Emodin stimulates the osteoblast differentiation via activating bone morphogenetic protein-2 gene expression at low concentration

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Emodin is one of the main active components of a traditional Korean medicine isolated from the root and rhizomes of Rheum palmatum L. In this study, of 222 natural compounds to evaluate the anabolic activities, emodin activated bone morphogenetic protein (BMP)-2 promoter in the differentiation process of mouse osteoblastic MC3T3-E1 cells. Emodin was shown to significantly stimulate the activity and expression of alkaline phosphatase, an earlyphase marker of osteoblastic differentiation, on the differentiation day 7, and induce the osteopontin mRNA expression from the differentiation day 14. In addition, low concentration (up to 5 M) of emodin dramatically promoted the induction of mineralization in MC3T3-E1 subclone 4 cells. The stimulatory effect of emodin on the osteoblast differentiation/mineralization could be associated with its potential to stimulate the BMP-2 gene expression. Although further studies are needed to determine the precise mechanism, this study suggests that the use of herbal medicine containing natural compounds with anabolic activity such as emodin could have a beneficial effect on bone health.

key words : Bone morphogenetic protein-2, emodin, osteoblast differentiation, mineralization, osteoporosis

I. Introduction

Bone mass is maintained through the repeated cycle of destruction and rebuilding[1]. The process in which old bone is removed from skeleton by multinucleated osteoclasts and new bone is added through the differentiation / mineralization by osteoblasts is referred as bone remodeling. Usually, a balance between osteoclastic bone resorption and osteoblastic bone formation can maintain the bone mass at its homeostatic steady state, but its imbalance that is caused by increasedbone resorption over bone formation can lead to most adult skeletal diseases including osteoporosis. The development and progress of osteoporosis can increase the risk for fractures (particularly in the hip) that are a serious problem with many adverse consequences such as substantial skeletal deformity, pain, and functional[2].

Until now, most therapies for osteoporosis have focused solely on the resorption side[3]. Therefore, treatments effective in increasing the bone mass, improving defects in bone microarchitecture and accelerating fracture healing are urgently needed.

Bone morphogenetic proteins (BMPs) play an important role in regulating osteoblast differentiation and bone formation. BMPs belong to the TGF- superfamily and genetic disruptions of BMPs have resulted in various skeletal and extraskeletal abnormalities during development[4]. Of 14 types of BMPs, BMP-2, BMP-6 and BMP-9 were shown to be the most potent inducers of osteogenic differentiation both in vitro and in vivo[5, 6]. Interestingly, by using the firefly luciferase reporter gene driven by the mouse BMP-2 promoter (-2736/+114 bp), lovastatin was identified to the only natural product stimulating luciferase activity[7]. These results suggest that the promoter of BMP-2 gene could be useful in a study for identifying agents stimulating/enhancing

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osteoblast differentiation. Therefore, here we used two green fluorescence protein (GFP) reporter genes with BMP-2 promoter and identified emodin with anabolic activity in bone metabolism from 222 natural compounds.

Ⅱ. Materials and methods

1. Cell culture

All materials for cell culture were purchased from HyClone (UT). Mouse osteoblastic MC3T3-El cells were kindly provided by Dr. Lim (Division of Endocrinology, Department of Internal Medicine, College of Medicine, Yonsei University, Seoul, Korea) and MC3T3-E1 subclone 4 (MC4) cells with high differentiation potential[8] were purchased from American Type Culture Collection (VA). Both cells were cultured in the growth medium [GM, -minimal essential medium (MEM) supplemented with 10% fetal bovineserum (FBS), 100 U/ml of penicillin, and 100 mg/ml streptomycin] with a change of medium every 3 days in humidified atmosphere of 5% CO_2 at 37C.

2. BMP-2 promoter cloning and transfection

Mouse BMP-2 gene transcription can be regulated by two transcription initiation sites within 2712 bp of the 5' flanking region of the BMP-2 gene[9] the distal start site was mapped to -736 bp in relation to the proximal start site (+1). Here, BMP-2 promoter clone 4 (-472 to -1562 bp) with distal start sites and clone 5 (-553 to +355 bp) with proximal start site were amplified by genomic DNA PCR with the primers; clone 4 sense: ggaccaggcagaaaattcaa, antisense: ctctagcttctcccggacac; clone 5 sense: gagggactggcacgacac, antisense: ggcacgtccattgagagagt. Amplified PCR products were cloned into pGlow-TOPO plasmid (Invitrogen, CA) according to the manufacturer's protocols. BMP-2 promoter-cloned plasmids were transfected into MC3T3-E1 cells by using Lipofectamine 2000 (Invitrogen) and transfected cells were selected by using Geneticin Selective Antibiotics (Invitrogen).

3. Osteoblast differentiation and screening of natural compounds

In order to screen the natural compounds with the potential to activate BMP-2 promoter in osteoblast cells, BMP-2 promoter-GFP plasmid-transfected MC3T3-E1 cells were plated in a 384-well plate at the density 0.5 10^3 cells/well and cultured in -MEM medium containing 10% FBS. After cells were > 95% confluent, medium was changed with the differentiation medium [DM, GM with 50 g/ml of ascorbic acid (Fluka, Germany), and 10 mM -glycerophosphate (Sigma, MO)] with a change of medium every 3 days. All 222 natural compounds used in this study were isolated as a single compound form or purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO). Dissolved natural compounds (2 mg/ml in DMSO) was then formatted in a 384-well plate and transferred into cell plates at day 0 and 3using QRep 384 Pin Replicator (Genetix, UK). At the differentiation day 4, BMP-2 promoter-GFP signals were observed using both IX51 inverted fluorescence microscope (Olympus, Japan) and Opera high-content screening instrument (Evotec Technologies, Germany). Nuclei were stained with SYTO59 (1.25 M/well; Molecular Probes, OR) for 30 min.This experiment was performed twice. Since compounds, which destructed the cell morphology, were considered as those with cytotoxicity, they were excluded in the next experiment. Compounds with low solubility were also excluded. In the next experiment, selected compounds on the activation of BMP-2 promoter were confirmed with BMP-2 promoter-GFP plasmid-transfected MC3T3-E1 cells plated in a 96-well plate.

4. Alkaline phosphatase(ALP) staining and its activity assay

Cells (1.5 10^4 cells/well) were plated in a 24-well plate and cultured in GM. After the cells reached confluence, they were cultured in DM in the absence or presence of 3-carene with a change of medium every 3 day. On the differentiation day 9, cells were washed with PBS twice, fixed with 10% formalin, rinsed with deionized water, and stained under the protection from direct light by using Alkaline Phosphatase

kit (Sigma). For measuring ALP activity, cells were washed with PBS twice and sonicated in the lysis buffer consisting of 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$ and 0.1% Triton X-100. After the centrifugation at 10,000 g for 20 min at 4C, the supernatant was transferred and the ALP activity was measured by using LabAssay ALP kit (Wako Pure Chemicals Industries). The protein concentration of each sample was measured by using BCA Protein Assay kit (Pierce, IL). Activity assay was performed in triplicate and significance was determined by Student's t-test. Differences were considered significant when $P < 0.001$.

5. Evaluation of mRNA expression levels

Primers were chosen with an on-line primer design program[10] and their sequences were presented in Table 1. Total RNA was isolated with TRIzol reagent (Life Technologies, MD) according to manufacturer's protocol. The concentration and purity of total RNA were calculated with absorbance at 260 and 280 nm. First strand cDNA was synthesized with 1 g of total RNA and 1 M of oligo-dT18 primer using Omniscript Reverse Transcriptase (Qiagen, CA). SYBR green-based quantitative PCR amplification was performed using the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, CA) with first-strand cDNA diluted 1:50 and 20 pmole of primers according to the manufacturer's protocols. The PCR reaction consisted of initial denaturation at 94C for 3 min, 3-step cycling (40 cycles) at 94C for 40 s, 60C for 40 s, and 72C for 1 min, and final extension at 72C for 5 min. All reactions were run in triplicate, and data were analyzed by the $2^{-\Delta\Delta CT}$method[11]. GAPDH was used as the control gene. Significance was determined by Student's t-test with GAPDH-normalized $2^{-\Delta\Delta CT}$ values. Differences were considered significant when $P < 0.05$.

5. Alizarin red S staining

The mineralized nodules present in the osteoblast cells were stained by using Alizarine red S solution. 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.The images of stained cells were captured under a microscope with DPController.

6. von Kossa staining

Cells were washed with PBS and fixed with 2.5% glutaraldehyde in PBS for 30 min. After washing with deionized water three times, cells were incubated with 5% silver nitrate at room temperature under UV light until calcium turns black. The images of stained cells were captured under a microscope with DPController.

7. Calcium formation assay

For decalcifying mineralized nodules, 1 N HCl was added to each well and after 24 hr, calcium contents in the supernatant was determined in triplicate by using Calcium C kit (Wako Pure Chemicals Industries, Japan). Significance was determined by Student's t-test and differences were considered significant when $P < 0.05$.

Table 1. Primer sequences used in this study

Target gene	Forward (5'-3')	Reverse (5'-3')
Alkaline phosphatase (ALP)	gctgatcattcccacgtttt	ctgggcctggtagttgttgt
Type I collagen (COLLI)	acgtcctggtgaagttggtc	cagggaagcctctttctcct
Osteocalcin (OCN)	aagcaggagggcaataaggt	tttgtaggcggtcttcaagc
Osteopontin (OPN)	cgatgatgatgacgatggag	tggcatcaggatactgttcatc
GAPDH	aactttggcattgtggaagg	acacattgggggtaggaaca

III. Results

In a 384-well plate, the effects of natural compounds on the activation of BMP-2 promoters were evaluated in MC3T3-E1 cells. Of 222 natural products, nine were shown to activate both BMP-2 subclone 4 and 5 promoters on the differentiation day 4 (data not shown). One of 9 was identified to emodin (Fig. 1).

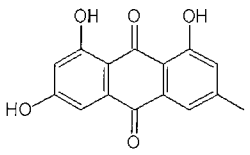

Figure 1. Structure of emodin

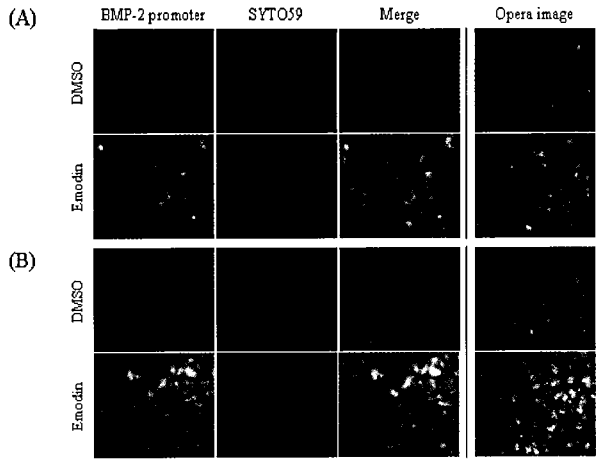

Figure 2. Effect of emodin on the activation of BMP-2 GFP signals in BMP-2 promoter subclone 4 (A) or subclone 5 (B)-transfected MC3T3-E1 cells 1 day after the treatment of emodin. BMP-2 GFP signals were observed by using IX51 inverted fluorescence microscope (merge images) and Opera high-content screening instrument.

The effect of emodin (2.5 g/ml, ~9 M) on the activation of BMP-2 promoters was confirmed in 96-well plate. The BMP-2 promoter-GFP signals were observed 1 day after the treatment of emodin as shown in Fig. 2; when observed using both IX51 inverted fluorescence microscope and Opera high-content screening instrument, BMP-2-GFP signals were higher in cells treated with emodin than those treated with DMSO.

To ascertain the anabolic activity of emodin in bone metabolism, its effect on the expression and activity of ALP, an early phase marker of osteoblastic differentiation, was evaluated in cells cultured with either GM or DM. In DM condition only, Emodin was shown to significantly stimulate both expression and activity of ALP on the differentiation day 7 when compared to controls (Fig. 3).

Next, the effect of emodin on the transcript expression levels of osteoblast differentiation/mineralization-related genes such as ALP, type I collagen (COLLI), osteocalcin (OCN) and osteopontin (OPN) was evaluated by quantitative real-time PCR. As shown in Fig. 4, In DM condition, emodinsignificantly induced COLLI on the differentiation day 14, and OPN from the differentiation day 14.

The anabolic activity of emodin was also evaluated in MC4 cells with high differentiation potential. On the

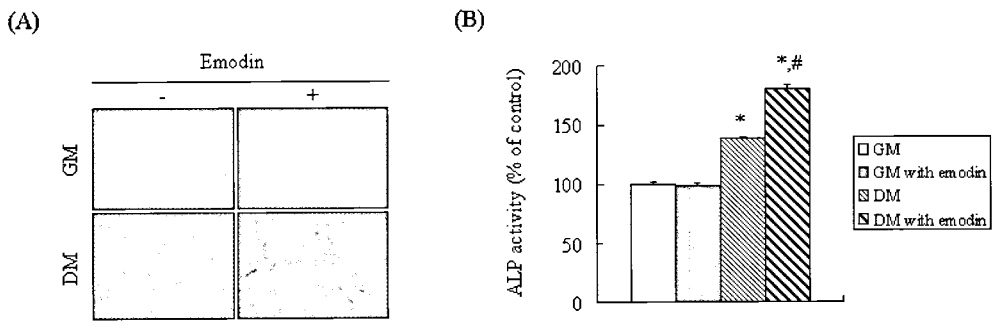

Figure 3. Effect of emodin (2.5 g/ml, ~9 M) on the ALP expression (A) and its activity (B) on the differentiation day 7. The ALP staining and its activity (units/mg) assay were performed in triplicate as described in 'Materials and Methods' and the data are expressed as the mean ± SD. *, p < 0.01 (GM vs. DM); #, p < 0.01 (DM vs. DM with emodin)

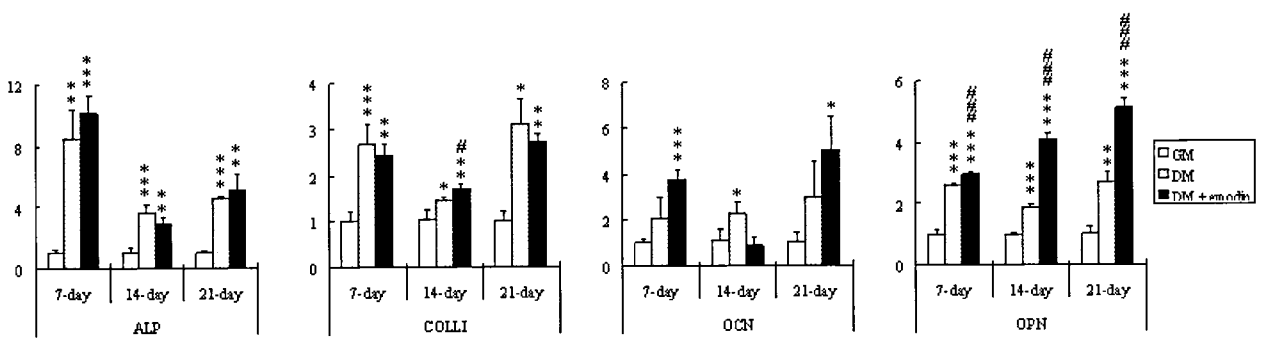

Figure 4. Effect of emodin on the transcript expression levels of osteoblast differentiation/mineralization genes. The effect of emodin on gene induction was evaluated by quantitative real-time PCR. The GAPDH-normalized fold changes are expressed as the mean ± SD. *, P < 0.05; **, P < 0.01; ***. P < 0.001 (GM vs. DM or DM + emodin); #, P < 0.05, ###, P < 0.001 (DM vs. DM + emodin)

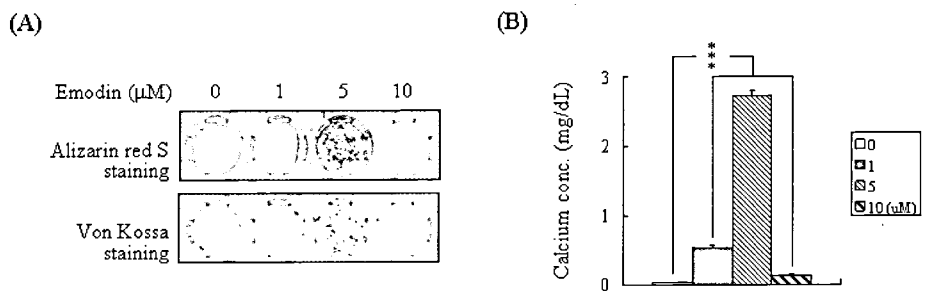

Figure 5. Effect of emodin on the calcium formation on the differentiation day 14. Alizarin red S staining and von Kossa staining were performed for the demonstration of mineralized nodule formation (A) and the amount of newly synthesized calcium was measured in triplicate as described in 'Materials and Methods' (B). The data are expressed as the mean ± SD. ***, $P < 0.001$ (compared with vehicle control).

differentiation day 14, the continuous treatment of emodin (up to 5 M) in MC4 cells was shown to strongly accelerate the calcium formation in the process of osteoblastic cell differentiation (Fig. 5).

Ⅳ. Discussion

Since natural compounds and their derivatives have historically been invaluable as a source of therapeutic agents, 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. Actually, a wide variety of natural compounds have beneficial effects on the skeleton[12].

Several natural compounds have been reported to enhance the osteogenic differentiation; one of flavonoids from the herb Epimedium, icariin stimulated the proliferation of bone marrow stromal cells and also increased ALP activity and calcium deposition during osteogenic induction13). Menaquinone-7 (in the fermented soybean), genistein, daidzein (in soybean) and epigallocatechin-3-gallate (in green tea) have been also shown to stimulate the osteoblastic bone formation14~18). Resveratrol, which is a phenolic compound found in the berry skins of most grape cultivars, directly stimulated cell proliferation and differentiation of osteoblasts[19].

Here, by using the cell-based screening, emodinwas identified to one of natural compounds with a potential to activate the BMP-2 promoter in MC3T3-E1 cells. Emodin induced the activation/expression of ALP in the early stage of differentiation and the expression of OPN from the middle stage of differentiation. In MC4 cells, the low concentration (up to 5 M) of emodin was shown to stimulate the mineralization.

Emodin is naturally occurring anthraquinone from the roots and rhizomes of Rheum palmatum L.[20]. Pharmacological studies have demonstrated that emodin possesses anti-bacterial[21], anti-inflammatory[22], immunosuppressive[23], vasorelaxant[24], anti-ulcerogenic[25] and anti-cancer effects[26]. Interestingly, two newly synthesized anthraquinone molecules have been shown to increase the trabecular bone volume and the trabecular thickness in in vivo study[27].

In conclusion, low concentration of emodin could stimulate the osteoblast differentiation/mineralization by the induction of osteoblast mineralization-related genes such as ALP and OPN via the activation of BMP-2 gene expression. Althoughstudies are needed to determine the precise mechanism, this study suggests that the use of herbal medicine containing natural compounds with anabolic activity such as emodin could have a beneficial effect on bone health.

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VI. References

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