SAMBUCUS FORMOSANA STIMULATES DIFFERENTIATION OF MC3T3-E1 OSTEOBLASTS THROUGH BMP-2 AND β-CATENIN PATHWAYS

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Sambucus formosana Nakai is used as a folk medicine in Taiwan to remedy bone fracture. Here we examined the effects of *S. formosana* on the differentiation and mineralization of MC3T3-E1 osteoblasts. Then, the impact of *S. formosana* on signaling pathways known to be implicated in osteoblastogenesis was explored. Results showed the chloroform soluble fraction prepared from the ethanol extract of *S. formosana* (defined as SF-C) concentration-dependently increased in alkaline phosphatase (ALP) activity, levels of osteopontin, osteocalcin and bone morphogenetic proteins-2 (BMP-2), and calcium nodules formation. SF-C evoked differentiation was significantly abrogated by noggin, wortmannin and SB2003580 treatment. SF-C also induced Smad1/5/8, Akt and p38 phosphorylation suggesting that these signal pathways were probably involved. β-catenin plays a pivotal role in regulating bone mass formation. The results further showed that transfection with β-catenin siRNA significantly attenuated SF-C induced ALP activity. Considering the indirect effect on the regulation of osteoclastogenesis, we found SF-E could stimulate osteoprotegerin (OPG) production although have no effect on RANKL synthesis from MC3T3-E1 preosteoblasts. In summary, our results proved the clinical application of *S. formosana* and indicated that SF-C has bone anabolic effect being probably through BMP-2- and β-catenin-dependant signal pathways. Further, repression of bone catabolism via up-regulation of OPG/RANKL ratio may also participate in cure bone fracture by *S. formosana*.

Key words: *Sambucus formosana*, osteoblast, BMP-2, β-catenin, p38, Akt

Introduction

Osteoblasts are the most important cells in bone tissues and are critical for bone formation and normal bone density. The cellular events involved in bone formation included the proliferation and differentiation of osteoblast precursors. During differentiation *in vitro*, osteoblast phenotypic

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markers appear in the following order: accumulation of collagenous matrix, expression of alkaline phosphatase (ALP), secretion of osteopontin (OPN) and osteocalcin (OCN), and finally mineralization of bone nodules[1].

Bone morphogenetic proteins (BMPs) have pivotal roles in the regulation of bone induction, maintenance and repair. In the BMP signaling pathways, the Smad proteins play a major role in osteoblastic differentiation[2]. MAP kinases also play an important role in osteoblast differentiation. ERK regulates ALP activity and the deposition of bone matrix proteins[3]. Activation of p38 is critical for ALP expression during differentiation of MC3T3-E1 cells[4]. Recently, the activation of Akt, which lie downstream in the PI3K signaling pathway, was shown to enhance osteoblast differentiation and bone formation[5]. Wnt/β-catenin is another crucial 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 series of molecular interactions that offer potential places for pharmacological intervention[6].

The genus *Sambucus* is widely distributed in Asia. In Taiwan, the root of *Sambucus formosana* is often used as folk medicine to cure bone fracture, contusion, and pain induced by rheumatism. Traditionally, an alcoholic drench of *S. formosana* roots and leaves is used for self-medication. However, at present, there is no direct experimental evidence of the therapeutic benefit of *S. formosana* in the bone healing. Therefore, this study was performed to clarify the effect of *S. formosana* on the differentiation and mineralization of MC3T3-E1osteoblastic cell. Then the impact of *S. formosana* on signaling pathways known to be implicated in osteoblastogenesis was further explored.

Materials and Methods

Extraction

Sambucus formosana Nakai (Caprifoliaceae) was purchased from a Chinese herbal drug store in Taipei city, and were authenticated by professor Chien-Chih Chen. A voucher specimen (NRICM-05-010) of this herbal drug was deposited in the National Research Institute of Chinese Medicine, Taiwan, R. O. C. The dried whole plant (10 kg) of *Sambucus formosana* Nakai (SF) was extracted with 95% ethanol (EtOH, 2 × 100 L) at 60 °C. The combined extracts were evaporated in vacuum to give a dark brown black residue, which was suspended in $CHCl_3$ (10 L) and centrifuged (10,000×g, 30 min) to give $CHCl_3$-soluble (defined as SF-C) and $CHCl_3$-insoluble portions (defined as SF-M) (Fig. 1). For the cell culture, extracts were dissolved in dimethyl sulfoxide (DMSO) at 200 mg/ml as a stock solution

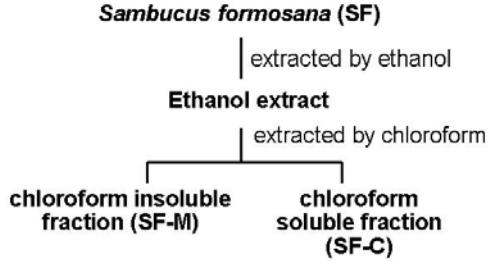

Fig. 1. Preparation diagram to obtain the $CHCl_3$-soluble fractions (defined as SF-C) and $CHCl_3$-insoluble fractions (defined as SF-M) from the ethanol extract of *Sambucus formosana* (SF).

and diluted with medium to give a final DMSO concentration of <0.1% in the culture.

Cell culture and drug treatment

The murine calvaria-derived osteoblastic cell line MC3T3-E1 were 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, 4th 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). At day 5, various concentrations of SF-C or SF-M was added to cells for further 3 to 14 days culture to assess drug's effect on cell differentiation and mineralization, respectively. For bone nodules formation, medium was changed every third day, and cells were re-stimulated with tested agent to ensure that tested agent was continuously present in the medium through the observation period. In another experiment, noggin (R&D Systems, Minneapolis, Minnesota, USA), SB203580, PD98059, SP600125 or wortmannin (Sigma-Aldrich) was added at 2 hr before SF-C stimulation to block BMP-, MAPKs- or PI3K- 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-nitrophenyl-phosphate (PNPP) for 30 min at 37ºC. The reaction was stopped by adding 1 M NaOH, and the absorbance was measured at 405 nm by an automatic biochemistry instrument (Hitachi, Japan)[7]. Protein concentration was determined by incubation in bicinchoninic acid (BCA) protein assay reagent and measured the absorbance 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 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 simultaneous 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.

Mineralized bone-like tissue observation

Cells were incubated in the differentiation medium for 14 days in the absence or presence of betulinic acid, respectively. On days 14, the cultures in the wells were rinsed using an 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 wash 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 viability assay

The cytotoxic effect of SF was evaluated by a cell viability test based on the cleavage of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases of metabolically active cells. Cells were cultivated in minimal medium for 24 h after the cells were adherent. The medium was removed and SF-C were added, then incubated in a humidified atmosphere with 5% CO_2 at 37 ºC for 72 h and harvested for MTT assay. The cells cultivated in minimal medium without SF served as the control.

Measurement of OPN, OCN, OPG and RANKL

MC3T3-E1 cells were maintained in differentiation medium for 3 days in the absence of presence of herbal extract, respectively. The concentrations of OPN, OCN, OPG and RANKL secreted into the culture medium were determined by ELISA kits (R&D Systems, Minneapolis, MN).

Western blot analysis

After treated with various concentrations of SF, MC3T3-E1 cells were washed quickly with cold PBS containing 5mM of EDTA and 0.1 mM of Na_3VO_4, and lysed with a lysis buffer consisting of 20 mM of Tris–HCl (pH 7.5), 150 mM of NaCl, 1% Triton X-100, 10 μM of NaH_2PO_4, 10 % glycerol, 2 μM of Na_3VO_4, 10 μM of NaF, 10 μg/ml leupeptin and 10 μg/ml aprotinin. Western blot was performed as above. Proteins were then transferred to a nitrocellulose membrane. The membrane was incubated with primary antibodies against BMP-2, Smad1/5/8, p-Smad1/5/8, p38, p-p38, Akt, p-Akt, actin, β-catenin or GAPDH for 1-2 h then incubated with goat anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase for 1 h. Blots were processed using an ECL Kit (Santa Cruz) and exposed to X-ray film.

Small interfering RNA (siRNA) and transfections

β-catenin (GenBank accession no. NM-007614) was targeted using a predesigned ON-TARGET plus SMARTpool siRNA (catalog no. L-040628-00-0005, Dharmacon). Non-targeting siRNA was used as a control in an identical manner as that of relevant siRNA. MC3T3-E1 cells were transfected at 70% confluence with a final concentration of 100 nM SMARTpool siRNA or nonspecific control pool using DharmaFECT-1 siRNA transfection reagents (Dharmacon) according to the manufacturer's instructions. After 24 h, the medium was replaced with differentiation medium and cells were treated for an additional 48 h with SF-C or left untreated. We then harvested protein for Western analysis. In another set of experiment, cell culture was collected for ALP activity measurement after siRNA transfection.

Statistical analyses

For each experimental series, data are presented as the mean ±SE. The significance of the concentrations and sample treatments was determined by one-way analysis of variance (ANOVA) with repeated measures. If there were significant interactions, the simple main effect of each factor was assessed using the Student–Newman–Keuls

test. A value of $p < 0.05$ was considered statistically significant.

Results

SF-C, but not SF-M, stimulates cell differe-ntiation in MC3T3-E1 cells

The effects of SF-C and SF-M on the differentiation by MC3T3-E1 cells were first clarified. We found that SF-C increased the ALP enzyme activity in culture in a concentration-dependent manner and a plateau effect occurred around 15 μg/ml. As shown in Fig. 2 (upper), 15 μg/ml SF-C increased the ALP activity of MC3T3-E1 cells to 2.3 folds of the control activity. By contrast, SF-M failed to influence cell differentiation under the same condition (Fig. 2, bottom). MTT assay showed that SF-C at the concentrations used did not markedly affect the cell viability. On the other hand, we also determined the effects of SF-C and SF-M on the proliferation of MC3T3-E1 cells. The results showed that neither SF-C nor SF-M enhanced cell proliferation at the concentrations between 1 to 25 μg/ml after 48 and 72h treatment (data not shown). Results obtained here revealed that the beneficial osteogenic activity of *S. formosana* might be attributable to SF-C. Thus, this fraction was employed to evaluate the other activity in the following study.

Effect of SF-C on bone matrix protein deposition and mineralization in MC3T3-E1 cells

The effect of SF-C on the medial and terminal differentiation of MC3T3 cells was also assessed by determining the production of OPN and OCN, respectively. As shown in Fig. 3, OPN level in medium was increased concentration-dependently in the presence of SF-C after 72 h culture, as compared to differentiation medium alone (represented as control). Significant increase was observed started at 5 μg/ml. The change pattern in OCN level was similar to that of OPN. Our preliminary study indicated that mineralization by MC3T3-E1 cells occurred in a time dependent manner after 14 days in culture. The addition of SF-C significantly stimulated the mineralization in a dose-dependent manner when

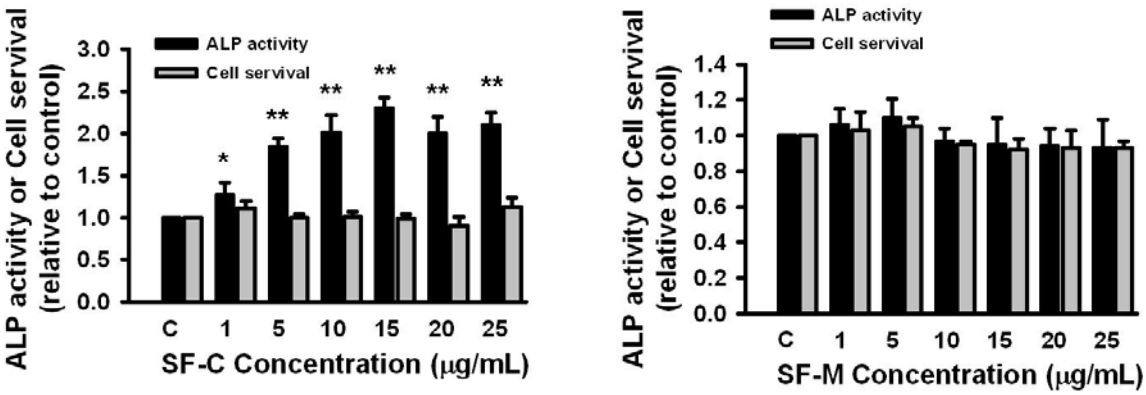

Fig. 2. The effects of SF-C and SF-M on the ALP activity and cell viability in MC3T3- E1 osteoblastic cells. The cells were incubated with herbal extract for 3 days. Results are expressed as relative ratio to control (C). Each value is the mean±SE of six independent experiments, each in triplicate.* $p<0.05$ and ** $p<0.01$, compared with the control group that without herbal extract treatment.

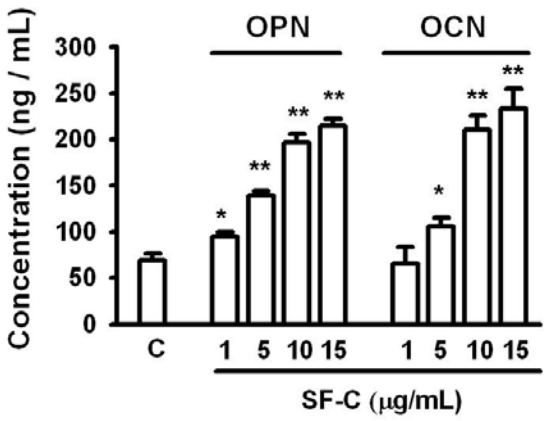

Fig. 3. The effects of SF-C on OPN and OCN synthesis in MC3T3-E1 osteoblastic cells. The cells were incubated with (1, 5, 10 and 15 μg/ml) or without SF-C for 3 days. OPN and OCN levels were measured by using ELISA as described in method. Each value is the mean±SE of four to six independent experiments. * p<0.05 and ** p<0.01, compared with group that without SF-C treatment.

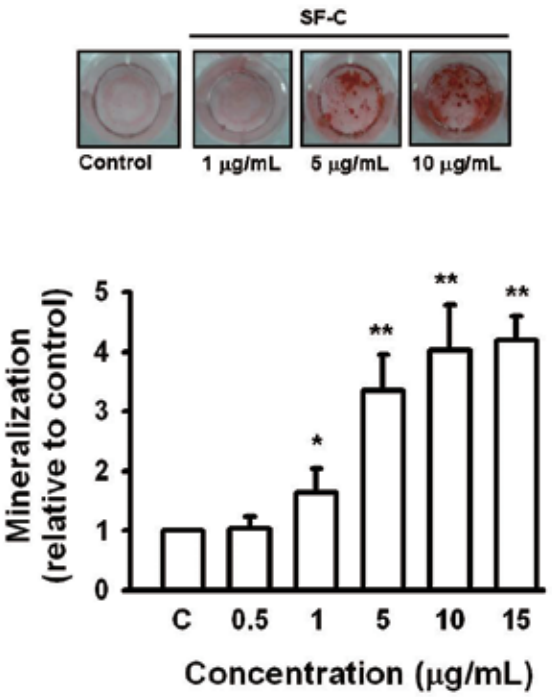

Fig. 4. (A) Mineral matrix deposition (Alizarin Red staining) in MC3T3-E1 osteoblastic cells cultured in differentiation medium with or without SF-C for 14 days (magnification = 10×). (B) Quantification of mineral matrix deposition was represented as relative ratio to control (C). Each value is the mean±SE of four independent experiments.* p<0.05 and ** p<0.01, compared with the control group that without SF-C treatment.

observed at day 14 (Fig. 4). Maximal effect was obtained at 10-15 μg/ml.

Roles of BMP/Smad in SF-C evoked differentiation

To understand the signal pathways involved in regulation of cell differentiation by SF-C, MC3T3-E1 cell was pretreated with a BMP inhibitor noggin for 2 h, then co-incubated with 10 μg/ml SF-C for further 3 days. Addition of noggin (0.05-1 μM) into differentiation medium seriously abrogated the stimulatory effect of SF-C on ALP activity (Fig. 5A). These suggested that SF-C-induced cell differentiation might operate by a BMP-dependent pathway. Cell viability in the presence of noggin was not different from that without noggin treatment. Since noggin blocked SF-C-induced cell differentiation, indicating BMP pathway is required in SF-C-mediated osteoblast differentiation. We therefore examined whether the Smad signal could be activated by this herbal remedy. The canonical Smad pathway in which receptor-specific Smad1, 5, and 8 are activated; form complexes with the common partner, Smad4, and translocated into the nucleus to regulate the transcription of target genes[2]. 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

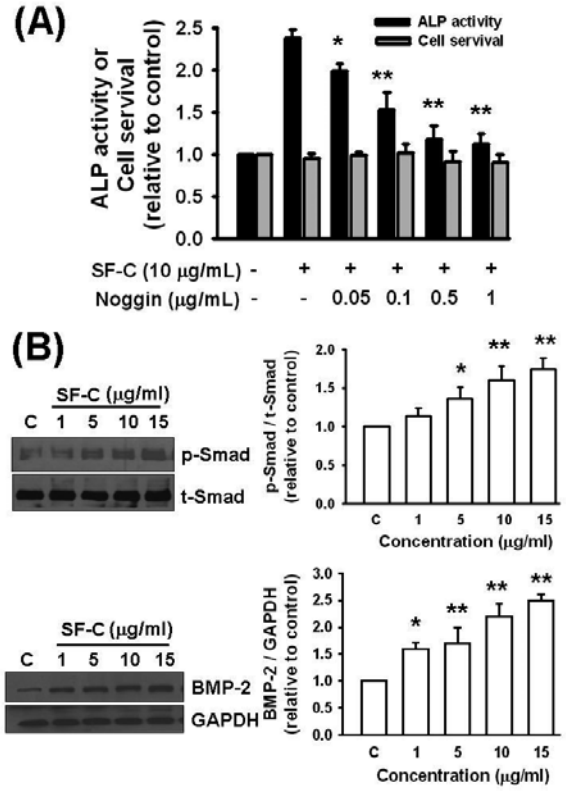

Fig. 5. (A) Effects of noggin (a BMP inhibitor) on SF-C induced ALP activity and cell viability in MC3T3-E1 osteoblastic cells. The cells were pretreated with noggin for 2 h then co-incubated with SF-C for further 3 days. The basal ALP activity in differentiation medium alone (without any drug treatment) was identified as control. Each value is the mean±SE of four independent experiments, each in triplicate. * p<0.05 and ** p<0.01, compared with SF-C group. (B) Concentration-dependent effect of SF-C on Smad1/5/8 phosphorylation and BMP-2 protein expression. Results are expressed as relative ratio to control. Each value is the mean±SE of four independent experiments. * p<0.05 and ** p<0.01, compared with control group.

(BMP-R2)[8], was examined in the SF-C-treated MC3T3-E1 cells by Western blotting using an anti-phosphorylated Smad1/5/8 antibody. Results showed stimulation of cells with SF-C for 30 min induced an activation of Smad1/5/8 in a concentration-dependent manner, as revealed by increased phosphorylation (Fig. 5B). The protein level of unphosphorylated Smad1/5/8 was not modified whatever the dose of SF-C.

To confirm whether the level of BMP-2 expression was influenced by the presence of SF-C, experiment was performed using Western blotting in the cultures grown for 24 h. The trace indicated in the bottom of Fig. 5B represented the concentration-dependent effects of SF-C on BMP-2 expression: the maximum stimulatory effect on BMP-2 expression was achieved at 15 μg/ml after 24 h treatment. The GAPDH protein level was analyzed in the same samples as a reference. Compared to BMP-2, the relative levels of GAPDH was equal in all cultures.

Roles of MAPKs in SF-C induced osteoblast differention

To elucidate the mechanism of action of SF-C in osteoblast differentiation, the effects of p38 inhibitor SB203580, MEK inhibitor PD98059 and JNK inhibitor SP600125 were employed to asses the roles of MAPKs by analyzing ALP activity. As shown in Fig. 6A (upper), pre-incubation with SB204580 (0.05, 0.1, 0.5 and 1 μM) caused a concentration-dependent inhibition of SF-C (10 μg/ml)-induced ALP activity by 57.9±5.4, 74.4±7.7, 91.7±3.5 and 99.8±4.4 %, respectively. MTT assay revealed that the repression effect of SB203580 on SF-C evoked osteoblastic differention was not due to cytotoxic effect. The effect of SF-C on the activation of p38 MAPK was also evaluated by Western blot analysis. Stimulation with SF-C for 30 min increased the phosphorylation level of p38 MAPK in a concentration-dependent manner;

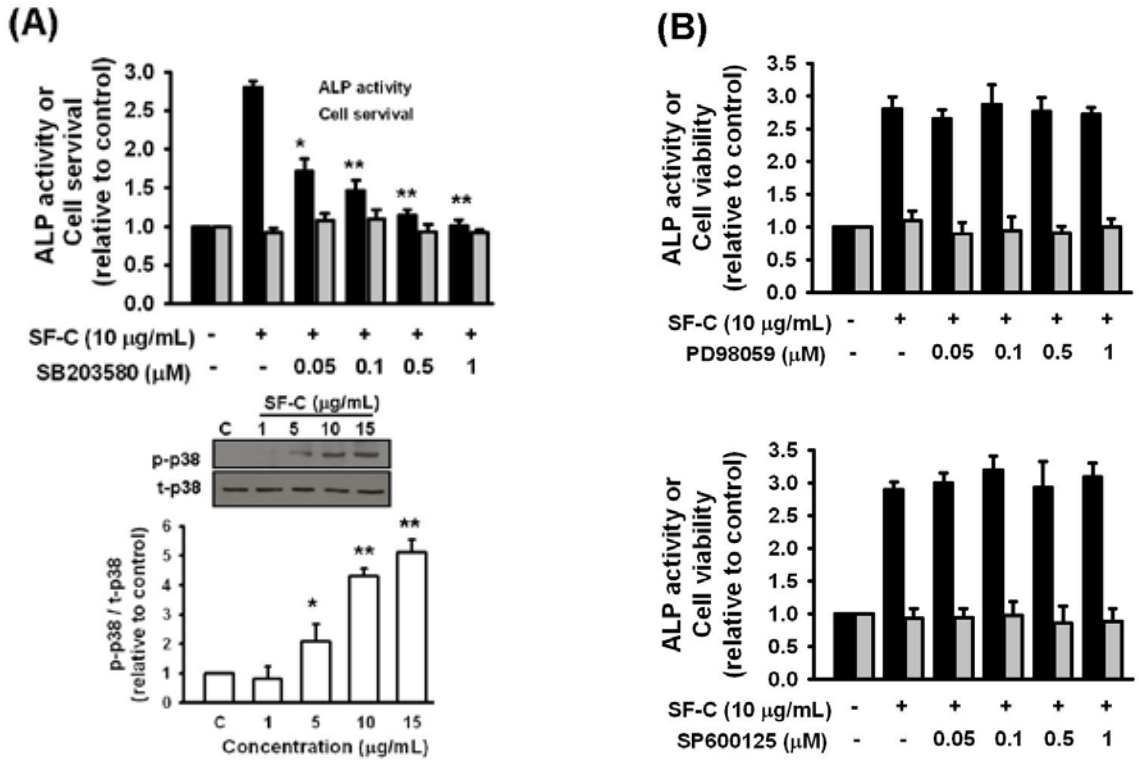

Fig. 6. Effects of (A) SB203580 and (B) PD98059 or SP600125 on SF-C induced ALP activity and cell viability in MC3T3-E1 osteoblastic cells. The cells were pretreated with individual MAPK inhibitor for 2 h then co-incubated with SF-C for further 3 days. The basal ALP activity in differentiation medium alone (without any drug treatment) was identified as control. Results are expressed as relative ratio to control. Each value is the mean±SE of three to four independent experiments, each in triplicate. * p<0.01 and ** p<0.001, compared with SF-C group. Concentration-dependent effect of SF-C on p38 phosphorylation was also shown in (A). Results are expressed as relative ratio to control. Each value is the mean±SE of three independent experiments. * p<0.05 and ** p<0.01, compared with control group.

significant phosphorylation was observed by 5 to 15 μg/ml SF-C stimulation. By contrast, PD98059 and SP600125 under the same concentration ranges, all failed to attenuate SF-C-induced increased in ALP activity (Fig. 6B).

SF-C activated Akt phosphorylation is associated with increase in ALP activity

The PI3K inhibitors wortmannin was used to determine whether SF-C-induced osteoblastic differention via activating the PI3K pathway. Wortmannin (0.05, 0.1, 0.5 and 1 μM) concentration-dependently repressed SF-C (10 μg/ml) stimulated ALP activity by 69.3±11.1, 82.2±4.6, 94.1±5.8% and 100%, respectively, but had no significant effect upon cell viability (Fig. 7, upper). The effect of SF-C on the activation of PI3K signal pathway was also evaluated by detection of Akt (a downstream target protein for PI3K) phosphorylation. Compared with un-stimulated conditions, stimulation with SF-C for 30 min indeed resulted in a respective 1.4, 2.2, 3.8 and 4.1 times increase in Akt phosphorylation by 1, 5, 10 and 15 μg/ml SF-C treatment (Fig. 7, bottom).

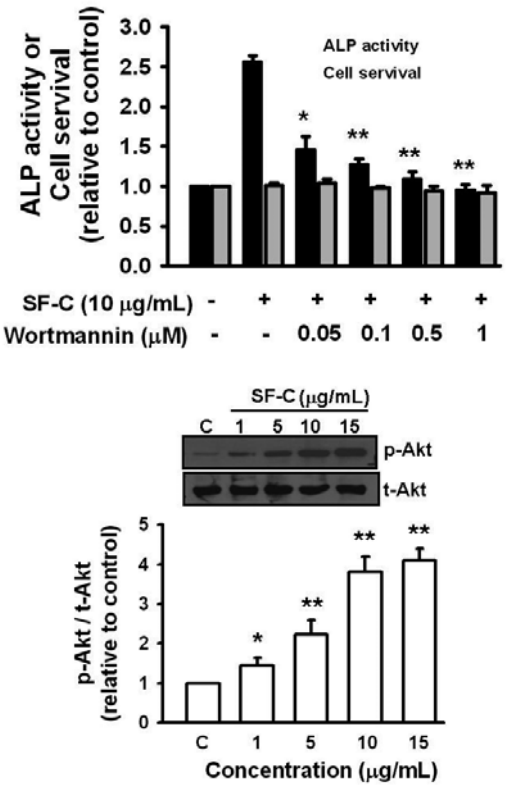

Fig. 7. Effect of wortmannin (a PI3K inhibitor) on SF-C induced ALP activity and cell viability in MC3T3-E1 osteoblastic cells. The cells were pretreated with the PI3K inhibitor for 2 h then co-incubated with SF-C for further 3 days. The basal ALP activity in differentiation medium alone (without any drug treatment) was identified as control. Each value is the mean±SE of three independent experiments, each in triplicate. * p<0.01 and ** p<0.001, compared with SF-C group. Concentration-dependent effect of SF-C on Akt (downstream of PI3K) phosphorylation was also shown. Results are expressed as relative ratio to control. Each value is the mean±SE of three independent experiments. * p<0.05 and ** p<0.01, compared with control group.

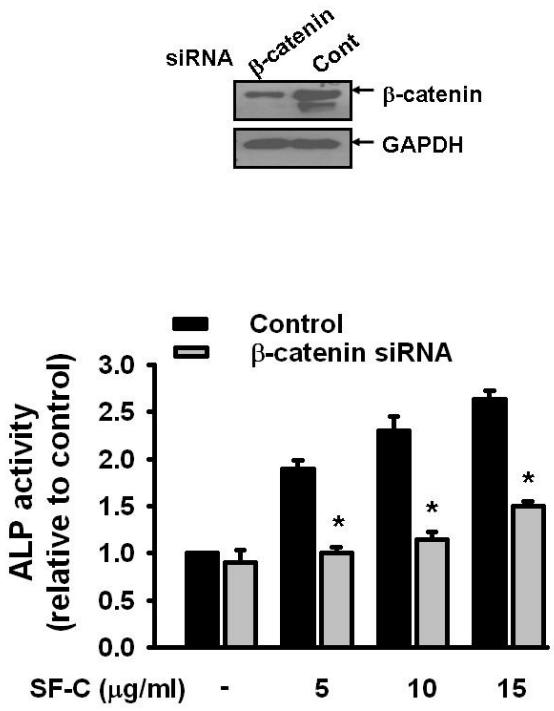

Fig. 8. Effects of SF-C (5, 10 and 15 μg/ml) on the ALP activity in MC3T3-E1 osteoblastic cells before (control) and after β-catenin siRNA transfection. β-catenin expression before and after siRNA transfection was also shown. The basal ALP activity in differentiation medium alone (without any treatment) was represented as 1. Results are indicated as relative ratio. Each value is the mean±SE of four independent experiments. * p<0.01, compared with control group.

Role of β-catenin in SF-C induced osteoblast differention

MC3T3-E1 cells were transfected with siRNA targeting β-catenin to assess the role of Wnt/β-catenin in SF-C induced osteoblastic differention. Protein trace illustrated in Fig. 8 indicated that transfection with β-catenin siRNA significantly knock down β-catenin expression by at lest 80%, as compared to those without transfection. We also confirmed that transfect with non-targeting control siRNA did not reduce β-catenin expression or cell viability (data not shown). After β-catenin siRNA transfection, SF-C increased ALP activity was markedly inhibited when compared with control situation. On the other hand, transfected with a non-targeting control siRNA

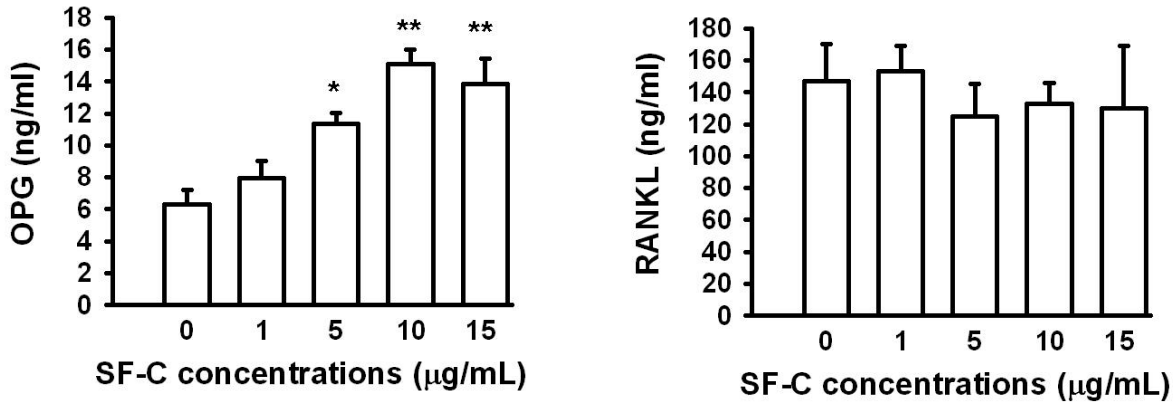

Fig. 9. Effects of SF-C on OPG and RANKL synthesis in MC3T3-E1 osteoblastic cells. The cells were incubated with (1, 5, 10 and 15 μg/ml) or without SF-C for 3 days. Levels of OPG and RANKL in the culture medium were measured by using ELISA as described in Materials and Methods. Each value is the mean±SE of two to three independent experiments, each in triplicate. * $p<0.05$ and ** $p<0.01$, compared with group that without SF-C treatment.

did not alter the increased ALP activity by SF-C stimulation (data not shown). These results proved the role of β-catenin in SF-C-induced osteoblastic differentiation.

Effect of SF-C on OPG and RANKL production

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, OPG/RANKL ratio is an important determinant of bone mass and skeletal integrity. The basal OPG level in differentiation medium was 6.3±0.9 pg/ml. The data showed that SF-C markedly up-regulated OPG production in a concentration-dependent manner to 7.4±1.1, 11.3 ±0.7, 15.1±0.9 and 13.8±1.6 pg/ml by 1, 5, 10 and 15 μg/ml treatment, respectively (Fig. 9). The basal RANKL level in differentiation medium was around 140~150 pg/ml. However, the RANKL level was not markedly attenuated in the presence of SF-C. These findings suggested that SF-C might be able to prevent bone resorption by increasing OPG/RANKL ratio then avoided the bone mass to flow away

Discussion

The root of *S. formosana* is often used as a folk medicine to cure bone fracture, contusion, and pain induced by rheumatism. Osteogenic cell proliferation and differentiation play a central role in adequate fracture healing to increase extracellular bone matrix production[9]. In this study, the effect of *S. formosana* extract on the osteoblastogenesis was evaluated in MC3T3-E1 cells. Results showed the chloroform soluble fraction prepared from the ethanol extract of *S. formosana* (SF-C) had the ability to stimulate osteoblast differentiation probably through BMP-2- and β-catenin-dependent signal pathways to promote mineralization process. Furthermore, activation of p38 MAPK and PI3K/Akt were also involved in the osteoblastogenetic mechanisms. 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 SF-C 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[10]. BMP has been proven to induce bone formation both *in vivo* and *in vitro*[11]. Its signaling is initiated by receptor binding, propagated by phosphorylation of Smad1/5/8 complex and finally translocated into the nucleus to regulate the transcription of target genes[12,13]. 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[14]. In the present study, we found that adding of noggin significantly repressed SF-C evoked differentiation, suggesting that SF-C may influence osteoblast functions potentially through BMP pathway. This was further proven by enhancement of BMP-2 protein expression and stimulation of Smad1/5/8 phosphorylation.

The genus *Sambucus* is often used as folk medicine to cure bone fracture[15,16]. 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[6]. In canonical Wnt signaling, binding of Wnt ligand to its transmembrane receptors frizzled and LRP5/6 leads to disruption of the β-catenin destruction complex, which allows accumulation of stabilized β-catenin and subsequent nuclear translocation. Although we did not assay whether SF-C could induce Wnt expression, the ability of siRNA targeted against β-catenin to decrease SF-C induced differentiation illustrates that Wnt/β-catenin is also an important mediator of SF-C action. Further, Wnt and BMPs are expressed in many overlapping tissues and dual regulation by Wnt and BMPs appear to be frequent in mammalian development[17]. Thus, we suggested that SF-C displayed bone anabolic effect might be through cooperative interactions with β-catenin and BMP signaling.

MAPKs are also essential signaling factors in the regulation of osteoblast differentiation. The activation of p38 appeared to be critical for the control of ALP expression during the differentiation of MC3T3-E1 cells[4]. In this study, SF-C induced increase in ALP activity was seriously repressed by SB203580. Further, SF-C also stimulated p38 phosphorylation in a concentration-dependent manner. Although ERK and JNK were activated by SF-C, but the patterns of their phosphorylations were quite different from p38. A significant phosphorylation of either ERK or JNK was observed at 24~48 h after SF-C stimulation (data not shown), suggesting a secondary regulation might be probably occurred. Base on the findings that PD98059 and SP600125 all failed to attenuate SF-C-induced increased in ALP activity precluding the direct role of either ERK or JNK in the early-stage differention by SF-C.

Extensive research has indicated that activation of the PI3K/Akt pathway enhances differentiation of osteoblastic cells[18-20]. The importance of Akt in bone formation was demonstrated in Akt-deficient mice, in which both differentiation and bone formation

were decreased and osteoblast-related certain genes were down-regulated[21]. We hypothesized that the PI3K/Akt pathway might also play a key role in SF-C induced differentiation of MC3T3-E1 osteoblasts. To test this hypothesis, we conducted experiments using wortmannin, a PI3K inhibitor. Results showed the enhanced ALP activity of osteoblastic cells by SF-C was largely blocked by wortmannin. Since SF-C also dose-dependently stimulated Akt phosphorylation. One explanation for this effect was that SF-C-induced differentiation of MC3T3-E1 osteoblasts were mediated by the PI3K/Akt pathway.

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 occurred[22] 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 modification of the RANKL/OPG dipole. Results obtained from Fig. 9 indicating that SF-C up-regulated the ratio of OPG/RANKL, especially raised in OPG production significantly. But how SF-C influenced the levels of OPG and RANKL? Sato et al noted that Wnt/β-catenin signaling enhanced expression of OPG but reduced that of RANKL[23]. Further, the increased 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[23]. 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 regulation of osteoclastic formation and activity. All this findings support us to speculate that the beneficial effect of SF-C on bone formation may act, at least in part, through synergistic activation of BMP and β-catenin pathways in osteoblast to modulate the balance of OPG/RANKL hence block the bone resorption.

In summary, our results provided evidences that BMP and β-catenin signals were involved in the bone anabolic effect of SF-C. These results further demonstrated that the p38 and PI3K/Akt pathways appear to play a predominant role in SF-C-induced differentiation of MC3T3-E1 osteoblasts. However, we still could not exclude additional signaling pathways involved in this process, which needs to be identified in the future study. On the other hand, SF-C may affect osteoclastogenesis indirectly via increase in OPG expression by osteoblast. The dual regulatory effect of SF-C on osteoblast and ostelclast indicated it may be useful for the treatment of common metabolic bone diseases. Nevertheless, further studies would be carried out in order to determine the biological efficacy of SF-C in *ex vivo* or *in vivo* studies.

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杇骨消 (*Sambucus formosana*) 藉由活化BMP-2及β-catenin訊息路徑刺激MC3T3-E1前驅造骨細胞分化

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臺灣民間草藥杇骨消（*Sambucus formosana*，SF）常用於治療骨折，本實驗研究杇骨消是否可促進MC3T3-E1前驅造骨細胞的分化及礦化，同時探討其對造骨新生（osteoblastogenesis）訊息路徑的影響。結果顯示杇骨消乙醇萃取物之氯仿可溶層製備物（SF-C）呈現濃度相關性地增加鹼性磷酸酶（ALP）活性、osteopontin濃度、osteocalcin蛋白及骨形態蛋白-2（BMP-2）表現、以及促進鈣化骨節的形成；同時SF-C促進造骨細胞分化的作用可被noggin、wortmannin、以及SB2003580所抑制，而SF-C也會增加磷酸化Smad1/5/8、磷酸化Akt、以及磷酸化p38的表現，猜測這些訊息分子均參與調控SF-C的造骨新生作用。另一方面，β-catenin在調控骨質生成作用上扮演相當重要的角色，本研究發現轉染β-catenin siRNA後可明顯抑制 SF-C促進分化的能力。此外，雖然SF-C並不影響前驅造骨細胞分泌RANKL的能力，但卻會促進osteoprotegerin（OPG）的生成。總言之，我們首先證實了杇骨消乙醇萃取物之氯仿可溶層製備物（SF-C）確實會促進前驅造骨細胞的分化及礦化，而根據機轉探討結果發現SF-C的骨生成作用可能與活化BMP-2-及β-catenin之訊息路徑有關，再者，SF-C也可藉由提高OPG/RANKL的比值來間接抑制蝕骨細胞的分化及活化。以上實驗驗證了杇骨消應用於改善骨折的臨床功效。

關鍵字：杇骨消、造骨細胞、骨形態蛋白-2（BMP-2）、β-catenin、p38、Akt

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