European Journal of Pharmacology 721 (2013) 225–230

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Molecular and cellular pharmacology

Silibinin promotes osteoblast differentiation of human bone marrow stromal cells via bone morphogenetic protein signaling

Xiaozhou Ying [a,*,1], Liaojun Sun [a,1], Xiaowei Chen [b], Huazi Xu [a], Xiaoshan Guo [a], Hua Chen [a], Jianjun Hong [a], Shaowen Cheng [c], Lei Peng [c]

[a] Department of Orthopedic Surgery, The Second Affiliated Hospital of Wenzhou Medical College, 109 Xue Yuan xi Road, Wenzhou 325000, China
[b] Department of Infectious Diseases, The First Affiliated Hospital of Wenzhou Medical College, Wenzhou 325000, China
[c] Trauma Center of the Affiliated Hospital of Hainan Medical College, Haikou 570100, China

ARTICLE INFO

Article history:
Received 7 April 2013
Received in revised form
31 August 2013
Accepted 11 September 2013
Available online 25 September 2013

Keywords:
Silibinin
Human bone marrow stromal cells (hBMSCs)
Osteogenic
RUNX2
Bone morphogenetic protein

ABSTRACT

Silibinin is the major active constituent of the natural compound silymarin; several studies suggest that silibinin possesses antihepatotoxic properties and anticancer effects against carcinoma cells. However, no study has yet investigated the effect of silibinin on osteogenic differentiation of human bone marrow stem cells (hBMSCs). The aim of this study was to evaluate the effect of silibinin on osteogenic differentiation of hBMSCs. In this study, the hBMSCs were cultured in an osteogenic medium with 0, 1, 10 or 20 μmol/l silibinin respectively. hBMSCs viability was analyzed by cell number quantification assay and cells osteogenic differentiation was evaluated by alkaline phosphatas (ALP) activity assay, Von Kossa staining and real time-polymerase chain reaction (RT-PCR). We found that silibinin promoted ALP activity in hBMSCs without affecting their proliferation. The mineralization of hBMSCs was enhanced by treatment with silibinin. Silibinin also increased the mRNA expressions of Collagen type I (COL-I), ALP, Osteocalcin (OCN), Osterix, bone morphogenetic protein-2 (BMP-2) and Runt-related transcription factor 2 (RUNX2). The BMP antagonist noggin and its receptor kinase inhibitors dorsomorphin and LDN-193189 attenuated silibinin-promoted ALP activity. Furthermore, BMP-responsive and Runx2-responsive reporters were activated by silibinin treatment. These results indicate that silibinin enhances osteoblast differentiation probably by inducing the expressions of BMPs and activating BMP and RUNX2 pathways. Thus, silibinin may play an important therapeutic role in osteoporosis patients by improving osteogenic differentiation of BMSCs.

1. Introduction

Bone formation is characterized by proliferation and formation of a properly laid-out collagenous extracellular matrix. Once matrix synthesis begins, osteoblast marker genes are activated in a clear temporal sequence; alkaline phosphatase and the parathyroid hormone (PTH)/PTH-related protein receptor are induced at early times while osteopontin and osteocalcin appear somewhat later (Wang et al., 1999). Once these marker genes are induced, mineralization of the collagenous extracellular matrix follows. Bone mass is controlled by continuous bone remodeling through osteoblastic bone formation and osteoclastic bone resorption. The balance between bone formation and bone resorption must be delicately maintained to ensure the integrity of the skeletal system. An imbalance brought about by increased bone resorption over bone formation can lead to most adult skeletal diseases including osteoporosis (Lee et al., 2008; Rodan and Martin, 2000). Until recently, however, most therapies for skeletal disorders are focused mainly on the resorption side and far less attention has been paid to promoting bone formation (Rodan and Martin, 2000). Effective therapeutic strategy is urgently needed to have satisfactory bone building (anabolic) agents that stimulate new bone formation and correct the imbalance of trabecular microarchitecture characteristic of established osteoporosis (Berg et al., 2003; Ducy et al., 2000). Bone marrow stromal cells (BMSCs) are the precursor cells of osteoblast lineage, they play an important role in bone modeling and remodeling, where they give rise to the essential osteoblasts for bone formation (Pittenger et al., 1999). They also serve to maintain a balance between bone formation and resorption (Manolagas, 2000), agents which regulate bone formation act by increasing the proliferation and inducing differentiation of the BMSCs.

Silibinin is the major active constituent of the natural compound silymarin, the isomeric mixture of flavonolignans extracted from milk thistle (Silybum marianum) consisting of silibinin A and B, isosilibinin A and B, silicristin, and silidianin. Several animal

* Corresponding author. Tel.: +86 13676497718.
E-mail address: yingxiaozhou@sina.com (X. Ying).
[1] Contributed equally to this work.

0014-2999/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejphar.2013.09.031

studies have shown that silymarin and silibinin have hepatoprotective activity against toxins and oxidative attack, in which these compounds act as a bioactive antioxidant (Al-Anati et al., 2009; Ha et al., 2010). Silibinin exerts anti-inflammatory and anti-fibrogenic effects on human hepatic stellate cells isolated from human liver (Trappoliere et al., 2009). In addition, silibinin possesses anti-cancer effects against various human carcinoma cells (Singh and Agarwal, 2005). Recently, Kim et al. (2011, 2012) reported that silibinin has a potential to enhance osteoblastogenesis in murine MC3T3-E1 pre-osteoblastic cells. Some studies also found that silibinin has the potential to inhibit osteoclast formation by attenuating the downstream signaling cascades associated with receptor activator of nuclear factor-kB ligand (RANKL) or tumor necrosis factor-α (TNF-α) (Kavitha et al., 2012; Kim et al., 2009, 2011, 2013).

Although a number of studies have established the various role of silibinin in both in vitro and in vivo models, the effect of silibinin on osteogenic differentiation of BMSC has yet to be revealed. In the present study, we found that silibinin promotes osteogenic differentiation and mineralization via BMP signaling pathways accompanied by upregulation of RUNX2 and Osterix.

2. Materials and methods

2.1. Isolation and culture of hBMSCs

Bone marrow (5 ml) aspirates were obtained from the posterior iliac crest of three healthy volunteers, aged 35–60 years (2 males and 1 female). Full ethical consent was obtained from all patients and the study was granted ethical approval by the Medical Ethical Committee of the Second Affiliated Hospital, Wenzhou Medical College. Cells from each of the three donors were cultured independently and experiments performed in triplicates. Bone marrow mononuclear cells (BMMNC) were prepared as previously described (Gronthos and Simmons, 1995). The bone marrow was washed with growth culture medium (DMEM, Gibco) supplemented with 10% (V/V) fetal bovine serum (FBS, Gibco), 1% (V/V) penicillin and streptomycin (Gibco). The mixture of bone marrow and medium was gently added to the 50% Percoll solution (Sigma) and centrifuged at 3000 rpm for 30 min. The cell suspension was obtained between the layer of Percoll and the supernatant liquid layer. Cells were plated and then incubated in a humidified atmosphere of 5% CO_2 at 37 °C. They were passaged every 3–4 days using 0.25% (w/v) trypsin-EDTA solution (Gibco) and the third passage cells were used in our experiments. To examine the effects of silibinin on terminal differentiation, the hBMSCs were cultured in an osteogenic medium [growth culture medium supplemented with 10^{-8} M dexamethasone (Sigma), 50 μg/ml ascorbic acid (Sigma) and 5 mM β-glycerol phosphate (Sigma)] at an initial density of 1×10^4 cells/cm^2 with 0, 1, 10 or 20 μmol/l silibinin (Sigma). Silibinin was dissolved in dimethyl sulfoxide for live culture with cells; its final culture concentration was 0.5%. The medium was changed every four days during osteogenic differentiation.

2.2. Cell viability by cell counting kit-8 (CCK-8) assay

For the cell viability assays, hBMSCs were cultured in 96-well plates at 1×10^4 cells per well with growth culture medium. Twenty-four hours later, cells were switched to silibinin containing media for up to 14 days. The viability of hBMSCs was determined by the CCK-8 (Kumamoto, Japan) and measured by microplate reader scanning (ELx800,BioTek) at 450 nm as previously described elsewhere (Liu et al., 2010).

2.3. Alkaline phosphatase (ALP) assay

ALP staining was performed at day seven after cells were cultured in 24-well tissue culture plates at a cell density of 1×10^4. Cells which were cultured in osteogenic medium were rinsed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde. ALP substrate mixture (ALP staining kit, Sigma) was then added and incubated for ten minutes. The ALP activity was determined at 405 nm using p-nitrophenyl phosphate (pNPP) (Sigma) as the substrate, and the total protein contents were determined with the bicinchoninic acid (BCA) method, which was previously described in the literature (Sun et al., 2006).

2.4. Calcium deposit analysis

To assess cell-mediated calcium deposition, hBMSCs which were cultured in osteogenic medium were visualized by von Kossa staining, which was performed at day 21 after cells were cultured in 24-well tissue culture plates at a cell density of 1×10^4. Cells in the well plates were fixed in 4% paraformaldehyde, then stained with 1% silver nitrate, placed under a UV lamp for 20 min and rinsed with distilled water before treatment with 5% sodium thiosulfate for two minutes. Von Kossa-positive (black) deposits were observed after alcohol washing. Pixel values were measured using imaging software (Sante DICOM viewer, Santesoft) as previously described (Hakki et al., 2010; Yoo et al., 2011). Measured pixel values were normalized versus image background pixel values: Normalized pixel value = pixel value of Von Kossa staining/pixel value of background image.

2.5. Real-time polymerase chain reaction (PCR) assay

Real-time PCR was used to detect the expression of several osteogenic differentiation related marker genes (COL-I, ALP, OCN, Osterix, BMP-2, RUNX2.) at 4th and 7th day respectively. Total RNA was extracted using TriZol (Invitrogen) according to the manufacturer's instructions and quantified. Its concentration was determined spectrophotometrically at 260 nm (HP 8452A Diode Array Spectrophotometer). First strand complementary DNAs (cDNAs) were synthesized from 0.3 mg of the isolated RNA by oligo (deoxythymidine) using DyNamoTM cDNA Synthesis Kit (Fermentas), and used as templates for real-time PCR. The expression of mRNAs was determined quantitatively using DyNamo SYBR1 Green qPCR kit (Takara, Japan).The PCR was performed on a final volume of 25 ml containing 2 ml cDNA, 7.5 pmol of each primer, 1 ml ROX reference dye and 12.5 ml of SYBR Green Master mix (TIANGEN),with ABI Prism 7300 (Applied Biosystems, Foster City, CA, USA). The samples underwent 40 cycles consisting of the following steps: initial denaturation at 95 °C for 5 min, followed by a set cycle of denaturation at 94 °C for 10 s, different annealing temperatures for each pair of primers (ranging between 53 and 62 °C) for 10 s, extension at 72 °C for 27 s and a final elongation at 72 °C for 5 min. Fold increment of any assayed gene was calculated by normalizing its expression level to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, which was used as an internal control. Each gene analysis was performed in triplicate. Primer's sequences of the targeted genes were listed in Table 1.

2.6. Reporter gene assays

hBMSCs were seeded onto 48-well plates and allowed to reach approximately 80% confluency. The cells were then transfected with 0.1 μg of reporter plasmids containing BMP-responsive elements (12xGCCG-Luc) (Kusanagi et al., 2000) or Runx2-responsive elements (6xOSE2-Luc) (Ducy and Karsenty, 1995) and 0.01 μg of control reporter plasmids encoding TK-Renilla luciferase (Promega, Madison, WI) using Lipofectamine LTX (Invitrogen) according to the