Panax Notoginseng Saponins Promote Endothelial Progenitor Cell Mobilization and Attenuate Atherosclerotic Lesions in Apolipoprotein E Knockout Mice

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Key Words
Atherosclerosis • Panax notoginseng saponins • Endothelial progenitor cell • Mobilization

Abstract
Background: Endothelial progenitor cells (EPCs) derived from the bone marrow (BM) play a key role in the homeostasis of vascular repair by enhanced reendothelialization. Panax notoginseng saponins (PNS), a highly valued traditional Chinese medicine, has been shown to reduce morbidity and mortality from coronary artery disease. The present research was designed to explore the contribution of progenitor cells to the progression of atherosclerotic plaques and the possible modulatory role of PNS in this process. Methods: PNS (60 or 120 mg/kg via intraperitoneal injection) was administered over 8 weeks in apolipoprotein E knockout mice on an atherogenic diet. The sizes and histochemical alteration of atherosclerotic lesions and numbers of EPCs in BM and peripheral blood were analyzed. The expression of chemokine stromal cell–derived factor 1α (SDF-1α) and its receptor, CXCR4, was monitored as well. Results: PNS significantly reduced the lesion area and intima-to-media ratio compared to vehicle treatment. PNS also augmented endothelialization and reduced the smooth muscle cell (SMCs) content of the lesions. The number of c-kit and sca-1 double-positive progenitor cells and flk-1 and sca-1 double-positive progenitor cells were significantly increased in the BM and the peripheral blood of the PNS-treated groups. PNS treatment increased the plasma levels of SDF-1α and SCF as well as the BM levels of matrix metalloproteinase-9 (MMP-9). Moreover, the mRNA levels of SDF-1α and protein levels of CXCR4 were both increased in the BM of mice treated with PNS, while SDF-1α expression decreased. Conclusion: PNS reduce the size of atherosclerotic plaques, and this effect appears to involve progenitor cell mobilization. SDF-1α–CXCR4 interactions and the possible modulatory role of PNS in this process may contribute to the increased progenitor cell mobilization.
Introduction

Atherosclerotic cardiovascular diseases are highly prevalent in Western populations and are the leading cause of death [1]. It is generally accepted that the progressive impairment of endothelial integrity and function constitutes the *primum movens* of the process, leading to atherosclerotic plaque formation. Therefore, to maintain the integrity of normal endothelium becomes an important therapeutic strategy aimed at reducing the high incidence of atherosclerotic vascular disease. In contrast to the traditional concept that postnatal tissue revascularization is achieved by neighboring endothelial replication, accumulating evidence suggests that endothelial progenitor cells (EPCs), a heterogeneous population of cells that can specifically differentiate into endothelial cells [2], play a key role in the homeostasis of vascular repair by enhanced reendothelialization [3-5]. EPCs have multiple origins, and bone marrow (BM) is the most defined source of circulating EPCs [6]. Several treatments cause a marked increase in the release of BM-derived EPCs into the periphery, a process termed mobilization, which may contribute to increased endothelial repair and reduced atherosclerosis development.

*Panax notoginseng* (sanqi or tienchi in Chinese), the root of *Panax notoginseng* (Burk.) F. H. Chen, is a highly valued and important traditional Chinese herb that belongs 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, such as *Panax notoginseng* saponins Injection (Xue Sai Tong Injection), freeze-drying powder of *Panax Notoginseng* Saponins for Injection are applied clinically in China and are well established to reduce morbidity and mortality from coronary artery disease [7, 8], meeting the criterion of the *Pharmacopoeia of the People’s Republic of China 2010*. In addition to the lipid-lowering effects associated with PNS, our previous studies on the anti-atherogenic effects of PNS have shown that it is capable of reducing vascular inflammation, modulating endothelial dysfunction, and decreasing the formation of foam cells via pleiotropic mechanisms [9-12]. However, the potential role of PNS on EPCs during atherogenesis has not been systematically evaluated. In the present study, we investigated the effects of PNS, administered daily over 8 weeks to apolipoprotein E knockout (apoE⁻/⁻) mice kept on atherogenic diet by assessing the size and composition of atherosclerotic lesions. The effect of PNS on EPCs contributing to the endothelialization of atherosclerotic lesions was examined. The expression of cytokines and their receptors known to be crucially involved in EPC mobilization was monitored as well.

Materials and Methods

*Chemical standardization of PNS*

A freeze-drying powder of *Panax Notoginseng* Saponins for Injection (without excipients; PNS content is 100%) was purchased from Kunming Pharmaceutical Corporation (Kunming, Yunnan province, China). Standard reagents, including notoginsenoside R1, ginsenoside Rg1 and ginsenoside Rb1 (purity>99%), were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The HPLC analyses of compounds in PNS were performed based on the method established by Zeng et al. [13] with some modification. In brief, the separation was carried out on an Elite LaChrom system (Hitachi, Tokyo, Japan) equipped with a Hypersil C18 column (5 μm; length, 200 mm; inside diameter, 4.6 mm, Interchim, Montlucon, France), and the column temperature was maintained at room temperature. A 20 μl sample was injected into the column and eluted with a constant flow rate of 1.0 ml/min. The mobile phase consisted of acetonitrile and H₂O (25:75, v/v), and a programmable UV detector (Model 526, ESA Inc, Chelmsford, MA) set at 203 nm was used for the analyses. The data acquisition was performed using the LaChrom Elite software (VWR, Darmstadt, Germany), and a standard curve was used to calculate the concentrations for samples based on the peak areas. As shown in Fig. 1, the notoginsenoside R1, ginsenoside Rg1 and ginsenoside Rb1 contents in the freeze-dried powder of Panax Notoginseng Saponins for Injection were 0.12 mg, 0.36 mg and 0.38 mg respectively.
Animal experiments

Male apoE\(^{-/-}\) mice (10 weeks of age) were obtained from the Experimental Animal Center of the Third Military Medical University (Chongqing, China). The present study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996; http://www.nap.edu/readingroom/books/labrats/index.html). All protocols were approved by the Ethical Committee for Animal Experimentation of the Third Military Medical University. After a 7-day adaptation period, apoE\(^{-/-}\) mice were fed an atherogenic high fat diet (normal diet supplemented with 0.5% cholesterol, 10% yolk powder, and 5% pork lard) and injected intraperitoneally (i.p.) daily with either a low dose (60 mg/kg; PNS(L); \(n=20\)) or high dose (120 mg/kg; PNS(H); \(n=20\)) of freeze-drying powder of Panax Notoginseng Saponins for Injection, or vehicle alone (0.9% NaCl; Control; \(n=20\)). Drinking water and food were available \textit{ad libitum} throughout the study. This treatment continued for 8 weeks. At the conclusion of the experiment, animals were fasted overnight and sacrificed under diethyl ether anesthesia for aorta, BM and blood analysis.

Histological and morphometric analysis of atherosclerotic lesions

The aorta was excised and immediately washed with saline, and the aortic root was cut into 0.5-cm sections, fixed in 10% buffered formalin. After 24 h, the sections were removed, embedded in paraffin, and performed by hematoxylin-eosin (H&E) staining and Weigert's staining, respectively. Histopathology alteration was observed by light microscopy and an automated computer based image analysis system (Image Pro Plus, version 4.5; Media Cybernetics, Silver Spring, MD) was applied to conduct the morphometric analysis.

Immunohistochemical analysis of atherosclerotic lesions

The paraffin-embedded arterial sections were immunostained with a polyclonal rabbit anti-\(\alpha\)-actin antibody (1:100; Abcam, Cambridge, MA, USA) and a polyclonal rabbit anti-vWF antibody (1:200; Abcam, Cambridge, MA, USA). Labeling was visualized using the streptavidin-biotin-peroxidase method plus diaminobenzidine (Dako, Japan) and counterstained with Mayer hematoxylin. The degree of plaque endothelialization was quantified by measuring the length of the vWF-positive endothelial cell layer, and the presence of SMCs in the vessel wall was quantitatively assessed by measuring the area that was immunopositive for \(\alpha\)-actin antigen within the internal elastic lamina using image analysis software (Image Pro Plus, version 4.5).
Flow cytometry analysis

BM was harvested by flushing the femurs and tibias with phosphate buffered saline. The blood samples were treated with a lysing solution (BD Bioscience, Heidelberg, Germany). Immunolabeling was performed by incubating cells together with directly conjugated mAbs (rat-anti mouse c-kit - phycoerythrin (PE), Sca-1-FITC, flk-1-PE (BD Biosciences, Heidelberg, Germany)) and their corresponding isotype-matched FITC or PE-conjugated rat immunoglobulins for 45 min at 4 °C in PBS/0.5% BSA. The double-stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), and the data were processed using Cell Quest software (Becton Dickinson, San Jose, CA). Each analysis included at least 10,000 events of mononuclear gated cells.

RNA preparation and real-time reverse transcription-PCR

Total RNA was isolated from the aorta and BM 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 DNase I (Takara, Shiga, Japan) for 30 min at 37 °C. The reaction was stopped by the addition of 25 mmol/L EDTA and a 15-min incubation at 65 °C. The total RNA concentration was quantified by measuring the absorbance at 260 nm. Total RNA (1 μg) was reverse transcribed using AMV reverse transcriptase (Promega, Madison, WI, USA) at 42 °C for 1 h. The PCR primers used were designed by Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA) based on published nucleotide sequences for mouse c-kit (forward: 5'-CTC GGA CAG CAC CAA GCA C-3'; reverse: 5'-GTC GGA GAC AGC AGC AAA GC-3'), mouse SDF-1α (forward: 5'-CTG TGC CCT TCA GAT TGT TGC-3'; reverse: 5'-TGG GCT GTT GTG CTT ACT TGT T-3'), mouse SCF (forward: 5'-AGG CTC ATC CGC TCT GTA GTT-3'; reverse: 5'-TAT GCC GTG GTA TTA TGC TGC-3) and mouse β-actin (forward: 5'-GCC TGT ATT CCC CTC CAT CG-3'; reverse: 5'-CCA GTT GGT AAC AAT GCC ATG T-3). Each real-time PCR reaction was carried out in triplicate in a total volume of 20 μl with the Quanti Tec SYBR Green PCR Master Mix (MJ Research, Waltham, MA, USA) using the following conditions: 5 min at 95 °C, 40 cycles at 95 °C for 10 s, annealing for 15 s (63 °C, 63 °C, 62 °C, 62 °C, for c-kit, SDF-1α, SCF and β-actin, respectively), 72 °C for 20 s, and 82.5 °C for 5 s (collecting fluorescence) with the ABI Prism 7700 sequence detection system (ABI, Oyster Bay, NY, USA). After amplification, a melting curve analysis was performed by collecting fluorescence data while increasing the temperature from 72 °C to 99 °C over 135 s. The Ct (cycle threshold) values were normalized to the expression levels of β-actin.

Enzyme-linked immunosorbent assay test

Blood plasma was obtained by the centrifugation of blood at 4°C and 1500×g for 20 min, and the BM was processed as previously described [14]. According to the manufacturer's instructions, an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, USA) was performed to determine SDF-1, SCF and MMP-9 levels in 100 μl of undiluted plasma or BM samples.

Western blot analysis

BM cells were resuspended in 100 μl of lysis buffer (Cell Signaling Technology, Boston, MA, USA), sonicated and cleared by centrifugation at 3000 rpm for 10 min at 4 °C. Equal amounts of protein (60 μg) were fractionated by electrophoresis on a 10% SDS polyacrylamide gel together with molecular weight standards and then transferred to nitrocellulose membranes (Roche, Indianapolis, IN, USA). The membranes were blocked in 3% non-fat dry milk (in TBS) prior to incubation with rabbit anti-SDF-1α antibody (1:1000; Abcam, Cambridge, MA, USA) or rabbit anti-CXCR4 antibody (1:1000; Abcam, Cambridge, MA, USA) overnight at 4 °C. The proteins were visualized with HRP-conjugated anti-rabbit IgG (1:2500; Dako, Denmark) followed by chemiluminescent detection and autoradiography (Fuji Medical X-Ray Film, Tokyo, Japan).

Statistical analysis

The data are expressed as the mean ± SD. The statistical significance of differences between the group means was determined by a one-way ANOVA (SPSS 13.0, Chicago, IL, USA). *P < 0.05 was considered significant.
**Results**

**PNS attenuates atherosclerotic lesion and increases endothelialisation**

After fed with an atherogenic diet for 8 weeks, the aortic root of apoE<sup>−/−</sup> mice exhibited typical atherosclerotic alteration, including an irregular lumen, impairment and...
desquamation of endothelial cells, thickening of the endarterium, aggregation of foam cells under endarterium, attenuation of tunica media, proliferation of SMA, and unclear even broken elastic fiber layers. In the 60 mg/kg PNS-treated group, the pathological changes observed were less serious than that in control group. In the 120 mg/kg PNS-treated group, however, only a portion of endarterium was slightly thickened. (Fig. 2A, B)

PNS dose-dependently reduced the atherosclerotic lesion area at the aortic root from $34580 \pm 5400 \, \mu m^2$ in the vehicle-treated group to $24380 \pm 4800 \, \mu m^2$ in the 60 mg/kg PNS-treated group ($P < 0.05$) and $17239 \pm 3347 \, \mu m^2$ in the 120 mg/kg PNS-treated group ($P < 0.01$). Similarly, the intima-to-media ratio was reduced from $0.55 \pm 0.091$ in the vehicle-treated group to $0.33 \pm 0.052$ in the 60 mg/kg PNS-treated group ($P < 0.01$) and $0.24 \pm 0.041$ in the 120 mg/kg PNS-treated group ($P < 0.01$) (Fig. 2 C,D)
The quantification of luminal vWF-immunopositive cells revealed that treatment with both 60 and 120 mg/kg PNS augmented plaque endothelialization ($P < 0.01$ vs. Control) (Fig. 3A). The lesions from 60 ($P < 0.05$ vs. Control) and 120 mg/kg ($P < 0.01$ vs. Control) PNS-treated mice were also characterized by reduced $\alpha$-actin-positive SMC content in aorta (Fig. 3B).

**PNS promotes the mobilization of progenitor cells from the bone marrow**

BM and peripheral blood were analyzed using flow cytometry to determine whether treatment with PNS affects the mobilization of BM-derived progenitor cells during atherogenesis. Because currently there is no conclusive marker for EPCs, it is believed that the
cells could be EPCs if they are double positive for both markers of progenitor and endothelial cells. As for mice, EPC surface marker combinations such as stem cell antigen-1 (sca-1) / VEGFR2 (flk-1 for rodents) and c-kit / flk-1 have been widely used to characterize EPCs [15-17]. In the present study, we found a significant increase in the proportion of c-kit and sca-1 double-positive cells in the 120 mg/kg PNS-treated group compared to the vehicle-treated group in both the BM (P < 0.01) (Fig. 4 A) and the peripheral blood (P < 0.01) (Fig. 4 C). However, this change was only detected in the BM (P < 0.05) of 60 mg/kg PNS-treated mice (Fig. 4 A). The proportion of flk-1 and sca-1 double-positive cells was significantly
increased in the 120 mg/kg PNS-treated mice compared to the vehicle-treated mice in both the BM \((P < 0.01)\) (Fig. 4 B) and the peripheral blood \((P < 0.01)\) (Fig. 4 D), while this increase was only detected in the BM \((P < 0.05)\) of the 60 mg/kg PNS-treated mice (Fig. 4 B).

**Messenger RNA Expressions of SDF-1α, SCF and c-kit in BM**

BM homogenates were analyzed for the mRNA expression of factors known to be involved in stem cell mobilization. The mRNA expression of SDF-1α (Fig. 5 A) and the SCF (Fig. 5 B) were both significantly increased in 60 and 120 mg/kg PNS-treated mice compared to vehicle-treated mice \((P < 0.01)\). However, the mRNA expression of c-kit in BM did not differ significantly between the treatment groups (Fig. 5 C).

**Expressions of CXCR4 and SDF-1α in BM**

Western blot analysis of the BM homogenates indicated an increase in the expression of CXCR4 in 120 mg/kg and 60 mg/kg PNS-treated mice \((P < 0.01 \text{ or } 0.05)\) (Fig. 6 A), whereas treatment with 120 mg/kg PNS appeared to decrease SDF-1α expression significantly by comparison of that of vehicle-treated mice \((P < 0.01)\) (Fig. 6 B).

**The level of SCF, SDF-1α and MMP-9 in plasma and BM**

Finally, the analyses of the SDF-1α, SCF and MMP-9 levels in plasma and BM revealed significantly elevated plasma SDF-1α (Fig. 7 A) and SCF (Fig. 7 B) levels in mice treated with 60 mg/kg \((P < 0.05)\) and 120 mg/kg PNS \((P < 0.01)\) compared with those in vehicle-treated mice. Plasma MMP-9 levels were similar and did not differ significantly between all the groups (Fig. 7 C). We also found the levels of SDF-1α, SCF and MMP-9 elevated significantly \((P < 0.01; \text{ Fig. 7 D, E, F})\) in the BM of 120 mg/kg PNS-treated mice, whereas treatment with 60 mg/kg PNS only elevated the levels of SCF and MMP-9 \((P < 0.05)\) compared to vehicle-treated mice.
Discussion

In the present study, we evaluated the effects of daily treatment with PNS on the size and composition of atherosclerotic plaques and the mobilization of BM-derived EPCs during atherosogenesis in hypercholesterolaemic apoE−/− mice. In accordance with our previous findings in rabbit and rat experiments [9, 18], satisfactory anti-atherogenic effect of PNS on apoE−/− mice was observed, displaying decreased size of aortic plaques, reduced ratio of intima-to-media, and alleviation of atherosclerotic pathological changes in arteries. Moreover, the quantitative analysis revealed that PNS treatment was associated with a reduction in intralesional SMCs, particularly in the high-dose PNS-treated mice. In this regard however, the effect of PNS on foam cells and SMCs might have contributed to its effect on plaque reduction [11, 19]. Interestingly, PNS treatment in the present study was found to enhance lesion endothelialization and rapid reendothelialization, which may effectively attenuate the aggregation of foam cells, proliferation of SMCs, and limit the extent of atherosclerotic plaque formation after vascular injury [20, 21]. All these suggested that effect of PNS at least partly resulted from enhancing lesion endothelialisation.

By performing flow cytometry analyses of blood and BM, we found that PNS treatment significantly increased the number of c-kit and sca-1 double-positive cells, the most primitive murine hematopoietic stem cells with multi-lineage potential, and the more differentiated fraction of flk-1 and sca-1 double-positive endothelial progenitor cells. Our findings also appear to show a pronounced effect of the high PNS dose compared to the low dose on the amount of EPCs in the BM and peripheral blood. The present data, namely the increase in EPC numbers, suggest a novel mechanism for PNS in enhancing progenitor mobilization from the BM. It is notable, however, that a single measurement of circulating EPC counts has prognostic implications, with low EPC levels associated with future cardiovascular events, such as stroke, acute coronary syndromes, heart failure, and even peripheral artery diseases [22, 23]. Accordingly, increasing the number of circulating EPCs has been shown to improve the neovascularization of ischemic hindlimbs, accelerate blood flow in diabetic mice, and improve cardiac function [24-27]. Other parallel lines of evidence have advanced the idea that BM-derived EPCs might be a source of the cells responsible, at least in part, for endothelial repair during the development of atherosclerosis [28-30]. Although the presence of BM-derived EPCs in atherosclerotic lesions remains to be confirmed directly, it is suggested that the mobilization of BM-derived EPCs by PNS may significantly contribute to atherosclerotic lesion regression.

The regulation of EPC mobilization from BM is a highly complex and interdependent process. Stromal cell-derived factor-1 (SDF-1) has been the primary focus of attention among the key players. The predominant isoform, SDF-1α, and its receptor, CXC chemokine receptor 4 (CXCR4), which are expressed at high levels for both vascular and hematopoietic progenitor cells, are of great importance in steady-state homeostatic processes within BM. In the present study, the increase in EPCs following PNS treatment is at least equipotent compared with the SDF-1α/CXCR4 axis, which is known to promote EPC mobilization in vitro and after gene transfer in vivo [31-34]. Analysis of the molecular pathways revealed that PNS treatment profoundly increased plasma SDF-1α levels, and evidence supporting the increased levels of plasma SDF-1α were significantly correlated with an elevated number of circulating EPCs [35, 36]. Of note, the SDF-1α level within the BM after PNS treatment for 8 weeks was significantly reduced in our study, indicating a possible dependence between the local and systemic changes in the levels of SDF-1α. Furthermore, several studies have shown that a newly formed gradient of SDF-1α towards the blood circulation is sufficient to induce stem cell mobilization [37, 38]. Decreased BM SDF-1α was further confirmed by western blot analysis, whereas high mRNA expression of SDF-1α was clearly detected after high-dose PNS treatment, which led us to the hypothesis that SDF-1α may be degraded by proteolytic cleavage and various matrix metalloproteinase (MMPs), including MMP-2 and MMP-9 [39-41]. The reduction of SDF-1α concentrations within the BM may interfere with retention and facilitate the egress of cells, which may suggest that a high degradation of BM SDF-1α is
required for the egress of progenitors. Consistent with our data, another study has shown a reduction of BM SDF-1 during cyclophosphamide and G-CSF mobilization in mice [42].

SDF-1α may not only promote the transfer of hematopoietic stem cells from a quiescent to a proliferative niche by mechanisms involving the activation of MMP-9 but may also induce the secretion of MMP-9 in BM [2, 43]. MMP-9 was also shown to cleave the membrane-bound Kit ligand (mKitL) and induce the release of soluble Kit ligand (KitL; also known as stem cell factor, SCF). Subsequently, sKitL induces the release of more SDF-1α, enhancing the mobilization of CXCR4+ and c-Kit+ cells into circulation [44]. In this regard, the MMP-9 level in the BM as well as the SCF level in the plasma and BM were significantly unregulated in mice treated with PNS. On the other hand, effects of PNS on the mRNA expression levels of SCF but not c-kit, within the BM were detected. In addition, we observed that PNS induced the up-regulation of BM CXCR4 expression during atherogenesis. The up-regulation of CXCR4 may serve to increase the sensitivity of cells to lower SDF-1α signals, which could be a consequence of the potent collapse of SDF-1α concentrations within the BM or induced by cytokines, such as SCF [44]. The present study suggests the regulation of SDF-1α and CXCR4 in the BM as targets for PNS to mobilize the BM-derived EPCs. Accumulating evidence has indicated that the SDF-1α–CXCR4 pathway also regulates several crucial aspects during the recruitment of progenitor cells to sites of vascular injury [45]. In the present study, we found that PNS significantly up-regulates the expression of SDF-1α and increases the number of CXCR4-positive cells within the vessel wall (not shown). However, further research is required to elucidate these mechanisms.

In conclusion, our study reveals new mechanistic insights into the capacity of PNS to protect apoE−/− mice from atherosclerosis, as characterized by a higher degree of endothelialization. These involve BM-derived EPC mobilization, a major role played by SDF-1α–CXCR4 interactions, and the possibility that PNS may have contributed to increased progenitor cell mobilization. Overall, this finding may shed light on the pharmacologic principles of the clinical use of PNS in the treatment of atherosclerosis.

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