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Betulinic Acid Stimulates the Differentiation and Mineralization of Osteoblastic MC3T3-E1 Cells: Involvement of BMP/Runx2 and β-Catenin Signals

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In the field of osteoporosis, there has been growing interest in anabolic agents that enhance bone formation. Here, we examined the effects of betulinic acid on cell proliferation, differentiation, and mineralization of MC3T3-E1 osteoblasts. Then, the impact of betulinic acid on signaling pathways known to be implicated in osteoblastogenesis was explored. Betulinic acid (1−20 μM) markedly increased alkaline phosphatase (ALP) activity and calcium nodule formation, although without a notable effect on cell proliferation. Stimulation with betulinic acid not only increased the osteopontin level and osteocalcin mRNA expression but also upregulated the osteoprotegerin (OPG)/RANKL ratio. Noggin, but not ICI 182780, significantly repressed betulinic acid-induced ALP activity, suggesting a possible action of betulinic acid through the bone morphogenetic protein (BMP) pathway. This was strengthened by the induction of BMP-2 expression, increases in Smad1/5/8 phosphorylation, and Runx2 expression. Furthermore, betulinic acid increased the nuclear level of the active form β-catenin. These results suggested that betulinic acid has the potential to enhance osteoblastogenesis probably through the activation of BMP/Smad/Runx2 and β-catenin signal pathways. Furthermore, upregulation of the OPG/RANKL ratio to repress bone catabolism may also indirectly contribute to the bone anabolic effect of betulinic acid.

KEYWORDS: Betulinic acid; osteoblast; BMP-2; Smad1/5/8; Runx2; β-catenin

INTRODUCTION

Osteoporosis is characterized by a reduction in bone mass and microarchitectural deterioration of bone tissue, resulting in skeletal fragility and susceptibility to fractures. Current drugs used to treat osteoporosis are all bone resorption inhibitors, which maintain bone mass by inhibiting the function of osteoclasts (*1*). However, the effect of these drugs in increasing or recovering bone mass is relatively small, certainly no more than 2% per year. It is desirable, therefore, to have satisfactory bone building (anabolic) agents that stimulate new bone formation of established osteoporosis.

Osteoblasts are the most important cells in bone tissues and are critical for bone formation. The cellular events involved in bone formation include the proliferation and differentiation of osteoblast precursors. Osteogenic differentiation of mesenchymal pluripotent cells is regulated by various soluble proteinous factors (*2*). Especially, bone morphogenetic protein (BMP) was originally identified as a molecule that promoted the differentiation of mesenchymal cells into an osteoblastic lineage, as suggested from its abilities not only to induce the expression of osteoblastic markers alkaline phosphatase (ALP), osteopontin (OPN), and osteocalcin (OCN) but also to stimulate mineralization (*3*). In the BMP signaling pathways, the Smad proteins play a major role in osteoblastic differentiation. The canonical Smad pathway in which receptor-specific Smad1, 5, and 8 are activated form complexes with the common partner, Smad4, and translocate into the nucleus to regulate the transcription of target genes (*4*). The other signaling pathways that are mediated by BMP include the runt-related transcription factor 2/core binding factor a1 (Runx2/Cbfa1) pathway and the Wnt/β-catenin system (*5*). Runx2 was shown to be indispensable for osteoblast differentiation and bone formation (*6*). Canonical Wnt/β-catenin is another signal pathway that regulates bone mass increase through a number of mechanisms including renewal of stem cells, stimulation of preosteoblast replication, induction of osteoblastogenesis, and inhibition of osteoblast and osteocyte apoptosis. This pathway is an enticing target for developing drugs to battle skeletal diseases as Wnt/β-catenin signaling is composed of a

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series of molecular interactions that offer potential places for pharmacological intervention (*7*).

Betulinic acid is a naturally occurring plant-derived triterpenoid present in many fruits and vegetables (*8*, *9*). This compound is also abundant in plants of the genus *Sambucus* (such as *Sambucus williamsii* Hance, *Sambucus nigra* Linn, *Sambucus formosana* Nakai, etc), a species widely distributed in Europe, Asia, and North Africa that has been used as an analgesic and anti-inflammatory agent, and as a herbal remedy for joint diseases, bone fractures, and osteoporosis (*10*, *11*). Although betulinic acid exhibited a variety of biological activities including anti-inflammatory, anticancer, and antioxidant properties (*9*, *12*, *13*), no osteogenic activity was reported for betulinic acid. Thus, we are interested in clarifying whether betulinic acid has a direct effect on osteoblast development and whether it is one of the active components responsible for the beneficial effect of the genus *Sambucus* on bone formation. Furthermore, the impact of betulinic acid on signaling pathways known to be implicated in osteoblastogenesis was explored. Our results showed that betulinic acid regulated osteoblast differentiation/mineralization through BMP/Smad/Runx2/ signaling pathways, forwarded the mineralization process by modulating OPN and OCN expression, and accelerated bone building via β-catenin activation.

MATERIALS AND METHODS

Cell Cultures and Drug Treatments. The murine calvaria-derived osteoblastic cell line MC3T3-E1 was maintained in an alpha modification of Eagle's minimum essential medium (α-MEM, Gibco BRL, Grand Island, NY 14072, USA) supplemented with 10% fetal calf serum (FCS, Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco) (defined as minimal medium) in a humidified 5% CO_2 balanced-air incubator at 37 °C. Cells were subcultured using 0.05% trypsin with 0.01% EDTA. To induce differentiation and/or mineral deposition, MC3T3-E1 cells (5×10^3/well or 1×10^5/well) were seeded into 96-well (Corning Costar, Tower 2, fourth Floor 900 Chelmsford Street, Lowell, NY, USA) or 24-well plates and cultured in minimal medium containing 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO) as well as 50 μg/mL ascorbic acid (Sigma-Aldrich) (defined as differentiation medium). Betulinic acid (Sigma-Aldrich, ≥98% HPLC) was dissolved in dimethyl sulfoxide (DMSO) at 0.1 M as a stock solution and diluted with medium. A final DMSO concentration in the culture was less than 0.05% and did not show observably artificial or cytotoxic effects. At day 5, betulinic acid was added to cells for further 3 to 14 days of culture to assess the drug's effect on cell differentiation and mineralization, respectively. For bone nodule formation, the medium was changed every third day, and cells were restimulated with the tested agent to ensure that the tested agent was continuously present in the medium through the observation period. In another experiment, noggin (R&D Systems, Minneapolis, Minnesota, USA) or ICI 182780 (Sigma-Aldrich) was added at 2 h before betulinic acid stimulation to block BMP- and estrogen-related signal pathways, respectively.

Quantitative Assay of ALP Activity. ALP activity in the cells was measured by incubation in 0.1 M $NaHCO_3-Na_2CO_3$ buffer (pH 10) containing 0.1% Triton X-100, 2 mM $MgSO_4$, and 6 mM *p*-nitrophenylphosphate (PNPP) for 30 min at 37 °C. The reaction was stopped by adding 1 M NaOH, and the absorbance was measured at 405 nm by an automatic biochemistry instrument (Hitachi, Japan) (*14*). Protein concentration was determined by incubation in bicinchoninic acid (BCA) protein assay reagent and the absorbance measured at 550 nm. ALP activity was calculated as PNPP concentration/protein concentration/time (nM PNPP/μg protein/h). ALP activity measured in differentiation medium alone was defined as the control. All results were expressed as relative ratio to control. To preclude the possibility that the attenuation in ALP activity was due to cytotoxicity, cell viability was simultaneously measured by a test based on the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases of metabolically active cells. The cells cultivated in differentiation medium without tested agents served as the control.

Observation of Mineralized Bone-Like Tissue. Cells were incubated in the differentiation medium for 14 days in the absence or presence of betulinic acid. On day 14, the cultures in the wells were rinsed using ice-cooled PBS and fixed with 95% ethyl alcohol. They were stained for 1 h with 0.1% Alizarin red S (Sigma-Aldrich) to detect the bone nodules (calcium precipitates). After washing with PBS, the samples were observed under light microscope, and the representative pictures were photographed. Finally, 0.1 N NaOH was added to dissolve the calcium precipitates, then the absorbance was measured at a wavelength of 548 nm.

Cell Proliferation. Effect of betulinic acid on cell proliferation was measured by 2,2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carbox-anilide (XTT) assay (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, cells cultured in minimal medium were seeded into 96-well culture plates (5×10^3 cells/well). After 24 h of incubation, the cells were treated with or without betulinic acid for 72 h. Fifty microliters of XTT test solution, which was prepared by mixing 5 mL of XTT-labeling reagent with 100 mL of electron coupling reagent, was then added to each well. After 4 h of incubation, absorbance was measured on an ELISA reader (Bio-Tek Instrument, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

Measurement of OPN, OPG, and RANKL. MC3T3-E1 cells were maintained in differentiation medium for 3 days in the absence or presence of betulinic acid. The OPN, OPG, and RANKL concentrations secreted into the culture medium were determined by ELISA kits (R&D).

RT-PCR Analysis. Expressions of OCN and BMP-2 mRNA were examined using RT-PCR, with the amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the control. MC3T3-E1 cells were seeded in a 35 mm dish and cultured for 3 days in the absence or presence of betulinic acid. After incubation, total RNA of cells was extracted using Trizol reagent. cDNA was synthesized using 5 μg of total RNA, 1 μL of random primer, 2 μL of dNTPs, and 200 U of M-MLV Reverse Transcriptase (Promega, USA) at 37 °C for 1 h. The specific primers of OCN were as follows: forward, 5′-GCAGCTTGGTGCA-CACCTAG-3′; reverse, 5′-GGAGCTGCTGTGACATCCAT-3′. The specific primers of BMP-2 were as follows: forward, 5′-CCAAGACA-CAGTTCCCTACA-3′; reverse, 5′-CACGGCTTCTAG- TTGATGGA-3′. The specific primers of GAPDH were as follows: forward, 5′-GCCAT-CAA- CGACCCCTTCATTGAC-3′; reverse, 5′-ACGGAAGGCCATG-CCAGTGAGC- TT-3′. Amplification was carried out for 30 cycles, each of which was at 94 °C for 15 s, 59 °C for 15 s, and 72 °C for 30 s in a 25 μL reaction mixture containing 3 μL of cDNA, 25 pmol of each primer, 0.25 mmol dNTPs, and 2 U of Taq DNA polymerase (Promega, USA). The products of PCR were analyzed with 1% agarose gel electrophoresis and visualized with ethidium bromide staining. The expected sizes for OCN, BMP-2, and GAPDH PCR products were 429 bp, 562 bp, and 609 bp, respectively. Band intensities were evaluated by densitometry.

Western Blotting. Whole cell lysates were prepared with lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EGTA, 0.24% sodium deoxycholate, and 1% IGEPAL CA-630, pH 7.5) containing 25 mM NaF and 2 mM Na_3VO_4. Aprotinin (1 μg/mL), leupeptin (1 μg/mL), pepstatin (1 μg/mL), and phenylmethylsulfonyl fluoride (1 mM) were added fresh prior to each lysis. For isolation of cytoplasmic and nuclear fractionates, cells were lysed for 15 min on ice in cytoplasmic lysis buffer (0.33 M sucrose, 1 mM $MgCl_2$, 0.1% Triton X-100, and 10 mM HEPES, pH 7.4, containing phosphatases and proteinases inhibitors as above) and nuclear lysis buffer (0.45 M NaCl and 10 mM HEPES, pH7.4) as described by Sen et al (*15*). Protein (5−20 μg) was loaded onto a 10% polyacrylamide gel for chromatography and transferred to polyvinylidene difluoride membranes. After blocking, the membrane was incubated with primary antibodies overnight at 4 °C. The primary antibodies used include anti-Smad1/5/8, antiphospho-Smad1/5/8, antiactive β-catenin (clone 8E7; Upstate Biotechnology, Inc., Lake Placid, NY), antitotal β-catenin (BD Biosciences), anti-Runx2, and antiactin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The antibody for active β-catenin was specific for the hypophosphorylated form of β-catenin (*16*). Secondary antibody conjugated with horseradish peroxidase was used to allow detection by the enhanced chemiluminescence plus kit (Amersham Biosciences Inc., Piscataway, NJ) and exposed to X-ray film.

Statistical Analyses. Statistical analysis was performed by Student's *t*-test. Values were expressed as mean ± SEM, and the difference between groups was considered to be significant at $P < 0.05$. Each experiment

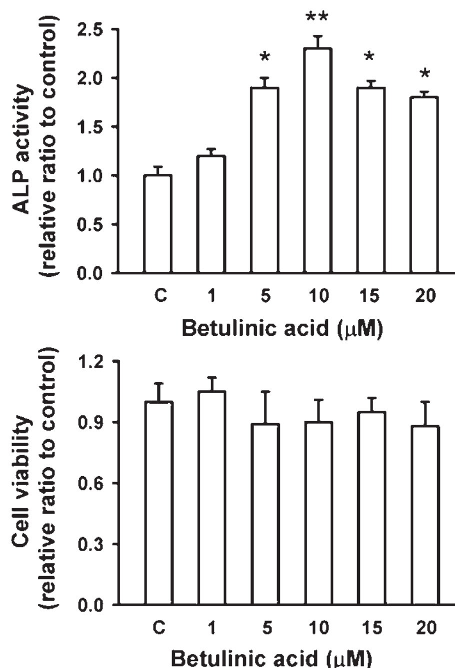

Figure 1. Effects of betulinic acid on the ALP activity and cell viability in MC3T3-E1 osteoblastic cells. The cells were incubated with betulinic acid for 3 days. Results are expressed as relative ratio to control. Each value is the mean ± SEM of six independent experiments, each in triplicate.* $P < 0.05$ and ** $P < 0.01$, compared with the control group without betulinic acid treatment.

was performed at least three times, and the representative graphs are shown.

RESULTS

Betulinic Acid Promoted Cell Differentiation and Mineralization of MC3T3-E1 Osteoblasts. To elucidate whether betulinic acid has an anabolic effect on bone, we first examined its effects on cell differentiation and mineralization of osteoblastic MC3T3-E1 cells. Results showed that betulinic acid (1, 5, 10, 15, and 20 μM) increased ALP enzyme activity in culture in a concentration-dependent manner. The increase in ALP activity was significant at concentrations of 5−20 μM and maximum at 10 μM. As shown in **Figure 1**, 10 μM betulinic acid increased the ALP activity of MC3T3-E1 cells to 2.3 times the control activity. Our preliminary study indicated that mineralization by MC3T3-E1 cells occurred in a time-dependent manner and can be easily observed after 14 days in culture. The addition of betulinic acid significantly and concentration-dependently enhanced bone nodule formation in MC3T3-E1 cells when compared with that in the control observed at day 14 (**Figure 2**). A plateau effect was noted at 10 μM betulinic acid. Furthermore, the effect of betulinic acid on cell proliferation on days 1, 2, and 3 was assessed by XTT assay (**Figure 3**). Results showed that the growth profiles observed at 1, 2, and 3 days of culture in the presence of betulinic acid were similar to those of the control, suggesting that betulinic acid failed to affect cell proliferation at the concentrations used.

Betulinic Acid Enhanced Bone Matrix Protein Deposition in MC3T3-E1 Osteoblasts. We examined the effects of betulinic acid (2.5, 5, and 10 μM) on the production of the medial and terminal differentiation markers OPN and OCN. As shown in **Figure 4**, OPN level in culture medium was increased concentration-dependently in the presence of betulinic acid after 72 h culture, as compared to that in differentiation medium alone (represented as 0). Significant increase was observed starting at 5 μM

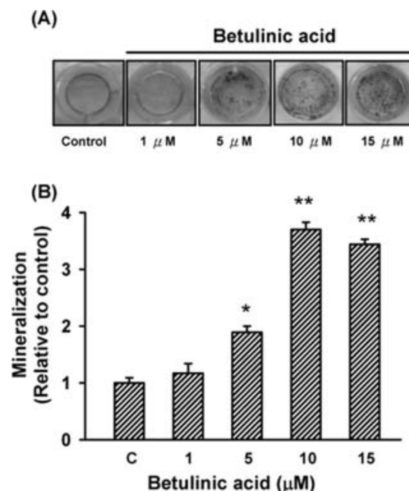

Figure 2. (**A**) Mineral matrix deposition (Alizarin Red staining) in MC3T3-E1 osteoblastic cells cultured in differentiation medium with or without betulinic acid for 14 days (magnification = 10×). (**B**) Quantification of mineral matrix deposition was represented as the relative ratio to control. Each value is the mean ± SEM of four independent experiments.* $P < 0.05$ and ** $P < 0.01$, compared with the control group without betulinic acid treatment.

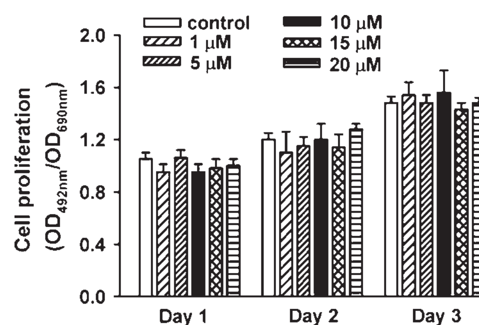

Figure 3. Effects of betulinic acid on the cell proliferation of MC3T3-E1 osteoblastic cells. Adherent cells that proliferated in 96-well plates (5×10^3 cells/well) were incubated with different concentrations (1, 5, 10, 15, and 20 μM) of betulinic acid for various time intervals (1, 2, and 3 days). Cell proliferation was determined by XTT assay as described in Materials and Methods. Each value is the mean ± SEM of five independent experiments, each in triplicate.

($P < 0.05$). The changes in the OCN mRNA abundances were quantitatively monitored in the absence (0) and presence of betulinic acid (2.5, 5, and 10 μM). As shown in **Figure 4** (bottom), there is no detectable band in the total RNA extract in the absence of betulinic acid. However, the cells treated with betulinic acid evoked a marked induction in OCN mRNA expression in a dose-dependent manner after 72 h of culture; a significant increase was also observed starting at 5 μM.

Noggin, but Not ICI 182780, Repressed Betulinic Acid Induced Cell Differentiation. To understand the signal pathways involved in the regulation of cell differentiation by betulinic acid, MC3T3-E1 cells were pretreated with a BMP inhibitor noggin for 2 h, then coincubated with 10 μM betulinic acid for a further 3 days. Addition of noggin (0.05−1 μM) protein into differentiation medium slightly diminished ALP activity, however, and seriously abrogated the stimulatory effect of betulinic acid on ALP activity (**Figure 5**). These suggested that betulinic acid-induced cell differentiation may operate by a BMP-dependent pathway. Cell viability in the presence of noggin was not significantly different from that without noggin treatment (data not shown). We also

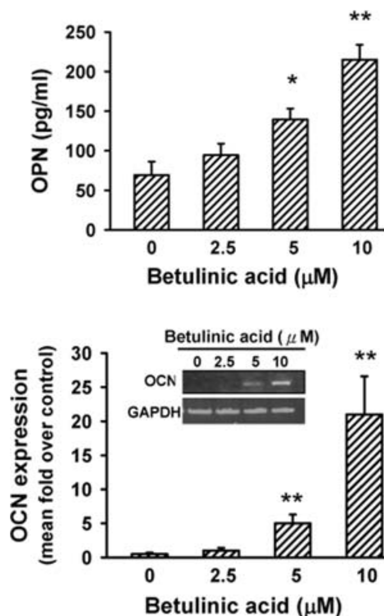

Figure 4. Effects of betulinic acid on OPN and OCN synthesis in MC3T3-E1 osteoblastic cells. The cells were incubated with (2.5, 5, and 10 μM) or without betulinic acid for 3 days. OPN level and OCN mRNA expression were measured by using ELISA and RT-PCR, respectively. Each value is the mean $\pm$ SEM of four to six independent experiments. * $P < 0.05$ and ** $P < 0.01$, compared with the group without betulinic acid treatment.

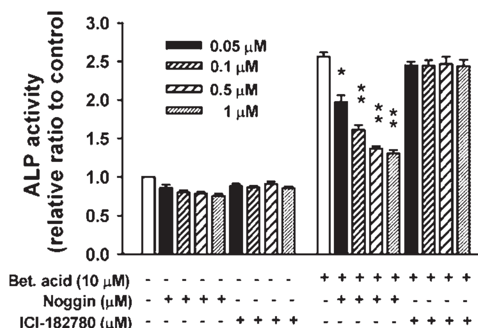

Figure 5. Effects of noggin (a BMP inhibitor) and ICI 182780 (a selective estrogen receptor antagonist) on betulinic acid induced ALP activity in MC3T3-E1 osteoblastic cells. The cells were pretreated with noggin or ICI 182780 for 1 h, then coincubated with betulinic acid for further 3 days. The basal ALP activity in differentiation medium alone (without any drug teatement) was identified as the control. Results are expressed as the relative ratio to control. Each value is the mean $\pm$ SEM of six independent experiments, each in triplicate. * $P < 0.05$ and ** $P < 0.01$, compared with the betulinic acid (Bet. acid) group.

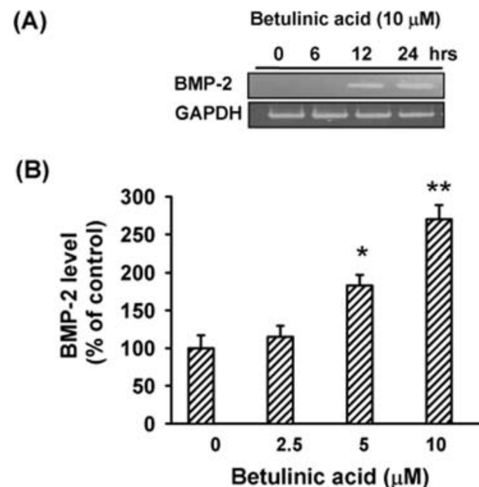

Figure 6. (**A**) Time-related effects of betulinic acid (10 μM) on BMP-2 mRNA expression in MC3T3-E1 osteoblastic cells. (**B**) Concentration-dependent effects of betulinic acid on BMP-2 mRNA expression in MC3T3-E1 cells cultured for 24 h. BMP-2 mRNA expression was measured by RT-PCR. The GAPDH mRNA level was analyzed in the same samples as a reference gene and visible by ethidium bromide stained on agarose gels. Each value is the mean $\pm$ SEM of five independent experiments. *$P < 0.05$ and **$P < 0.01$, compared with group that without betulinic acid treatment.

examined the effect of ICI 182780, the selective estrogen receptor (ER) antagonist (*17*), on betulinic acid induced increase in ALP activity. Results showed that ICI 182780 (0.05−1 μM) failed to attenuate betulinic acid-induced cell differentiation, suggesting that ER(s) or ER-related signal pathways might be precluded in the observed effect by betulinic acid.

Suppression of BMP-2 mRNA Expression by Betulinic Acid. As shown above, betulinic acid-induced cell differentiation was abolished by noggin treatment, suggesting that a BMP-dependent mechanism may be involved. BMP-2 has been shown to play an important role in bone formation. To confirm whether the level of BMP-2 expression was influenced by the presence of betulinic acid, an experiment was performed using RT-PCR in the cultures grown for 24 h (**Figure 6A**). The results indicated that betulinic acid caused a significant increase in BMP-2 mRNA levels in MC3T3-E1 cells. The upregulation of BMP-2 started to increase at 12 h after 10 μM betulinic acid treatment, and maximum expression was observed at 24 h. The GAPDH mRNA level was analyzed in the same samples as a reference gene. Compared to the BMP-2 mRNA level, the relative levels of GAPDH mRNA was equal in all cultures, as can be seen on the ethidium bromide-stained agarose gels. **Figure 6B** indicated the concentration-dependent effect of betulinic acid on BMP-2 expression; the maximum stimulatory effect on BMP-2 mRNA expression was achieved at 10 μM after 24 h treatment.

Betulinic Acid Upregulated Smad1/5/8 Phosphorylation and Runx2 Expression. Since noggin blocked betulinic acid-induced cell differentiation, this indicated that the BMP pathway is required in betulinic acid-mediated osteoblast differentiation. We therefore examined whether the Smad signal could be activated by betulinic acid. The phosphorylation of Smad1/5/8, which is usually triggered by the BMP-dependent oligomerization of BMP receptors type I (BMP-R1) and type II (BMP-R2) (*18*), was examined in betulinic acid-treated MC3T3-E1 cells by Western blotting using an antiphosphorylated Smad1/5/8 antibody. Results showed that the stimulation of cells with betulinic acid for 60 min induced a strong activation of Smad1/5/8 in a concentration-dependent manner, as revealed by increased phosphorylation (**Figure 7**, upper panel). The phosphorylation of Smad1/5/8 was moderately increased by 2.5 μM and reached a maximum by 5−10 μM betulinic acid treatment. However, betulinic acid did not affect the expression levels of unphosphorylated Smad1/5/8.

Runx2 is essential for the differentiation of osteoblasts from mesenchymal precursors, which can directly stimulate transcription of osteoblast-related genes such as those encoding OCN, OPN, and type I collagen by binding to specific enhancer regions (*19*). Furthermore, BMPs function by activating Smad proteins and other signal transduction pathways to stimulate the expression of many target genes including Runx2. This knowledge led us to hypothesize that betulinic acid may exert its osteogenic effects by interacting with this factor. We found that

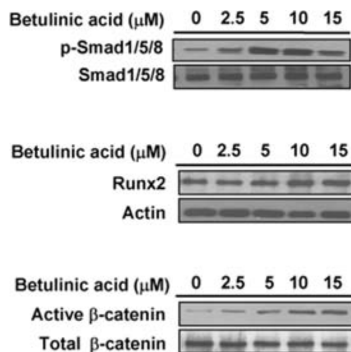

Figure 7. Western blot analysis of Smad1/5/8 phosphorylation, Runx2 induction, and active β-catenin nuclear translocation in MC3T3-E1 osteoblastic cells before and after betulinic acid (2.5, 5, 10, and 15 μM) stimulation for 60 min, 2 days, and 60 min, respectively. Bands were visualized by an ECL method. Similar results were obtained in four independent experiments. p-Smad1/5/8: phosphorylated Smad1/5/8 complex; Smad1/5/8: nonphosphorylated Smad1/5/8 complex.

Runx2 expression was upregulated by betulinic acid treatment in a concentration-dependent manner at 48-h culture conditions. As shown in **Figure 7** (middle), betulinic acid (10 and 15 μM) increased the levels of Runx2 expression 2- to 2.5-fold.

Betulinic Acid Increased in the Nuclear Level of Active β-Catenin. The level of β-catenin in the cytoplasm is regulated by a destruction complex consisting of adenomatous polyposis coli, axin, and GSK3β (glycogen synthase kinase 3β). Constitutive phosphorylation of β-catenin by GSK3β, targets β-catenin for degradation by the ubiquitin/proteosome pathway, limiting the levels of cytoplasmic β-catenin under resting conditions. In canonical Wnt signaling, binding of the Wnt ligand to its transmembrane receptors leads to the disruption of the β-catenin destruction complex, which allows the accumulation of stabilized β-catenin and subsequent nuclear translocation. Here, betulinic acid (2.5−15 μM) significantly activated β-catenin in the MC3T3-E1 cells after a 60 min culture period, as determined by using an antibody to detect β-catenin that is dephosphorylated at serine 37 and threonine 41. This active form of β-catenin has been shown to be increased in response to Wnt stimulation and by mechanical loading (*16*, *20*). As shown in **Figure 7** (bottom panel), active β-catenin was strongly increased in the nuclei of betulinic acid-treated cells as compared with that in untreated control cells. The increase in active nuclear β-catenin reached a plateau by 15 μMl betulinic acid. A significant change of total β-catenin level was not observed in response to betulinic acid.

Upregulation of OPG/RANKL Ratio by Betulinic Acid. Osteoblasts can secret OPG to protect the skeleton from excessive bone resorption by binding to RANKL and preventing it from binding to its receptor, RANK. Thus, the OPG/RANKL ratio is an important determinant of bone mass and skeletal integrity. The results showed that the basal OPG level in differentiation medium culture for 3 days was 6.4 ± 1.7 pg/mL. We found that betulinic acid treatment markedly increased OPG production in a concentration-dependent manner: a significant increase in OPG level was observed starting at 2.5 μM and peaking at 10 μM betulinic acid (**Figure 8**). The basal RANKL level in differentiation medium was around 140−150 pg/mL. Betulinic acid was tended to repress basal RANKL production, however, without statistical significance. Generally, the OPG/RANKL ratio was upregulated by betulinic acid.

DISCUSSION

Osteogenic cell proliferation and differentiation play a central role in adequate fracture healing to increase extracellular bone

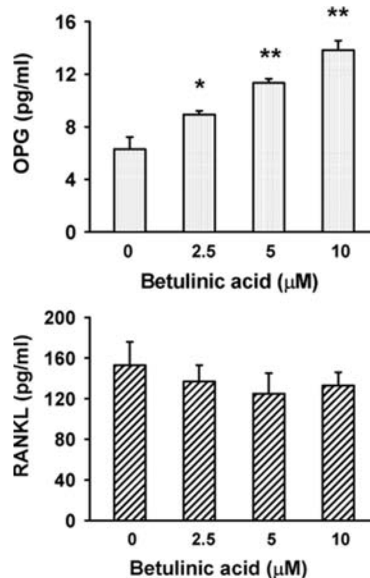

Figure 8. Effects of betulinic acid on OPG and RANKL synthesis in MC3T3-E1 osteoblastic cells. The cells were incubated with (2.5, 5, and 10 μM) or without betulinic acid for 3 days. Levels of OPG and RANKL in the culture medium were measured by using ELISA as described in Materials and Methods. Each value is the mean ± SEM of three to four independent experiments, each in triplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with group that without betulinic acid treatment.

matrix production (*21*). In this study, we found that betulinic acid has the ability to stimulate osteoblast differentiation/mineralization, although it failed to enhance osteoblast proliferation. During differentiation in vitro, osteoblast phenotypic markers appear in the following order: accumulation of collagenous matrix, expression of ALP, secretion of OPN and OCN, and finally mineralization of bone nodules. Our results showed that betulinic acid indeed markedly raised the levels of OPN and OCN, two bone matrix proteins which are implicated in osteoblast mineralization. Certain bone formation agents such as statins have been shown to induce osteoblast differentiation by stimulating the expression of BMP-2 in MC3T3-E1 cells, leading to positive effects on bone formation (*22*). BMP has been proven to induce bone formation both in vivo and in vitro (*23*). Its signaling is initiated by receptor binding, propagated by the phosphorylation of Smad1/5/8 complex, and finally translocated into the nucleus to regulate the transcription of target genes (*24*, *25*). Besides, the BMP pathway can be regulated by a negative feedback loop. Noggin is one of the osteoblast-secreted proteins that can limit the level of BMP signals through complexation with BMPs and prevention of their receptor binding (*26*). In the present study, we found that the adding of noggin significantly repressed betulinic acid evoked differentiation, suggesting that betulinic acid may influence osteoblast functions potentially through the BMP pathway. This was further proven by enhanced BMP-2 mRNA expression and stimulated Smad1/5/8 phosphorylation.

Among the downstream targets of BMPs are Runx2 and other osteoblast-related transcription factors such as DLX5 and osterix (*27*,*28*). Runx2 is the main transcription factor required for the activation of osteoblast differentiation and is crucial for the regulation of genes responsible for the production of bone specific proteins such as collagen type 1, OCN, OPN, and bone sialoprotein (*19*). Our results showed that the stimulation of MC3T3-E1 cells with betulinic acid resulted in 2- to 2.5-fold increases in Runx2 expression; this might be helpful in elevating its binding

level to enhancer regions in target genes. Therefore, it is possible that upregulation of Runx2 expression participated in the bone anabolic effect by betulinic acid. These results support our hypothesis that one of the pathways that betulinic acid may activate during osteoblast differentiation could be BMP\Runx2.

The genus *Sambucus* is often used as folk medicine to cure bone fracture (*10*, *11*). Our results showed that betulinic acid had the ability to stimulate cell differentiation and mineralization suggesting that betulinic acid may be one of the active components responsible for the osteogenic effect of *Sambucus*. Bone is one of the few tissues in the body with the capacity to regenerate and repair itself. Fracture repair and bone regeneration require the localized reactivation of signaling cascades that are crucial for skeletal development. An important role for Wnt/β-catenin signaling has been recognized in promoting bone anabolism during fracture healing (*7*). In canonical Wnt signaling, binding of the Wnt ligand to its transmembrane receptors frizzled, and LRP5/6 leads to the disruption of the β-catenin destruction complex, which allows the accumulation of stabilized β-catenin and subsequent nuclear translocation. Small molecules and biologics aimed at this pathway are now being tested as potential new anabolic agents. Since β-catenin acts as a transcription factor to modulate bone fracture regeneration, it should also be considered in the potential mechanism of action of betulinic acid in stimulating new bone formation. Although we did not assay whether betulinic acid could induce Wnt expression, it is worthwhile noticing that betulinic acid treatment indeed increased in the nuclear level of active β-catenin. It had been reported that stabilized β-catenin synergizes with BMPs to stimulate in vitro osteoblast differentiation and in vivo new bone formation (*29*, *30*); that genetic ablation of β-catenin blocks the osteogenic effect of BMP-2 in ex vivo mouse calvaria cultures (*31*); and that the canonical Wnt signaling is induced by BMPs via an autocrine loop (*32*, *33*). These data suggested a model whereby canonical Wnt signaling, via β-catenin, is part of the downstream events activated by BMPs to induce osteogenesis. Furthermore, Wnt and BMPs are expressed in many overlapping tissues, and dual regulation by Wnt and BMPs appear to be frequent in mammalian development. Thus, we suggested that betulinic acid might display bone anabolic effect through cooperative interactions with β-catenin and BMP signaling.

In the bone remodeling process, the members of the molecular triad OPG/RANK/RANKL are closely linked to each other. RANKL is synthesized either in membranous or soluble form, primarily by the osteoblastic lineage cells, the immune cells, and some cancer cells. The binding of RANKL to the extracellular RANK (a cell surface receptor located on osteoclasts) leads to the activation of specific signaling pathways involved in the formation and survival of osteoclasts, hence bone resorption (*34*). OPG is secreted by the stromal cells and other cell types, including osteoblasts, and acts as a soluble decoy receptor for RANKL. OPG, by interacting with RANKL, inhibits the binding of RANKL to RANK, thereby preventing RANK activation and subsequent osteoclastogenesis, and, as a result, inhibits bone resorption. Current therapies used to prevent or treat metabolic bone diseases are thought to act, at least in part, through the modification of the RANKL/OPG dipole. Results obtained from **Figure 8** indicated that betulinic acid reciprocally regulated OPG and RANKL levels especially significantly raised in OPG production. But how did betulinic acid influence the levels of OPG and RANKL? Spencer et al. reported that activation of Wnt/β-catenin signaling down-regulated RANKL expression in osteoblasts (*35*). Conversely, Sato et al. noted that Wnt3a enhanced the expression of OPG but reduced that of RANKL (*36*). Furthermore, the increase in OPG level by Wnt3a was significantly enhanced after BMP-2 addition suggesting that Wnt/β-catenin signaling, in combination with BMP-2, regulates OPG expression (*36*). Overall, net changes in bone mass induced by Wnt/β-catenin signaling may result from changes in the balance between bone formation and bone resorption through the regulation of osteoclast formation and activity. All these findings support the assumption that the beneficial effect of betulinic acid on bone formation may act, at least in part, through synergistic activation of BMP and β-catenin pathways in osteoblasts to modulate the balance of OPG/RANKL, hence blocking bone resorption.

Recently, Huh et al. (*37*) studied the effect of KHBJ-9B (a herbal remedy containing 15.4% betulin, 10.1% pimaradieonic acid, 5.1% betulinic acid, 2.8% (−)-epicatechin, and 2.2% (±)-catechin hydrate) and its major compound triterpenoids in human cartilage culture and in a rabbit model of collagenase-induced osteoarthritis. An in vitro experiment was performed to demonstrate the inhibition of GAG and type II collagen release in human cartilage culture challenged by IL-1β. They found that betulinic acid in doses ranging from 50 to 200 μg/mL (around 10^{-4}–4×10^{-4} M) showed dose-dependent inhibition ranging from 22% to 70% of GAG release and 15% to 56% of type II collagen degradation. On the cartilage degradation of tibial plateau in the KHBJ-9B treated rabbits, a significant decrease in histologic grading was observed, which were about 29.1%, 43.0%, and 51.1% in the 50, 100, and 200 mg/kg KHBJ-9B groups, respectively. After calculation, the content of betulinic acid in 50, 100, and 200 mg/kg KHBJ-9B were equal to 2.5, 5, and 10 mg/kg, respectively. In other words, about $1-3 \times 10^{-3}$M concentration can be found in the rabbit serum when rabbits (~4 kg body weight) were exposed to 2.5, 5, and 10 mg/kg betulinic acid. On the basis of these observations, we suggested an effective concentration of betulinic acid can be achieved by exposure to betulinic acid containing the diet/herb remedy.

In summary, our results provided evidence that BMP/Runx2 and β-catenin signals were involved in the bone anabolic effect of betulinic acid. However, the mechanisms for gene expression are complex, and the results of this study only begin to clarify these mechanisms. Furthermore, betulinic acid may affect osteoclastogenesis indirectly via an increase in OPG expression by osteoblasts. The dual regulatory effect of betulinic acid on osteoblasts and ostelclasts indicated that it may be useful for the treatment of common metabolic bone diseases. Nevertheless, further studies would need to be carried out in order to determine the biological efficacy of betulinic acid in ex vivo or in vivo studies.

ABBREVIATIONS USED

ALP, alkaline phosphatase; BCA, bicinchoninic acid; BMP, bone morphogenetic protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OCN, osteocalcin; OPG, osteoprotegerin; OPN, osteopontin; RANKL, receptor activator of nuclear factor κB (NF-κB) ligand; Runx2, runt-related transcription factor 2; XTT, 2, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carbox-anilide.

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