Phytomedicine 19 (2012) 1029–1034

Contents lists available at SciVerse ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Canonical Wnt signaling is required for *Panax notoginseng* saponin-mediated attenuation of the RANKL/OPG ratio in bone marrow stromal cells during osteogenic differentiation

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ARTICLE INFO

Keywords:
Panax notoginseng saponins
Bone marrow stromal cells
Wnt/β-catenin signaling pathway
Osteoprotegerin (OPG)
Receptor activator of NF-κB ligand (RANKL)

ABSTRACT

Panax notoginseng saponins (PNS) are known to regulate the osteogenic differentiation of bone marrow stromal cells (BMSCs). In the present study, we investigated whether PNS could promote the osteogenic differentiation of BMSCs through modulating the Wnt/β-catenin signaling pathways, which are implicated in BMSCs osteogenesis. We found that PNS enhanced the mRNA expression of OPG, β-catenin, and cyclin D1 while decreased the mRNA expression of RANKL and PPARγ2. The actions of PNS on BMSCs were reversed (or partially) by DKK-1, a classical inhibitor of Wnt/β-catenin signaling. These results suggest that PNS stimulating bone formation by promoting the proliferation and osteogenic differentiation of BMSCs, and could also protect the skeletal system by decreasing bone resorption through reduction of RANKL/OPG expression via Wnt/β-catenin signaling pathways.

Introduction

The adult skeleton undergoes continuous remodeling through tight coupling of opposing bone-resorbing osteoclasts and bone-forming osteoblasts. This balance is critical for maintenance of skeletal integrity, bone homeostasis, and calcium homeostasis. An imbalance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation can result in osteoporosis. Bone formation/resorption balance is governed by a wide variety of cell signals, hormones, and growth factors that are essentially regulated by the receptor activator of NF-κB ligand (RANKL) and its two receptors, receptor activator of NF-κB (RANK) and osteoprotegerin (OPG) (Simonet et al. 1997; Lacey et al. 1998; Wu et al. 2009; Mulcahy et al. 2011). RANKL is highly expressed on the surface of bone marrow stromal cells (BMSCs) and preosteoblasts (Ikeda et al. 2001). When RANKL binds to RANK, osteoclast differentiation and function are enhanced (Hofbauer et al. 2000). OPG, produced by BMSCs and osteoblasts, is a soluble decoy receptor to inhibit RANK–RANKL-mediated osteoclastogenesis (Simonet et al. 1997; Arron and Choi 2000). Thus, changing the ratio of RANKL/OPG to alter bone turnover can potentially provide a mechanism for osteoanabolic therapy (Thomas et al. 2001). Panax notoginseng saponin is a potential anti-osteoporotic agent shown to enhance bone mineral density and bone strength, and prevent bone loss in ovariectomized rats (Shen et al. 2010). Based on recent studies demonstrating that Panax notoginseng saponins could stimulate bone marrow stromal cell osteogenesis by modulating gap junction intercellular communication activity (Li et al. 2010, 2011a,b), we hypothesize that Panax notoginseng saponins exert their effects by altering the RANKL/OPG ratio and associated underlying signaling pathway(s). In the present study, we investigated the signaling pathways responsible for the attenuating effect of Panax notoginseng saponins on the RANKL/OPG ratio in BMSCs during osteogenic differentiation.

Materials and methods

Reagents and antibodies

PNS (Lot No. 08105830) was provided from Wu-Zhou Pharmaceutical (Group) Co., Ltd. (Wuzhou, China). The contents of the main ingredients of PNS are notoginsenoside R1 10.0%,

Abbreviations: ALP, alkaline phosphatase; BMSCs, bone marrow stromal cells; DKK-1, Dickkopf-1; DMEM, Dulbecco's modified eagle medium; ELISA, enzyme-linked immunosorbent assays; FBS, fetal bovine serum; MTT, methyl thiazolyl tetrazolium assay; OIM, osteogenic induction medium; OPG, osteoprotegerin; PNPP, p-nitrophenyl phosphate; PNS, Panax notoginseng saponins; PPARγ2, peroxisome proliferator-activated receptor-γ2; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; TNFR family, tumor necrosis factor receptor family.

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http://dx.doi.org/10.1016/j.phymed.2012.06.002

ginsenoside Rg1 37.9%, ginsenoside Re 5.8%, ginsenoside Rb1 38.4%, and ginsenoside Rd 4.9%, respectively. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL Life Technologies (Gaithersburg, MD). Fetal bovine serum (FBS) was purchased from ThermoScientific (Beijing, China), TRIzol was purchased from Invitrogen (USA). MTT was purchased from Sangon Biotech (Shanghai, China). The Bradford Protein Assay Kit was purchased from Generay Biotech Co., Ltd. (Shanghai, China). Dexamethasone, ascorbic acid, β-glycerophosphate, and DMSO were purchased from Sigma (USA). RANKL and OPG ELISA kits were purchased from Boster Biological Technology, Ltd. (Wuhan, China).

Bone marrow stromal cell isolation, expansion, and culture

Four-week-old male Sprague-Dawley rats were purchased from the Experimental Animal Center of Shantou University Medical College (Shantou, China). All animal protocols were approved by the Animal Care and Use Committee of Shantou University Medical College. Primary BMSCs were collected as previously described (Lennon and Caplan 2006; Fu et al. 2008). Briefly, under aseptic conditions, the epiphyseal regions of the femora and tibia were removed and marrow plugs were flushed out using DMEM containing 10% (v/v) FBS. A suspension of single bone marrow cells was obtained by repeated aspiration. Cells were inoculated into 25 ml cell culture flasks at a density of 1×10^6 cells/ml and cultured in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin, and kept in a humidified 37 °C incubator under 5% CO_2 and 95% air. After 2 days, the culture medium and non-adherent cells were removed. The medium was changed two or three times a week. As the culture reached almost complete confluence, cells were subcultured. For experiments, cells at passage three to six were used.

Osteogenic differentiation protocol and treatment with PNS

For osteogenic differentiation, BMSCs were inoculated at approximately 1×10^4 cells/cm^2 on culture dishes, and induced in an osteogenic induction medium (OIM: DMEM, 0.1 μM dexamethasone, 50 μM ascorbic acid, and 10 mM sodium β-glycerophosphate) 24 h after plating. PNS was prepared as stock solutions in PBS at concentrations of 100 mg/ml, stored at −20 °C, and sterilized by filtration through a 0.22 μm filter. PNS was then added to the OIM to provide final concentrations of 0, 10, 50, and 100 μg/ml. Medium was changed two or three times every week. Differentiation was evaluated by measuring alkaline phosphatase (ALP) activity and mineralization.

Analysis of calcium deposits

Mineralization of BMSCs was determined using alizarin red S staining after culturing in basal medium (control), OIM, and OIM with PNS at final concentrations of 10, 50, 100, and 200 μg/ml for 21 days. Cells were fixed with ice-cold 70% (v/v) ethanol for 10 min, rinsed thoroughly with distilled water, then stained with 40 mM alizarin red S in deionized water (pH 4.2) for 10 min at room temperature. After removing alizarin red S solution by aspiration, cells were rinsed with PBS and dried at room temperature. Alizarin red S concentrations were calculated by comparison with an alizarin red S dye standard curve and expressed as nmol/ml (Guan et al. 2009).

Alkaline phosphatase (ALP) assay

Osteogenesis of BMSCs was induced by OIM with or without PNS for various times, then ALP activity was determined as previously described (Li et al. 2009). Briefly, cells were lysed by sonication in 0.5 ml of 10 nM Tris–HCl (pH 7.5) containing 0.1% Triton X-100. ALP activity in the lysates was determined using p-nitrophenyl phosphate (PNPP) as a substrate. Absorbance was measured by a microtiter plate reader (KHB Labsystems Wellscan K3, Finland) at 520 nm. Four replicates from each PNS concentration were analyzed. ALP activity was normalized to total protein measured with the Bradford protein assay method.

Cell proliferation assay (MTT)

Cells (1×10^4 per well) were plated in a 96-well plate and allowed to attach in basal medium for 24 h. Cells were then refed with either OIM or OIM containing 100 μg/ml PNS, cultured for 1–5 days, and harvested for MTT assay following the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using a Total RNA Kit (Tiangen, Beijing, China) and first strand cDNAs were generated by reverse transcription using oligo-dT as a primer. Template cDNA was then used in gene-specific PCR for rat β-catenin, cyclin D1, osteoprotegerin (OPG), peroxisome proliferator-activated receptor-γ2 (PPARγ2), and receptor-activated NF-κB ligand (RANKL). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as a housekeeping gene. Primer sequences (Generay Biotech Co., Ltd., Shanghai, China) and PCR conditions are shown in Table 1. PCR products were subjected to electrophoresis in 2% agarose gels and visualized with ethidium bromide. Relative expression was quantified densitometrically using a Gel Image Ver. 3.74 System (Tianon, Shanghai, China).

Enzyme-linked immunosorbent assays (ELISA)

Cell culture supernatants were collected and cell monolayers were lysed to extract cell-associated protein. RANKL and OPG protein levels present in cell culture supernatants and cell lysates were measured by commercially available ELISA kits according to the manufacturer's instructions. The total levels of RANKL and OPG produced by the cells were calculated as lysates plus supernatants. The results of the ELISA were normalized to total protein determined by the Bradford protein assay and expressed as ng/mg protein.

RANKL and OPG level analysis after addition of DKK-1

BMSCs induced to undergo osteogenesis were treated with 100 μg/ml *Panax notoginseng* saponin in the presence or absence of 0.2 μg/ml DKK-1 (Peprotech, USA). Cells were collected for RANKL/OPG analysis at Day 14.

Statistical analyses

Results are expressed as means ± SD for three or more independent experiments. Statistical significance was estimated by one-way ANOVA with Bonferroni's post test (multiple comparisons) and the Student–Newman–Keuls test (comparisons between two groups) as appropriate.

Results

PNS increases the mineralization of bone marrow stromal cells

We first investigated whether Panax notoginseng saponin could promote the mineralization of BMSCs *in vitro*. BMSCs maintained a fibroblast-like morphology (spindle-shaped) in basal medium (control). Following culture in OIM, BMSCs began to change from an elongated to a polygonal morphology by Day 4, with most of