Biochemical and Biophysical Research Communications 405 (2011) 256–261

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Psoralen stimulates osteoblast differentiation through activation of BMP signaling

De-Zhi Tang [a,b,c], Feng Yang [a,b], Zhou Yang [a,b], Jian Huang [c], Qi Shi [a,b], Di Chen [c,*], Yong-Jun Wang [a,b,*]

[a] Spine Research Institute, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, PR China
[b] Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, PR China
[c] Department of Orthopaedics, Center for Musculoskeletal Research, University of Rochester, Rochester, NY 14642, USA

ARTICLE INFO

Article history:
Received 1 January 2011
Available online 8 January 2011

Keywords:
Psoralen
BMP-2
BMP-4
Osteoblast
Osteoporosis

ABSTRACT

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. In order to improve the treatment of osteoporosis, identification of anabolic and orally available agents with minimal side effects is highly desirable. Psoralen is a coumarin-like derivative extracted from Chinese herbs, which have been used to treat bone diseases for thousands of years. However, the role of Psoralen in osteoblast function and the underlying molecular mechanisms remain poorly understood. In this study, we found that Psoralen promoted osteoblast differentiation in primary mouse calvarial osteoblasts in a dose-dependent manner, demonstrated by up-regulation of expressions of osteoblast-specific marker genes including type I collagen, osteocalcin and bone sialoprotein and enhancement of alkaline phosphatase activity. We further demonstrated that Psoralen up-regulated the expression of Bmp2 and Bmp4 genes, increased the protein level of phospho-Smad1/5/8, and activated BMP reporter (12xSBE-OC-Luc) activity in a dose-dependent manner, as well as enhanced the expression of Osx, the direct target gene of BMP signaling. Deletion of the *Bmp2* and *Bmp4* genes abolished the stimulatory effect of Psoralen on the expression of osteoblast marker genes, such as *Col1*, *Alp*, *Oc* and *Bsp*. Our results suggest that Psoralen acts through the activation of BMP signaling to promote osteoblast differentiation and demonstrate that Psoralen could be a potential anabolic agent to treat patients with bone loss-associated diseases such as osteoporosis.

1. Introduction

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with consequent increases in bone fragility and susceptibility to fracture [1]. There are about 44 million people in the United States affected by osteoporosis and low bone mass [2]. The clinical complications include fractures, disability and chronic pain. It is estimated that 54% of women aged 50 and older will sustain an osteoporotic fracture during their lifetime [3]. Further, approximately 24% of patients who experience a hip fracture will die within a year [4]. Osteoporosis has been a major public health threat for the elderly, particularly for postmenopausal women.

The current clinical treatment regimens for osteoporosis are anti-resorptive drugs, which maintain bone mass by inhibiting osteoclast function, such as estrogen, estrogen receptor analogues, calcitonin and bisphosphates. The effect of these drugs in increasing bone mass or recovering bone loss is relatively minor, probably no more than 2% per year [5]. The potential complications, including breast cancer, uterine bleeding and cardiovascular events, also restrict their usage for osteoporosis. It is desirable, therefore, to identify better and safe anabolic agents with low cyto-toxicity. Since new bone formation is primarily mediated by osteoblasts, agents that act by either increasing the osteoblast proliferation or inducing osteoblast differentiation can enhance bone formation [6,7].

Chinese herbal medicine has been widely used to prevent and treat diseases for thousands of years. *Psoralea corylifolia* fruit (Buguzhi) is one of the commonly used herbs in formulas that are prescribed for the treatment of fractures, bone and joint diseases. Psoralen (molecular formula: $C_3H_6O_3$, molecular weight: 186.16) is the main active ingredient extracted from the fruits of *P. corylifolia* L. It has been reported that Psoralen when applied with collagen matrix had stimulatory effect on local new bone formation in vivo [8]. However, the effects of Psoralen on osteoblast

Abbreviations: OP, steoporosis; BMP, bone morphogenetic protein; Smad, signaling mothers against decapentaplegic; Oc, osteocalcin; Col1, collagen type 1; Bsp, bone sialoprotein; Alp, alkaline phosphatase.

* Corresponding authors. Address: Spine Research Institute, Shanghai University of Traditional Chinese Medicine, 725 South Wan-Ping Road, Shanghai 200032, PR China (Y.-J Wang), Department of Orthopaedics, Center for Musculoskeletal Research, University of Rochester School of Medicine, Rochester, NY 14642, USA (D. Chen).

E-mail addresses: di_chen@urmc.rochester.edu (D. Chen), yjwang88@hotmail.com (Y.-J. Wang).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.01.021

function and the underlying molecular mechanisms remain poorly understood. In this study, we clarified the detailed molecular mechanisms of Psoralen on osteoblast function using primary osteoblast cells. The results demonstrated that Psoralen induced osteoblast differentiation through activation of BMP signaling in osteoblasts.

2. Materials and methods

2.1. Materials and reagents

Psoralen was obtained from the company of Sigma (St. Louis, MO) and the purity of this compound is more than 99%. Stock solutions of Psoralen were prepared in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) and stored at −20 °C.

2.2. Cell culture

Calvariae from neonatal mice were removed, washed with sterile PBS solution, digested in 0.2% collagenase A (Roche, Indianapolis, IN) for 1 h at 37 °C with shaking. Cells were isolated and cultured on 25 cm^2 flasks in a-Minimum Essential Medium (a-MEM; Gibco–BRL, GrandIsland, NY) supplemented with 10% fetal bovine serum (FBS; Gibco–BRL, GrandIsland, NY) and antibiotics (100 U/ml of penicillin–streptomycin; Invitrogen, Carlsbad, CA). Cell culture medium was replaced every 3 days. When osteoblast cells reached 80% confluence, they were harvested with 0.25% trypsin–EDTA solution. The cells were seeded in 96-well plates and 6-well plates at a density of 1×10^4 and 1×10^6 cells/well, respectively, and cultured in a humidified atmosphere of 5% CO_2 and 95% air, at 37 °C. All animal protocols were approved by the University Committee on Animal Resources of the University of Rochester.

2.3. Cell viability assay

Primary calvarial osteoblasts were seeded in 96-well plates at a density of 1×10^4 cells/well. After 2-day culture, cells were treated with Psoralen at concentrations of 0, 1, 10, 100 and 1000 μM for 48 h. The supernatant was then removed and 100 μl of Cell-Titer Blue buffer (Promega, Madison, WI) was added to each well. After incubation at 37 °C for 4 h, the cell viability assay was performed and the fluorescence activity was detected at excitation/emission wavelengths of 560/590 nm in a FLUO-STAR plate reader (Promega, Madison, WI).

2.4. Cell proliferation assay

Inhibition of cell proliferation by Psoralen was measured by 3-[4,5-dimethylthiazol]-2,5-diphenylterazolium bromide assay (MTT assay). Osteoblasts cells were plated in 96-well plates at a density of 1×10^4 cells/well. After 2-day culture, the cells were treated with Psoralen (0, 1, 10 and 100 μM) for 48 h. The supernatant was then removed and 20 μl of 0.5% MTT (Sigma, St. Louis, MO) was added to each well. After 4-h incubation at 37 °C, the supernatant was again removed and acid–ethanol (100 ml of 0.04 N HCl in ethanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. The plates were read on an ELISA reader (Multiskan EX; Thermo Electron Corporation, Waltham, MA) at a wavelength of 490 nm and a reference wavelength of 690 nm was also used.

2.5. Quantitative PCR (qPCR)

Osteoblasts cells were seeded in 6-well plates at a density of 1×10^6 cells/well. After 2-day culture, Cells were treated with Psoralen at concentrations of 0, 10, 50 and 100 μM for 48 h. Total RNA from the cells of each well was isolated respectively using RNeasy Mini Kit (Qiagen, Valencia, CA). One microgram of total RNA was reverse-transcribed separately into cDNA using the iScriptcDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative PCR (qPCR) analysis was carried out using Absolute QPCR SYBR Green Master Mix (Thermo Scientific, Waltham, MA) in a total volume of 20 μl of buffered solution containing 1 μl of the diluted (1:5) reverse transcription product in the presence of 10 pM of sense and antisense primers specific for the genes listed in Table 1. The cycling conditions were 15-min polymerase activation at 95 °C followed by 45 cycles, 95 °C for 20 s, 58 °C for 20 s and 72 °C for 30 s. All reactions were performed in triplicate.

2.6. ALP activity/staining assay

After cultured in 6-well plates with Psoralen (0, 1, 10 and 100 μM) for 48 h, osteoblast cells were lysed with Passive Lysis Buffer (Promega, Madison, WI). Protein concentrations were determined by Coomassie Protein Assay Kit (Bio-Rad, Hercules, CA) and ALP activities were analyzed with solutions containing 0.5 mg/ml of *p*-nitrophenyl-phosphate (Sigma, St. Louis, MO) in AMP buffer (0.5 M 2-methyl-1, 2-aminopropanol and 2 mM magnesium chloride, pH 10.3) at 37 °C for 15 min. The reaction was stopped by a solution containing 0.3 M sodium phosphate (pH 12.3). The collected medium was determined by reading the absorption value at 405 nm and the enzyme activity were normalized by the protein concentration. For the ALP staining, the cells were fixed in 10% Neutral Buffered Formalin for 15 min, washed and then incubated with ALP staining buffer, NBT-BCIP (Bio-Rad, Hercules, CA) at 37 °C for 30 min. All reactions were performed in triplicate independently.

2.7. Western blot analysis

Osteoblasts cells were cultured in 6-well plates with Psoralen at concentrations of 0, 1, 10 and 100 μM. After 2- and 48-h culture, cells lysates were, respectively, extracted with E-PER protein extraction reagents (Thermo Scientific, Waltham, MA) according to the manufacturer's protocol. Proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA) and the membrane was blocked with 5% non-fat milk in PBST solution for 1 h at room temperature (RT). After incubation with the primary antibody overnight at 4 °C and the HRP-conjugated secondary antibodies (Thermo Scientific, Waltham, MA) for 1 h at RT, the protein expression was detected using a SuperSignal West Femto Maximum Sensitivity Substrate Kit (Thermo Scientific, Waltham, MA).

The anti-β-actin polyclonal mouse antibody (Sigma, St. Louis, MO), anti-osterix polyclonal rabbit antibody (Abcam, Cambridge, MA), anti-phospho-Smad1(Ser463/465)/Smad5(Ser463/465)/Smad8 (Ser426/428) and anti-total-Smad1 polyclonal rabbit antibodies (Cell Signaling Technologies, Beverly, MA) were used as primary antibodies.

2.8. Dual luciferase assay

Osteoblast cells were seeded at a density of 1×10^4 cells/well in 96-well plates. The BMP signaling reporter construct, 12xSBE-OC-Luc, and the SV40-*Renilla* luciferase construct was co-transfected into the cells using FuGENE HD Transfection Reagent (Invitrogen, Carlsbad, CA). Cells were incubated with Psoralen at concentrations of 0, 10, 50 and 100 μM for 48 h. Cell lysates were then extracted and the relative amounts of Renilla and firefly luciferase were measured using a dual luciferase assay kit (Promega, Madison, WI). The *Renilla*/firefly luciferase ratio was calculated and normalized against the control.