Panax notoginseng Saponins Attenuate Atherogenesis Accelerated by Zymosan in Rabbits

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Panax notoginseng saponins (PNS) are highly valued traditional Chinese medicine. The effects of PNS (120 mg/kg, once daily administrated intragastrically (i.g.)) on atherosclerosis induced by a high-cholesterol diet and chronic inflammation, which was derived through zymosan (10 mg/kg, once every 2 d) administration intraperitoneally, were evaluated in rabbits for 8 weeks. A normal group, a simple high-fat diet group, and a zymosan plus high-cholesterol diet group (Zym) were used as controls. Typical pathologic changes associated with atherosclerosis in rabbits following induction by zymosan were alleviated by PNS treatment. After 2, 4, 6, and 8 weeks of treatment, PNS decreased the serum levels of total cholesterol, triglyceride, low-density lipoprotein cholesterol, interleukin-6 and C-reactive protein as well as increased high-density lipoprotein cholesterol level significantly in comparison with those in the Zym group, except for triglycerides at week 2. In addition, PNS treatment significantly decreased the mRNA expression levels of monocyte chemoattractant protein-1 and nuclear factor-kB/p65 in the aorta wall after 8 weeks of treatment compared with the Zym group. In conclusion, PNS attenuates atherogenesis through an antiinflammatory action and regulation of the blood lipid profile.

Key words atherosclerosis; inflammation; Panax notoginseng saponin; monocyte chemoattractant protein-1; nuclear transcription factor-kB

Atherosclerosis (AS) and its complications such as stroke, myocardial infarction, and peripheral vascular disease are still the major causes of morbidity and mortality world wide.1) Although circulating lipoproteins and triglyceride levels remain important factors in atherogenesis,2) these classical risk factors do not account for all cases, because many patients who experience a myocardial infarction have normal blood lipid levels.3,4) Most patients continue to have events even when treated successfully with cholesterol-lowering medications.5,6) Studies of populations and cohorts have clearly demonstrated that the patients with chronic inflammatory disease have a higher prevalence of subclinical atherosclerosis compared with controls.7,8) In addition, the increased cardiovascular morbidity and mortality in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) cannot be entirely explained by traditional risk factors, suggesting that the systemic inflammation that characterizes these diseases may accelerate AS.9—11) Accumulating evidence indicates that chronic inflammation of the arterial wall in specific locations is the process that causes atheroma lesion formation and, eventually, thrombosis.12—14) Therefore, in addition to lowering serum cholesterol levels, it is considered to be of benefit to maintain vascular homeostasis and prevent AS to inhibit inflammatory cytokines and other mediators.

Panax notoginseng (sanqi or tienchi in Chinese), the root of Panax notoginseng (Burk.) F. H. Chen, is a highly valued and important traditional Chinese medicine, belonging to the family Araliaceae. Panax notoginseng saponins (PNS) were reported to be the biologically active constituents responsible for the therapeutic action of this medicine. Various reagents of PNS are commercially available and widely applied clinically in China, meeting the criterion of “Pharmacopoeia of the People’s Republic of China 2005.”

PNS have been demonstrated to have extensive effects on the cardiovascular system, including, among others, inhibition of platelet aggregation, increasing blood flow through the coronary arteries, improving left ventricular diastolic function in hypertensive patients, protecting against damage resulting from myocardial ischemia, an antiinflammatory effect due to a reduction of intracellular free calcium levels in neutrophils, reducing myocardial oxygen consumption and an antiarrhythmic effect.15—19) However, there has not been a detailed investigation of PNS in the treatment of AS and the mechanisms involved.20) The present studies were designed to address the role of PNS in the progression of AS and to explore the possible relevant molecular mechanisms.

MATERIALS AND METHODS

Materials PNS were purchased from Kunming Research Institute of Botany, Chinese Academy of Sciences (Kunming, Yunnan province, China). Zymosan A was obtained from Sigma Chemical (St. Louis, MO, U.S.A.). A zymosan A suspension was prepared by high-frequency oscillation in liquid medical paraffin (10 mg/ml), with the suspension then disinfected in a 100 °C water bath for 80 min prior to storage at 4 °C for future use. Before use, the zymosan A suspension was warmed to 40 °C and vibrated at high frequency for 15 min. Sterility was verified by incubation on blood-agar culture plates.

Animals Male Japanese White rabbits (2 months old, weight 1.6—2.2 kg) were obtained from the Experimental Animal Center of the Third Military Medical University (Chongqing, P. R. China). The present study conforms to the “Guide for the Care and Use of Laboratory Animals” published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996; http://www.nap.edu/reading-room/books/labrats/index.html). The study protocol was approved by the Ethical Committee for Animal Experimentation of the Third Military Medical University.

Experimental Protocol After 1 week of adaptation, the
rabbits were randomly divided into four groups (n=10 in each group): (1) Normal control group rabbits were fed a normal diet as well as injected with 2 ml/kg of sterilized liquid medical paraffin, intraperitoneally (i.p.), once every 2 d. In addition to the normal control group, the other rabbits were all fed a high-cholesterol diet (normal diet supplemented with 0.5% cholesterol, 10% yolk powder, 5% pork lard), and treated with different placebo. (2) The high-cholesterol group was injected with 2 ml/kg of sterilized liquid medical paraffin, i.p., once every 2 d. (3) In the zymosan plus high-cholesterol diet (Zym) group, inflammation was induced by injection of 10 mg/kg, i.p., of zymosan A once every 2 d. (4) PNS-treated group was administered 10 mg/kg, i.p., of zymosan A once every 2 d and 120 mg/kg of PNS once daily by oral gavage. Drinking water and food intake were available ad libitum throughout the study. Treatment was continued for 8 weeks.

**Preparation of Serum and Tissue Samples** Before and at the end of weeks 2, 4, 6, and 8, peripheral blood was collected from the ear vein into 3.8% trisodium citrate (1/10 volume) after 12-h fasting; then plasma was obtained by centrifugation (15 min, 1200 g) and stored at −80 °C until analysis. Real-time PCR was performed as described previously. Briefly, total RNA was isolated from arteries using TriPure reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. The RNA samples were dissolved in nuclease-free water and treated with 5 U of DNase I (Takara, Shiga, Japan) for 30 min at 37 °C. The reaction was stopped by the addition of 25 mmol/l of ethylenediamine tetraacetic acid (EDTA) and 15-min incubation at 65 °C. The total RNA concentration was quantified by measuring absorbance at 260 nm. Total RNA (1 μg) was reverse-transcribed using AMV reverse transcriptase (Promega, Madison, WI, U.S.A.) at 42 °C for 1 h. The PCR primers used were designed by Premier 5.0 (Premier Biosoft International, Palo Alto, CA, U.S.A.) based on published nucleotide sequences for rabbit Monocyte chemotactic protein (MCP-1) (forward: 5'-AAT CAA CAG CAC CAA GTG TC-3'; reverse: 5'-TTT TTG TTC AGG TTG GCA AT-3', with an amplified product of 120 bp), rabbit Nuclear Factor (NF)-κB (forward: 5'-TCC GTT ACA AGT GCG AGG-3'; reverse: 5'-TCC CGT GTA GCC ATT GAT-3', with an amplified product of 104 bp), and rabbit β-actin (forward: 5'-CGT GCT GTC CCT GTA CGC CTC T-3'; reverse: 5'-CGC TTC TGT TTC CCG ATG ATG AT-3', with an amplified product of 348 bp). Each real-time PCR reaction was carried out in triplicate in a total volume of 20 μl with Quanti Tect SYBR Green PCR Master Mix (MJ Research, Waltham, MA, U.S.A.) under the following conditions: 5 min at 95 °C; 40 cycles at 95 °C for 10 s; annealing for 15 s (58 °C, 63 °C and 59.8 °C for MCP-1, NF-κB, and β-actin, respectively), 72 °C for 20 s; and 82.5 °C for 5 s (collecting fluorescence) using the ABI Prism 7700 sequence detection system (ABI, Oyster Bay, NY, U.S.A.). After amplification, melting curve analysis was performed by collecting fluorescence data while increasing the temperature from 72 to 99 °C over 135 s. The cycle threshold (Ct) values were normalized to the expression levels of β-actin.

**Statistical Analysis** Data are expressed as mean±S.D. The statistical significance of differences between group means was determined using one-way ANOVA. p<0.05 was considered significant.

**RESULTS**

**General Status** Rabbits in the normal control group were in good condition during the entire experimental period. However, coprorrhrea and infection in claws were observed in some rabbits in the other three groups due to the high-cholesterol diet in the middle of the experimental period. In addition, rabbits challenged with Zym were in poor physical condition, with torpor and fur, disorder, and were less active than the control groups. During the experimental period, rabbits in each group gained body weight, and this was especially true in high-cholesterol group, but there was no significant difference between groups at the same time points (data not shown).

**Macrosopic Observations** In normal controls, the sur-
face of endarterium was smooth and glossy. In the Zym group, extremely obvious and extensive plaques were formed, with foci and fragments even protruding from the surface of the endarterium. Nevertheless, there were only spots and lipid streaks on aortas from the high cholesterol and PNS group. The results indicate that the rabbits in the Zym group developed the widest lesions (48.14 ± 9.15%) which showed a dramatic difference from the cholesterol-fed rabbits (36.52 ± 6.45%) (p < 0.05). On the other hand, rabbits treated with PNS showed an obvious decrease (25.05 ± 4.81%), which was significantly different from the Zym group (p < 0.01) (Fig. 1).

Histopathologic Observation Staining with H&E revealed significant changes in the endothelium, foam cells, and vascular smooth muscle cells, representing typical atherosclerotic alterations. As shown in Fig. 2, all the cells and structure mentioned above were normal in the normal control group. In the Zym group, however, obvious thickening of the endarterium, denudation of endothelial cells, aggregation of foam cells and lymphocytes under endarterium, proliferation of vascular smooth cells, and collagen fibers were found. In the high-cholesterol group, the pathologic changes observed were less marked than in the Zym group. After treatment with PNS, only a portion of the endarterium was slightly thickened.

Effects of PNS on Serum Lipid Level There was no difference in serum lipid levels before treatment among all groups. After feeding with a high-cholesterol diet, TG, TC, and LDL-C levels were all increased, but the HDL-C level was decreased significantly compared with the normal control group (p < 0.01). In comparison with the high-cholesterol group, Zym treatment produced sequential increases in TG and showed more striking differences at weeks 4, 6, 8 (p < 0.05). Moreover, the LDL-C level in the Zym group increased and demonstrated differences at all time points (p < 0.05). However, the HDL-C level clearly decreased at week 4, 6 and 8 (p < 0.05 or 0.01). Compared with the Zym group, administration of PNS markedly decreased the level of TC and LDL-C at each time point (p < 0.05 or 0.01) as well as significantly increased the level of HDL-C at each time point (p < 0.05 or 0.01) (Fig. 3).

Effects of PNS on IL-6 and CRP Serum Levels As shown in Fig. 4a, the basal level of serum IL-6 was not significantly different among the groups. However, IL-6 levels increased gradually after feeding the high-cholesterol diet and exhibited a significant difference compared with that in the normal control group (p < 0.01). The Zym treatment group displayed a more striking increase at the end of weeks 2 (p < 0.05) and 4, 6, 8 (p < 0.01). The IL-6 level in the PNS group decreased significantly compared with that in the Zym group at all time points (p < 0.01). The CRP level increased significantly from the base line value in all high-cholesterol diet rabbits. Zym treatment resulted in a sequential and
marked increase at all time points \((p<0.01)\). However, PNS administration resulted in a sharp decrease \((p<0.01)\) in the CRP level in comparison with that in the Zym group (Fig. 4).

**DISCUSSION**

In recent years, there has been a surge in literature documenting the key role of inflammation and inflammatory factors in the pathogenesis of atherosclerosis. Elevated lev-
els of circulating inflammatory markers, such as CRP, tumor necrosis factor alpha (TNF-α), IL-6, and cellular adhesion molecules (CAM) are associated with an increased risk of cardiovascular events. Clinical evidence also indicates that inflammation can promote atherogenesis. Although the AS model accompanying inflammation in rats has been observed previously, in this study we evaluated the relative contribution of inflammatory stimuli and hypercholesterolemia to atherogenesis in the rabbit model. Rabbits are the most widely used animal model in AS research due to their sensitivity to the inducement of atheromatous lesions, and many researchers use this type of animal model to test the effectiveness of drugs because of their similarity to human fatty streaks. In addition, unlike rats, rabbits can provide sufficient aortic material for detailed study. Zymosan A was used as the inflammatory stimulus in the present study, which is a substance derived from the cell wall of the yeast Saccharomyces cerevisiae. It is composed of polysaccharide chains of various molecular weights, containing approximately 73% polysaccharides, 15% proteins, and 7% lipids and inorganic components. Intraperitoneal injection of zymosan A is widely used as a trigger of a wide range of inflammatory mediators, including proinflammatory cytokine, such as TNF, proinflammatory lipid mediators such as platelet-activating factor, and cyclooxygenase metabolites.

The present study showed that compared with rabbits that received a high-cholesterol diet alone, atherosclerotic plaque area and pathologic alteration in thoracic aorta was more striking in Zym-treated rabbits, and the concentration of IL-6 and CRP in peripheral blood were sequentially increased during atherogenesis. Although CRP is mainly produced by hepatocytes in response to IL-6 with synergistic enhancement of IL-1 and TNF, accumulating experimental data indicates that in patients with acute coronary syndromes, CRP is derived from nonhepatic organs, such as the heart and blood vessels. Among numerous circulating inflammatory markers implicated in atherogenesis, CRP has emerged as the most powerful signal of future cardiovascular risk, even in apparently healthy men. Furthermore, studies suggested that the concentration of CRP determined with traditional lipid screening may significantly improve cardiovascular risk prediction, particularly when LDL-C is low. Beyond its role as a predictor, CRP plays a pathogenic role in the atherosclerotic process, stimulates the production of IL-6 by endothelial cells, and increases the expression of MCP-1. All these indicate that systemic inflammation induced by zymosan A promotes the development of AS, which is in line with previous clinical investigations. Cardiovascular effects elicited by zymosan A, including impaired vascular function (vasoconstriction and relaxation), endothelial dysfunction, and macrophage activation, might be one important pathway promoting atherogenesis. A new appreciation of the role of inflammation could yield considerable new insights into the mechanism framework and innovative therapeutic strategies for AS.

Recently, there has been increased interest in herbal medicines and their safety and efficacy in many inflammatory diseases. Previous experiments in vitro indicated that the zymosan-induced formation of macrophage-foam cells is inhibited following the administration of PNS. In the present study, satisfactory effects of PNS on zymosan A challenged rabbits was observed, with decreased size of atherosclerotic plaque and alleviation of atherosclerotic pathologic changes in arteries seen. In aortic plaques from rabbits treated with PNS, the lipid content, number of lymphocytes, and foam cells under the endarterium, and proliferation of vascular smooth muscle cells in the vessel walls were reduced, accompanying a decrease in CRP and IL-6 in peripheral blood, which suggest that the effects of PNS at least partly resulted from its antiinflammatory activity. PNS could not only directly affect systemic inflammation, but also the inflammatory milieu of atheroma.

It was found that PNS protects the rabbit iliac artery against balloon endothelial denudation injury by promoting the regeneration of the endothelium, decreasing intimal thickness, and down regulating the expression of vascular endothelial growth factor and matrix metalloproteinase-2. PNS also inhibits the proliferation of cultured aortic smooth muscle cells stimulated by hypercholesterolaemic serum. Thus PNS provides a protective effect on the cardiovascular system. In the study, MCP-1 mRNA expression in arteries during atherogenesis was down regulated by PNS.

MCP-1 is classified as the C–C subfamily of chemokines and acts as a potent monocyte chemoattractant. MCP-1 was shown to be expressed on atheromatous lesions and to regulate the directed monocyte chemotaxis of firmly attached monocytes to the arterial intima through interaction with its ligand on monocytes, chemokine receptor-2 (CCR-2), which is a crucial element for foam cell formation. A recent study has demonstrated decreased monocyte recruitment and consequent impairment in LDL clearance in the CCR2−/− knockout (KO) mouse. When AS-prone mice such as apoE-KO or LDL receptor (LDLR)-KO mice were reared on with a diet deficient in MCP-1 or CCR2, lesion formation was reduced drastically. Thus regulating the expression of MCP-1 is crucially important for the initiation of a lesion in the arterial wall. The stimulation of MCP-1 mRNA and protein in vascular smooth muscle cells treated with certain proatherogenic lipoproteins via mitogen-activated protein kinase (MAPK) activation was reported.

Our studies also demonstrated the involvement of NF-κB in the actions of PNS. NF-κB is a ubiquitous transcription factor that, by regulating multiple inflammatory and immune gene expression, plays a significant role in the immune response. NF-κB activation in endothelial cells has been considered as a critical event in the pathogenesis of AS. A recent study has demonstrated the activation of NF-κB in cells within atherosclerotic lesions but not in cells of normal vessels. Furthermore, NF-κB activation was demonstrated in an arterial injury model. In endothelial cells, NF-κB regulates the inducible expression of genes encoding chemokines, adhesion molecules, and growth factors. Activated NF-κB may also modulate the endothelial cell production of chemotactic substances such as MCP-1. In the present study, the expression of NF-κB and its mRNA in arteries of rabbits challenged with zymosan A were strikingly elevated, while treatment with PNS significantly reduced their expression. NF-κB not only affects MCP-1 but also induces a coordinated upregulation of other proinflammatory cytokines, such as adhesion molecules and chemoattractants, which provides molecular linkers between inflammation and
atherogenesis.

In addition to the effects mentioned above, PNS also modulates lipid profile. Zymosan A challenge-induced lipid profiles were characterized by an increased concentration of serum TG and LDL-C, which was persistent and striking, as well as decreased HDL-C level. Recent studies have shown that inflammation can cause lipid metabolic disturbances that, in turn, can aggravate the inflammatory reaction. Lipid metabolism disorders and inflammation promote each other to create a vicious circle. Improvement of lipid metabolic disturbances is beneficial to the recovery from inflammation and, in turn, antiinflammatory effects are conducive to maintaining a balanced lipid metabolism. In this study, inflammation occurring in the liver and aortas caused by zymosan A clearly aggravated the vicious cycle. PNS administration blocked the cycle through modulation of blood lipids as well as anti-inflammatory activity.

Previous studies reported that PNS are a mixture of more than 20 dammarane-type saponins, including ginsenoside Rg1, Rg2, Rb1, Rb2, Rb3, Re, Rd, Re, Rh, F2 and notoginsenoside R1, R2, R3, R4, R6, Fa, Fc, Fe. However, which compounds from PNS extract correspond to the lipid-lowering effect and, in turn, antiinflammatory effects are conducive to protecting the rabbit aorta from AS, and antiinflammation and decreased HDL-C level. Recent studies have shown that inflammation can cause lipid metabolic disturbances well as decreased HDL-C level. Further research is required is to elucidate this.

In summary, our results show that PNS have the capacity to protect the rabbit aorta from AS, and antiinflammation and lipid modulation may be the potential mechanism. Furthermore, these two mechanisms not only work independently but also crosstalk with each other, and the complex link between them needs further research. Overall, this finding may shed light on the pharmacologic principle of the clinical use of PNS in the treatment of AS.

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