005; Eckardstein et al., 2005). Lowering lipid levels has been shown to decrease the risk of coronary artery disease and to help prevent additional coronary events. It has been suggested that immune mechanisms, such as inflammatory mediators induced by the lipopolysaccharide (LPS)-cell wall component of Gram-negative microorganisms, play an important role in the development of hyperlipidemia (Plutzky, 2001; Takahashi et al., 2005). However, the details molecular was unclear, Therefore, it is necessary to improve our understanding of molecular mechanisms responsible for hyperlipidemia development, progression, and metastasis, and in turn, develop novel strategies for the early detection, prevention, and treatment of hyperlipidemia.

Proinflammatory cytokines such as tumor necrosis factor-a (TNF) and interleukin-1ß (IL-1) are considered to be the main mediators of LPS toxicity (Lopes-Virella et al., 1993). Reports investigating the effect of ginsenosides on cytokine production capacity *in vitro* demonstrated a ginsenosides effect on the production of several cytokines (Saikku et al., 1988). Ginsenoside Rg1, one of the richest and active ingredients in ginsenosides (Chen et al., 2008), have potential benefits on the cardiovascular system through various mechanisms, such as antioxidant, modifying vasomotor function, reducing platelet adhesion, altering autonomic neurotransmitters release, improving lipid profiles, and involving glucose metabolism (Zhou et al., 2004), however, the effect of ginsenosides Rg1 on cytokine production capacity *in vitro* had little report, thus, in the present study, we used rats with hyperlipidemia as study objective to evaluate the cytokine production after using

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ginsenosides Rg1 at both the protein and mRNA levels were studied as well, which might be a possible beneficial hyperlipidemia treatment for this therapy.

MATERIALS AND METHODS

Experiment rat and sample preparation

120 specific pathogen free (SPF) Wistar rats (6 weeks age, 180 to 200 g) were studied after 6 weeks of observation. Wistar rats were randomly divided into control group (n=40) and experiment group (n=80) after six weeks. Hyperlipidimia model was established by high cholesterol diet plus Vitamin D3 method in experiment group. Experiment group were randomly divided into ginsenosides Rg1 (purity >99%, Jilin University, China) group (100 μmol/L, 100 μl/day), and hyperlipidemia group (n=40 in each group). Liver tissue and serum were collected from rats after fed six weeks.

RNA preparation and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from new blood of rats using Blood RNA Isolation Kit (OMEGA, USA) according to the manufacturer's protocol and as described. Using mRNA as template, single-stranded cDNAs were generated by Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's directions. The TNF-α primer sequences were as follows: sense prime: 5′- ACAAGCCTGTAGCCCATGTT -3′; anti-sense prime: 5′-AAAGTAGACCTGCCCAGACT -3′. GAPDH (Applied Biosystems) served as the internal control. The PCR conditions were 94°C for 3 min, followed by 32 cycles of DNA amplification (40 s at 94°C, 45s at 61°C, and 1 min at 72°C) and 5 min incubation at 72°C. PCR products were separated by electrophoresis at a constant voltage (2 V/ cm) in a 1.2% (w/v) agarose gel. Images were captured using a Gel Print 2000i/VGA (Bio Image), and the integrated densities value (IDV) was analyzed with computerized image analysis system (Motic Images Advanced 3.2). All DNA manipulations were performed as described by Sambrook et al. (2001).

Enzyme-linked immunosorbent assay (ELISA) analysis

To detect expression of TNF-α in serum of rats, ELISA was conducted by using assay kit. The rats TNF-α ELISA kit (Leikai, china) is an in vitro ELISA for the quantitative measurement of rats TNF-α in serum, plasma, cell culture supernatants and urine. This assay employs an antibody specific for rats TNF-α coated on a 96-well plate. Standards and samples are pipetted into the wells and rats TNF-α present in a sample is bound to the wells by the immobilized antibody. The wells are washed, and then biotinylated anti-rats TNF-α antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of rats TNF-α bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm Ray Biotech, Inc.

Immunohistochemical analysis

To detect expression and localization of TNF-α in liver tissue, Immunohistochemical was performed. Frozen liver tissue samples obtained from rats with hyperlipidemia and control subjects were processed and cut at 4 μm for slide preparation. The sections were deparaffinized in xylen and rehydrated with graded alcohols. For heat-induced epitope retrieval, the sections were immersed in 0.01 M citrate buffer solution (pH 6.0) for 10 min. Then, they were cooled for 1 h at room temperature and washed in water and phosphate-buffered saline (PBS). Next, 0.3% hydrogen peroxide was applied to block endogenous peroxidase activity, and the sections were incubated with normal goat serum to reduce nonspecific binding. They were then incubated overnight at 4°C with primary rabbit polyclonal anti-rats antibody (1:100; Santa Cruz). Biotinylated goat anti-rabbit serum IgG was used as a secondary antibody. After the sections were washed 3 times in PBS, the sections were stained using the ABC Kit (Santa Cruz Biotechnology, Inc), and the color was developed with diaminobenzidine (DAB). Negative controls were conducted by exchange of primary antibody for PBS.

Statistics analysis

To calculate the statistical differences between the control and hyperlipidemia the statistical package SPSS13.0 (SPSS Incorporated, Chicago) was used for all analysis. One-way ANOVA followed by Bonferroni's post hoc test were utilized to determine the significant difference among multiple groups. Student's *t* test was used to determine the significance of differences between the groups. All values were expressed as mean ± SD. In general, p values less than 0.05 were considered statistically significant.

RESULTS

Semi-quantitative RT-PCR analysis of TNF-α expression

In order to detect the mRNA expression of TNF-α in rat with hyperlipidemia by ginsenoside Rg1 treatment, reverse transcription PCR was conducted. As shown in Figure 1, TNF-α mRNA levels in ginsenoside Rg1 group were significantly decreased after ginsenoside Rg1 treatment group compared to hyperlipidemia group ($P<0.05$), moreover, hyperlipidemia group were significantly higher than control group($P<0.05$), and ginsenoside Rg1 group and control group had no significantly difference. These results showed that ginsenoside Rg1 treatment could down-regulate TNF-α mRNA expression.

Serum levels of TNF-α

Serum levels of TNF-α were decreased in ginsenoside Rg1 group as compared with that of hyperlipidemia group. Meanwhile, the Serum levels of TNF-α in all hyperlipidemia treatment group was higher than that of healthy control, and ginsenoside Rg1 treatment group and control group were no significantly difference. All serum cytokine levels are illustrated in Table 1.

Immunohistochemistry analysis of TNF-α expression

The immunostainings were performed to detect TNF-α protein expression of using a multiheaded microscope. TNF-α was expressed in liver tissue with hyperlipidemia

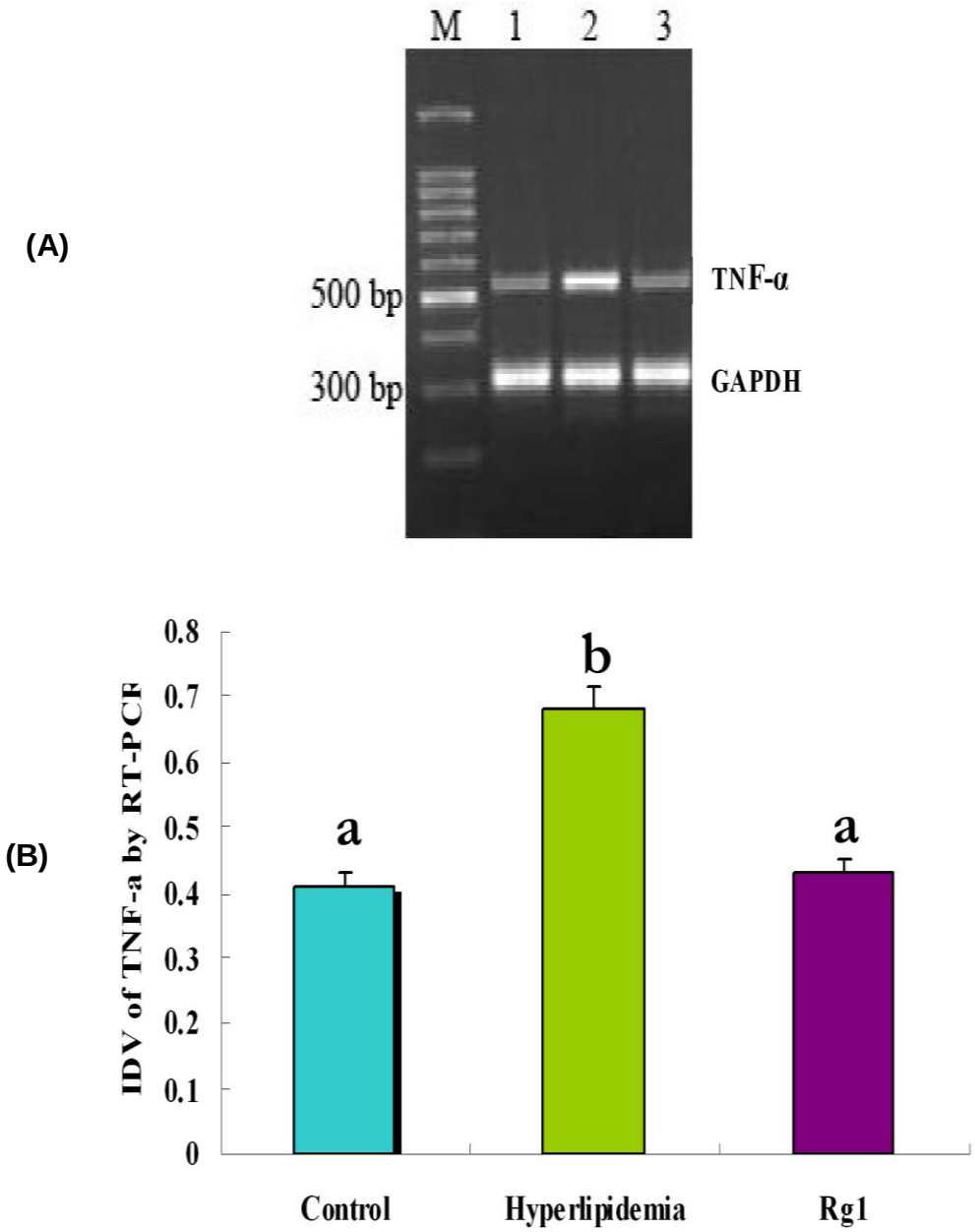

Figure 1. The mRNA expression of TNF-α at serum of rats from each group after Ginsenoside Rg1 treatment. A: The expression of TNF-α in different group by RT-PCR: M: Marker; 1: control group; 2: hyperlipidemia group.3, Ginsenoside Rg1 treatment group. B: The integrated density value of TNF-α mRNA at serum of rats from each group after Ginsenoside Rg1 treatment, different letter represent the significant difference at $p<0.05$.

Table 1. The TNF-α of serum at different group by ELESA. A: control group; B: hyperlipidemia group; C: Ginsenoside Rg1 treatment group; different letter represent the significant difference at $p<0.05$.

Group	TNF-α
Control group	18.46 ± 0.51^{a}
Hyperlipidemia group	42.25 ± 0.75^{b}
Ginsenoside Rg1 treatment	19.18 ± 0.57^{a}

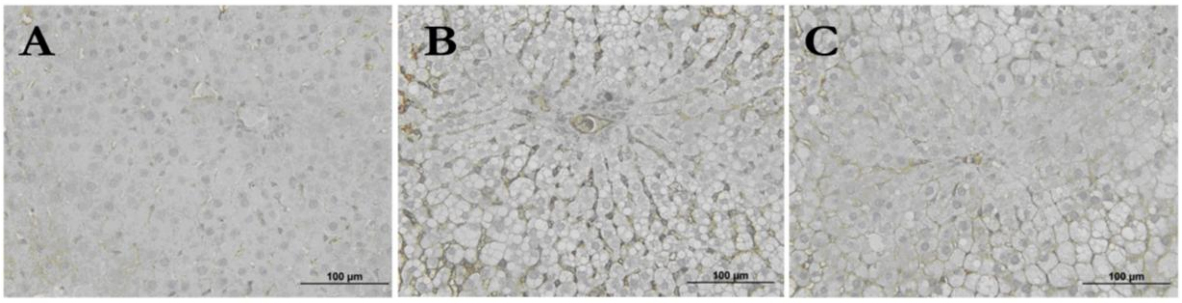

Figure 2. The expression of TNF-α by immunohistochemical staining at different group in liver tissue. A: control group; B: hyperlipidemia group. C: Ginsenoside Rg1 treatment group.

(Figure 2). Brown-yellow TNF-α staining was observed in the cell membrane and portal area (Figure 1B) for hyperlipidemia group, but TNF-α staining was scarce in portal area or cell membrane for ginsenoside Rg1 treatment group (Figure 1C). The control did not show non-specific staining (Figure 1A). These results indicated that TNF-α expression was decreased in ginsenoside Rg1 treatment group, which was compatible with result of TNF-α mRNA expression decreasion using Ginsenoside Rg1 treatment.

DISSCUSSION

Proinflammatory cytokines are suggested to have a role in LPS-mediated endothelial damage, in the uptake of oxidized LDL through increased expression of macrophage scavenger receptors (Li et al., 1995), and in regulating the plaque stability (Libby et al., 1995). Each of these may be important in the process of atherosclerosis (Ross et al., 1993). Nevertheless, it is important in which compartment the cytokines are produced. In plasma, TNF and IL-1 may induce endothelial cell damage (Van-Zee et al., 1992), whereas cytokines produced in the atherosclerotic plaque can stimulate cell proliferation and migration of smooth muscle cells and macrophages (Ross et al., 1993), and decrease the stability of the plaque (Libby et al., 1995).

In present study, we found that the serum levels of TNF-α in rat with hyperlipidemia group was higher than that of healthy control rats, which demonstrated that hyperlipidemia could cause proinflammatory cytokines up-regulated and lead to liver tissue damage.

Ginsenoside are one of the most important agents in the contemporary therapy of patients at high risk for cardiovascular disease. Administered to subjects with coronary artery disease, dyslipidemia and/or overt diabetes, ginsenoside have been found to diminish procoagulant activity by influencing different stages of the coagulation cascade (Lee et al., 2007; Santini et al., 2008; Chen et al., 2008). In some studies, ginsenoside have been shown to reduce fibrinogen levels (Calabres et al., 2003; Tonelli et al., 2005).

Ginsenoside Rg1, an important ginsenoside, can drastically improve the recovery of patients after a heart attack and low hyperlipidemia, however, Ginsenoside Rg1 *in vivo* effects exerted by hyperlipidemia lowering therapy on cytokine production had no details report. Hence, it is important to study change of cytokine production for hyperlipidemia treatment by Ginsenoside Rg1. In this study, we found that the expression level of TNF-α in Ginsenoside Rg1 group rats were significantly decreased ($P<0.05$) compared with that in hyperlipidemia group, which was consistent with that hypocholesterolemic treatment did alter TNF production by monocytes *in vivo* (Li et al., 2003).

In conclusion, the present study demonstrated that TNF-α was significantly down-regulated in rats with hyperlipidemia after Ginsenoside Rg1 treatment in comparison to the hyperlipidemia rats, which demonstrated that Ginsenoside Rg1 could release anti-inflammatory cytokine and decrease TNF-α expression by some effective drug component. The current study provides an effective approach for studying the mechanism underlying the pathogenesis of hyperlipidemia and treating hyperlipidemia method.

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