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Anabolic activity of ursolic acid in bone: Stimulating osteoblast differentiation *in vitro* and inducing new bone formation *in vivo*

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ABSTRACT

In the field of osteoporosis, there has been growing interest in anabolic agents that enhance bone mass and improve bone architecture. In this study, we demonstrated that the ubiquitous plant triterpenoid, ursolic acid, enhances differentiation and mineralization of osteoblasts *in vitro*. We found that ursolic acid induced the expression of osteoblast-specific genes with the activation of mitogen-activated protein kinases, nuclear factor-κB, and activator protein-1. Additionally, noggin, an antagonist of bone morphogenetic proteins (BMPs), inhibited ursolic acid-induced osteoblast differentiation. Noggin also inhibited the activation of Smad and the induction of BMP-2 mRNA expression by ursolic acid in the late stage of osteoblast differentiation. Importantly, ursolic acid was shown to have bone-forming activity *in vivo* in a mouse calvarial bone formation model. A high proportion of positive immunostaining of BMP-2 was found in the nuclear region of woven bone formed by ursolic acid. These results suggested that ursolic acid has the anabolic potential to stimulate osteoblast differentiation and enhance new bone formation.

1. Introduction

Bone mass is maintained through the process of bone remodeling: old bone is resorbed by osteoclasts and new bone is formed by osteoblasts [1,2]. However, an imbalance in bone remodeling, caused by an increase in bone resorption over bone formation, may lead to skeletal diseases including osteoporosis. Pharmacological treatments available are divided into two categories, anti-resorptive agents including bisphosphonates, selective oestrogen receptor modulators, calcitonin, anti-receptor activator of NF-κB ligand (RANKL) antibody and hormone replacement therapy, and anabolic agents including parathyroid analogues, vitamin D analogues and fluoride. Anti-resorptive agents with beneficial effects on preventing bone loss have been the most prominent therapeutic advances, but they can only prevent further bone loss once the process has begun. Therefore, the development of anabolic agents that enhance bone mass and improve bone architecture has become a growing area of interest [3].

The bone-forming osteoblasts are derived from mesenchymal precursor cells; the maturation of pre-osteoblasts committed from mesenchymal precursor cells plays a role in the rebuilding of the resorbed bone by elaborating matrix that then becomes mineralized [1]. Committed pre-osteoblasts by signals for activation of osteogenic genes are recognizable near the bone surface by their proximity to surface osteoblasts and by histochemical detection of alkaline phosphatase (ALP) enzyme activity, one of the earliest markers of the osteoblast phenotype. The active mature osteoblast on the bone surface is distinguished by its morphological properties and the temporal expression of several non-collagenous enriched proteins such as osteocalcin (OCN) and osteopontin (OPN), providing markers of the mature osteoblast [4].

Ursolic acid (Fig. 1A) is one of many ubiquitous triterpenoids in medicinal herbs. It is found throughout the plant kingdom and constitutes an integral part of the human diet. Pharmacological effects of ursolic acid include anti-cancer [5], pro-differentiation [6], antiviral [7], and anti-invasion activities [8]. Recent approaches in the early stage of drug discovery and development include the development of therapeutic agents from natural substances, which retain the beneficial effects while minimizing the adverse side effects, but effects of ursolic acid on bone metabolism have not been determined. Therefore, in this study, we examined the effects of ursolic acid on the formation of bone using both *in vitro* and *in vivo* models.

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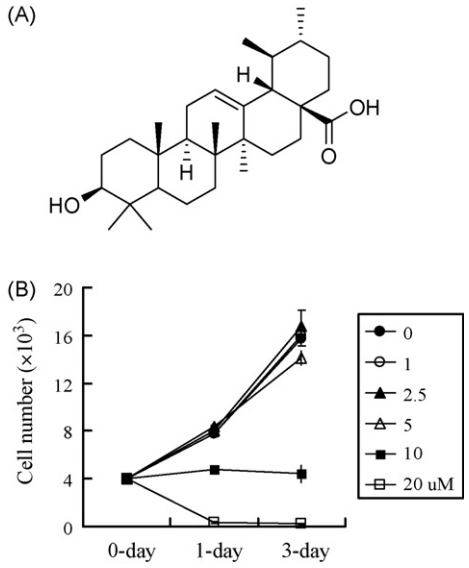

Fig. 1. (A) Chemical structure of ursolic acid. (B) Effect of ursolic acid on cell proliferation. Cells (1000 cells/well) were cultured in a 96-well plate for 1 day, then treated with ursolic acid, and incubated for 1 or 3 days.

2. Materials and methods

2.1. Cell culture

All materials for cell culture were purchased from HyClone (UT). Mouse osteoblastic MC3T3-E1 subclone 4 cells with high differentiation potential [9] were purchased from American Type Culture Collection and cultured in growth medium (GM; α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 mg/ml streptomycin). The medium was changed every 3 days and cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Recombinant murine noggin was purchased from Peprotech Inc. (Korea) and co-treated with ursolic acid in this study.

2.2. Cell viability assay

Cells were plated in 96-well plates in GM at a density of 1×10^3 cells/well. After 24 h, cells were incubated with ursolic acid for 1 or 3 days. Cell viability was then measured in triplicate with a Cell Counting Kit-8 (Dojindo Molecular Technologies, ML) according to the manufacturer's protocol. Absorbance was measured with a Wallac EnVision microplate reader (PerkinElmer, Finland) and measured absorbance was converted to the cell number with a standard curve.

2.3. Osteoblast differentiation

Cells (1.5×10^4 cells/well) were plated in 24-well plates and cultured in GM. After cells reached confluence, cells were cultured in differentiation medium (DM; GM with 50 μg/ml of ascorbic acid (Fluka, Germany), and 10 mM β-glycerophosphate (Sigma, MO)) in the absence or presence of ursolic acid. Medium was changed every 3 days.

2.4. Alkaline phosphatase (ALP) staining and activity assay

For measuring ALP expression levels, cells were washed with PBS twice, fixed with 10% formaldehyde in PBS for 30 s, rinsed with deionized water, and stained under protection from direct light with an Alkaline Phosphatase kit (Sigma). For measuring ALP activity, cells were washed with PBS twice and sonicated in lysis buffer consisting of 10 mM Tris–HCl (pH 7.5), 0.5 mM $MgCl_2$, and 0.1% Triton X-100. After centrifugation at 10,000 × *g* for 20 min at 4 °C, the ALP activity in the supernatant was measured in triplicate with the LabAssay ALP kit (Wako Pure Chemicals Industries). The protein concentration was measured with a bicinchoninic acid (BCA) Protein Assay kit (Pierce). Significance was determined by the Student's *t*-test, and differences were considered significant at $P < 0.05$.

2.5. Alizarin red S staining and measurement of calcium content

For measuring mineralization, cells were washed with PBS twice, stained with 40 mM Alizarin red S solution (pH 4.2) for 10 min at room temperature, and washed with deionized water twice. For measuring the amount of calcium deposited, cells were washed with PBS twice, fixed with 3.7% formaldehyde in PBS for 30 s, and decalcified with 300 μl of 1N HCl for 24 h. The calcium content was then measured with a Calcium C kit according to the manufacturer's protocol. Additionally, Alizarin red S was released from the cell matrix by incubating with 10% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) for 15 min. The concentration of released Alizarin red S was determined by measuring the absorbance at 562 nm [10,11] Significance was determined by Student's *t*-test and differences were considered significant when $P < 0.01$.

2.6. Evaluation of mRNA expression levels

Primers were chosen with an on-line primer design program [12]; primer nucleotide sequences are presented in Table 1. Isolation of total RNA, synthesis of cDNA, and quantitative real-time PCR were carried out as described previously [13]. In brief, total RNA was isolated with TRIzol reagent. Then first strand cDNA was synthesized with Omniscript Reverse Transcriptase (Qiagen, CA). Next, SYBR green-based quantitative PCR amplification was performed with the Brilliant SYBR Green Master Mix (Stratagene, CA) and the Stratagene Mx3000P Real-Time PCR system. All reactions were run in triplicate, and data were analyzed by the $2^{-\Delta\Delta C_T}$ method described previously [14]. GAPDH was used as a control for gene amplification. Significance was determined with the Student's *t*-test using GAPDH-normalized $2^{-\Delta\Delta C_T}$ values, and differences were considered significant at $P < 0.05$.

2.7. Western blot analysis

Nuclear proteins were extracted with the NucBuster Protein Extraction kit (Novagen, Germany). Western blot analysis was car-

Table 1
Primer sequences used in this study

Target gene	Forward (5′-3′)	Reverse (5′-3′)
ALP	GCTGATCATTCCCACGTTTT	CTGGGCCTGGTAGTTGTTGT
OPN	CGATGATGATGACGATGGAG	TGGCATCAGGATACTGTTCATC
OCN	AAGCAGGAGGGCAATAAGGT	TTTGTAGGCGGTCTTCAAGC
COLI	ACGTCCTGGTGAAGTTGGTC	CAGGGAAGCCTCTTTCTCCT
c-Jun	TCCCCTATCGACATGGAGTC	TGAGTTGGCACCCACTGTTA
c-Fos	CCAGTCAAGAGCATCAGCAA	AAGTAGTGCAGCCCGGAGTA
JunD	CGACCAGTACGCAGTTCCTC	AACTGCTCAGGTTGGCGTAG
Fra-1	AGAGCTGCAGAAGCAGAAGG	CAAGTACGGGTCCTGGAGAA
Fra-2	ATCCACGCTCACATCCCTAC	GTTTCTCTCCCTCCGGATTC
NFATc1	GGGTCAGTGTGACCGAAGAT	GGAAGTCAGAAGTGGGTGGA
BMP-2	GCTCCACAAACGAGAAAAGC	AGCAAGGGGAAAAGGACACT
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA