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Effects of *Rubus coreanus* Miquel Extracts on the Proliferation and Differentiation of Mouse Osteoblast-like MC3T3-E1 Cells

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ABSTRACTS

Rubus coreanus Miquel (RCM), one a type of edible red raspberry, grows wild in Korea and China and its unripe fruit has been used as a folk medicine for the treatment of impotence and as a diuretic agent. The goal of this study was to investigate the effects of RCM on the proliferation and differentiation of mouse osteoblast-like MC3T3-E1 cells. In this study, RCM extract (1-10 μg/mL) enhanced the viability of osteoblast cell. RCM extract also increased alkaline phosphatase (ALP) activity and osteocalcin secretion of MC3T3-E1 cells (significant at 1-10 μg/mL). Especially, at a concentration of 10 μg/mL ALP activity was increased by approximately 4.3 times in comparison with the control. Alizarin red staining and osteocalcin secretion assay showed that RCM extract stimulates differentiation of osteoblast cells by inducing the activation of mineralization. Moreover, reverse transcriptase polymerase chain reaction and western blotting assay indicated that RCM extract induced the differentiation at mRNA and protein level. These results suggest that RCM extract enhanced the osteoblast function by increasing the proliferation and differentiation of osteoblast like MC3T3-E1 cells.

Key words: *Rubus coreanus* Miquel, osteoblast, bone formation, proliferation, alkaline phosphatase, mineralization

INTRODUCTIONS

Bone is mineralized connective tissue in vertebrates that supports body weight, enables locomotion of the organism, protects internal organs from external force, and maintains mineral homeostasis. Bone homeostasis is ensured by the balance between bone formation and bone resorption, which depend on osteoblasts and osteoclasts, respectively. Thus, control of the recruitment, proliferation and differentiation of these cells is essential for the maintenance of bone mass. Disturbances in any of these processes result in systemic or local bone diseases such as osteoporosis, osteomalasia and periodontal diseases, and impair the healing of fractures[1-3]. Osteoporosis, which is associated with estrogen deficiency after menopause, is the most common cause of age-related bone loss in women[4]. Estrogen replacement therapy, which is recommended only for women who are at high risk of osteoporosis and without any contraindication, appears to be the most effective method for reducing the rate of postmenopausal bone loss, although some side-effects may occur[5]. Attempts have been made to use a combination of anti-resorptive agents, such as estrogen, and bone formation-stimulating agents, such as a growth hormone, in the treatment of osteoporosis[6]. However, the potential bone-forming agents currently available either may have serious side-effects, or may not improve bone quality to reduce the susceptibility to fracture. Thus, the discovery of a natural dietary substance that minimizes bone loss in postmenopausal women would be of great interest.

Plants that are used in folk medicine have been recognized as one of the main sources in drug discovery and development. Because natural products of plant origin are still a major part of traditional medicinal systems, a resurgence of interest in herbal medicines has occurred in western countries as an alternative source of drugs often for intractable diseases such as rheumatoid arthritis[7]. From ancient times in China, Japan and Korea, women who have had lower back pain in climacteric and senescent periods have been treated with traditional medicines[8].

One of the traditional medicines, *Rubus coreanus* Miquel (RCM), a type of red raspberry, grows wild

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in Korea and China and its unripe fruit is used as a folk medicine for the treatment of impotence and as a diuretic. The botanical origin of the *Rubi Fructus* in the Korean crude drug market is mostly derived from fruits of *R. crataegifolius* and is sometimes mixed with the fruit of R. parvifolius which is closely related to *R. phoenicolasius*[9]. The fruits are rich in sugars, organic acids and several vitamins, as well as triterpenoids[10,11], and also include various anti-oxidants, tannins[12] and phenolic acids[13]. Extract of the fruit have been found to show considerable antioxidant activity in various test systems regardless of the degree of ripeness[14].

Unripe fruits of RCM are used, not only as food, but also to remit diabetes mellitus and sexual disinclination as a drug component in herbal medicine[15,16], therefore, it is thought to contain diverse functional substances. In studies on RCM, flavonoids complexes have been isolated from its leaves and stems[17], and it has been reported to contain antioxidants such as quercetin, gallic acid, 2,3-(S)-HHDP-D-glycopyranose, sanguine, etc.[18,19]. Therefore, based on analyses of its components and biological activities, we examined the relationship between bone cells and the RCM extract, which may contribute to the justification for the clinical application in the treatment of metabolic bone disease. It is shown that an acceleration effect is exerted by extract of RCM on the proliferation and differentiation of murine osteoblast like MC3T3-E1 cells.

MATERIALS AND METHODS

I. *Cell Culture*

MC3T3-E1 cells derived form newborn mouse calvaria and purchased from ATCC (CRL-2593, USA), were grown in α-MEM (Gibco BRL, NY, USA) supplemented with 10% fetal calf serum (Gibco) at 37°C in a humidified atmosphere of 5% CO_2, and the medium was changed at 3-day intervals. After the cells reached confluence, they were switched to the differentiation medium (α-MEM containing 50 μg/mL of the phosphate ester of ascorbic acid (Sigma, MO, USA) and 10 mM β-glycerophosphate (Sigma) in the absence or presence of various doses of RCM, which was dissolved in distilled water.

II. *Preparation of Rubus coreanus Miquel Extract*

Unripe fruits of *R. coreanus* Miquel were purchased from Yakryeong (Drug) Market (Dae-gu, Korea). The dried plant material (4.0 kg) was extracted three times with 80% methanol under reflux for 5 hr and the supernatant was filtered with a 10-μm paper cartridge. The filtered extract was concentrated on a rotary evaporator (Eyela, Tokyo, Japan) under reduced pressure to give a viscous methanol extract (450 g). The concentrated extract was freeze-dried. Brown powder (230 g) was generally produced in this process. A voucher specimen has been deposited at the Department of Food Science and Technology, Keimyung University, Daegu, Korea.

III. *Cell Viability Assay*

Effect of RCM extract on the proliferation of osteoblasts was investigated by MTT assay, as described by Green *et al.*[20] where the reduction of (3-(3,4-dimethylthiazolyl-2)- 2,5-diphenyltetrazolium bromide reagent (MTT) was measured. Cultured cells were counted using 0.4% trypan blue and adjusted to 1×10^4 cells/well. RCM extract was added in at final concentrations of 1, 5, 10, and 100 μg/mL, and the incubation lasted for 48 hr. Vehicle of 20 μL of α-MEM media with no supplement was added to the culture medium as a control. After the incubation, 10 μL of MTT reagent of 5 mg/mL was added, to each well, followed by 4-hr incubation. The medium was then removed and the wells were washed twice with PBS. One hundred microliter of DMSO was then added in to solubilize the generated insoluble formazan crystals and the optical density (OD) was measured at 550 nm in an ELISA reader (Spectra MAX 340 pc, Molecular Device, USA). Cell proliferation is indicated as the percentage of the OD of samples to that of the control group.

IV. *Determination of Alkaline Phosphatase Activity and Staining*

MC3T3-E1 cells were grown in 96-well plates and treated with RCM extract for 12 days. After incubation, the cells were rinsed twice with ice cold phosphate buffered saline (PBS, pH 7.4) and resuspended in Tris / glycine / triton buffer (50 mM Tris, 100 mM glycine, 0.1% triton X-100, pH 10.5). The supernatant was collected by centrifugation at 5,000 ×g for 15 min at 4°C. Ten microliter of p-nitrophenyl-phosphate (p-NPP) substrate (100 mM) mixed with 20 μL of 0.1N glycine NaOH (pH 10.4) was added to 20 μL of the supernatant and incubated at 37°C for 30 min. The enzymatic reaction catalyzed by alkaline phosphates (ALP) was stopped by adding 200 μL of 0.1 N NaOH. The OD of p-nitrophenol (p-NP) product was measured at 405 nm within 1 hr. ALP activity was standardized as the relative control %. MC3T3-E1 cells grown in 24-well plates were treated with RCM extract for 12 days, and ALP was stained using an alkaline phosphatase kit (Sigma). Briefly, after the culture medium was removed, fixative (citrate-acetone-formaldehyde) was added and kept for 30 sec at room temperature, and the cells were rinsed with distilled water for 45 sec. A diazonium solution (sodium nitrite: FRV-alakline : naphthol AS-BI alkaline solution = 1:1:1) was added, followed by incubation for 15 min at room temperature, and the cells were washed with distilled water for 2 min. The cell cultures were stained again with a hematoxylin solution for 2 min, and then washed with running water and examined under a microscope[21]. To determine the protein expression of ALP in osteoblast cells, the western blot analysis was performed. The

cultured cells were treated with RCM for 12 days. The cells were washed twice with PBS and then lysed, homogenized and sonicated in a RIPA lysis buffer containing 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40 and 10 mM EDTA. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 ×g for 10 min at 4°C. Twenty microgram protein was denatured in SDS sample buffer and resolved on the 10% SDS-polyacrylamide gel. Proteins were transferred in 25 mM Tris, 192 mM glycine, and 20% methanol to polyvinylidene difluoride (PVDF) membrane. Blots were blocked with Tris-buffered saline [20 mM Tris-HCl (pH 7.5) and 137 mM NaCl] plus 0.1% Tween 20 containing 3% dried milk powder. After blocking, the membrane was probed with anti-alkaline phosphatase (Abcam Ltd, Cambridge, UK), and then with peroxidase-labeled antibodies raised against rabbit IgG being used as second antibodies. Peroxidase activity on the PVDF sheet was visualized on X-ray film using an ECL western blotting detection system.

V. *Determination of the Level of Calcification by Alizarin-Red Staining*

After 12 days of incubation with RCM extract in 96-well plate, the calcium deposition of MC3T3-E1 cell cultures were revealed by staining with alizarin-red (AR) solution. The cells were rinsed with PBS, and fixed with 70% EtOH at 4°C for 1 hr. AR solution was freshly prepared as follows: 40 mM of alizarin red was dissolved in 10 mL distilled water, and the pH adjusted to 4.2. After fixing, the cells were stained with the AR solution for 10 min, washed twice with distilled water. The unstained area was washed with PBS and soaked with PBS to prevent drying. The level of the nodule formation was examined under a microscope. AR staining was released from the cell matrix by incubation in 10% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) for 15 min. The AR concentration was determined by measuring the absorbance at 562 nm[22].

VI. *Measurement of Osteocalcin*

After the cells were cultured to 1×10^4 cells/well in a culture dish for 12 days, the culture medium was replaced with α-MEM containing 5% CD-FBS. The cells were then cultured with RCM extracts in the presence of β-GP (10 mM) and vitamin C (50 ng/mL) and the osteocalcin content in the culture medium was measured using a sandwich ELISA assay kit (Biomedical Technologies Inc., USA). Two mouse osteocalcin antibodies were employed, each directed toward one end (C- or N-terminal) of the osteocalcin molecule. The N-terminal antibody is attached bound to the well, which in turns binds the mouse osteocalcin in standard or sample. The biotin-labeled C-terminal mouse osteocalcin antibody completes the sandwich. This sandwich ELISA kit was specific for intact mouse osteocalcin only. Both carboxylated and decarboxylated mouse osteocalcin were recognized. The osteocalcin secretion was normalized by total protein content. The total protein was determined using of BCA protein assay kit (Bio-Rad, CA, USA).

VII. *Reverse Transcription-polymerase Chain Reaction (RT-PCR)*

Total RNA was extracted from osteoblast-like cells grown in 100 mm tissue culture dishes of modified surfaces using Trizol reagent (GibcoBRL, Grand Island, NY). RT-PCR was performed using the TAKARA RT-PCR kit (Takara). The PCR products were analyzed by electrophoresis using a 2% agarose gel. Primer pairs for ALP (F:5'-CCA AGACGTACAACACCAACGC-3', R: 5'-AAATGCTGATGAGGTCCAGG-3'), OCN(F: 5'-AAATGCTGATGAGGTCCAGGC-3', R: 5'-ACCGTAG-ATGCGTTTGTAGGC-3'), Collagen type I (F: 5'-CTCCG-GCTCCTGCTCCTCTTA-3',R:5'-GCACAGCACTCGCCCTCCC-3'), OPN (F: 5'-GATGAATCTGACGAATCTAC-3', R: 5'-CTGCTTAATCCTCACTAACAC-3'), GAPDH (F: 5' – TGAGAACGGGAAGCTTGTCA-3', R: 5'-GGAAGGC-CATGCCAGTGA-3') were used.

VIII. *Statistical Analysis*

The data were expressed as mean ± S.D. Statistical analysis was performed by one-way ANOVA ($p < 0.05$) using SAS statistical software.

RESULTS

I. *Effect of RCM on the Proliferation of MC3T3-E1 Cells*

The effect of RCM at concentrations of 1, 5, 10, 100 μg/mL on the growth of mouse osteoblast-like MC3T3-E1 cells was investigated by an MTT assay[23]. The proliferation of MC3T3-E1 cells cultured with 5 μg/mL RCM extract was boosted by up to 152% in comparison with the control. The following values were found for other concen-

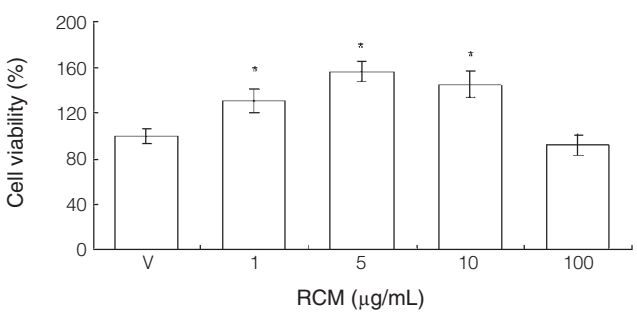

Figure 1. Effect of RCM on the proliferation on MC3T3-E1 cells. Viable cells, as determined by MTT assay. MC3T3-E1 cells (1×10^4 cell/well) were plated and cultured 48 hr in the absence (V) or presence of different concentrations of RCM (1-100 μg/mL). Each bar represents means ± SD. *$p < 0.05$ vs. control. V, vehicle control.

trations: 1 μg/mL, over 131%; 10 μg/mL, 142%; and 100 μg/mL, lower than 90% (Figure 1). Therefore, the optimal concentration of RCM extract without any inhibitory effect was determined to be in the range of 1-10 μg/mL.

II. *Effect of RCM on the Alkaline Phosphatase Activity of MC3T3-E1 Cells*

Osteoblast cells were adjusted to 1×10^4 cells/well in the differentiation medium, which was treated with the RCM extract at concentrations of 1-100 μg/mL, and incubated for 12 days, and the ALP enzyme activity was then determined. As shown in Figure 2A, ALP activity of MC3T3-E1 cells cultured with RCM at 10 μg/mL increased by 4.3 folds at 12 days ($p < 0.05$). Total protein extracts were also examined for ALP protein expression by Western blotting (Figure 2B).

III. *Effect of RCM by the Staining*

To confirm the induction of the ALP enzyme in osteoblasts, the AZO staining method was employed. Naphthol AS-BI phosphate is hydrolyzed into orthophosphate and naphthol by ALP present in tissues and the released naphthol binds to the diazonium compound present in the reaction mixture, forming an AZO dye, which thus stains the active site of the enzyme red. The RCM extract was added at a concentration of 10 μg/mL, cells were cultured for 12 days and the staining level of the ALP enzyme in cells was assessed. As shown in Figure 3A, in the control group without any RCM treatment, the red enzyme was not formed abundantly. On the other hand, in the group treated with the RCM extract, the red stained enzyme was generated in large quantity, and the cells were found changed to red overall. Therefore, it is confirmed that the activation of ALP is induced in osteoblasts by the RCM extract. Also, calcification formation was examined by the Alizarin-Red staining method. Consistent with ALP activity result, increased mineralization/bone nodule formation was observed upon the treatment with RCM (Figure 3B). The Alizarin-Red concentration was determined by measuring the absorbance at 562 nm (Figure 3C).

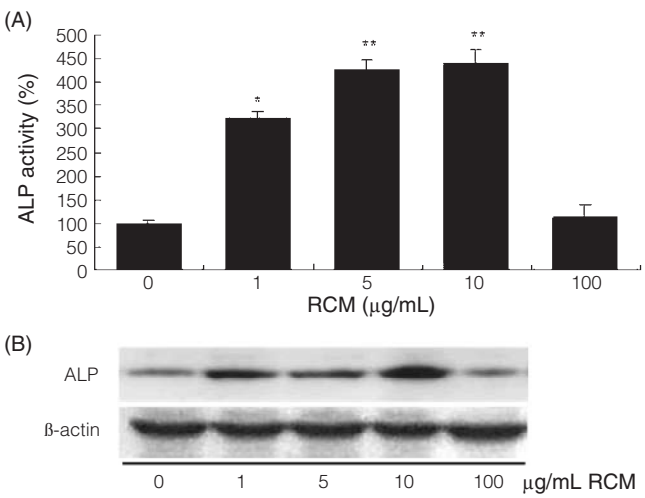

Figure 2. Effect of RCM on the alkaline phosphatase activity in MC3T3-E1 cells. (A) ALP activity was determined with extracellular enzyme. MC3T3-E1 cells were seeded at 70~80% density in 96 well plate and grown for 12 days in α-MEM containing 10% FBS, vitamin C (50 ng/mL), and β-glycerophosphate (10 mM). Cells were treated with the indicated concentrations of RCM extract. (B) Total protein extracts were also examined for ALP protein expression by Western blotting. MC3T3-E1 cells were seeded at 70~80% density in 6well plate. After 24 hr incubation, the medium exchanged with α-MEM containing 10% FBS, vitamin C (50 ng/mL), and β-glycerophosphate (10 mM). Cells were also treated with the indicated concentrations of RCM extract for 12 days. β-actin was used as an internal control. Each bar represents means ± SD. *$p < 0.05$ vs. control.

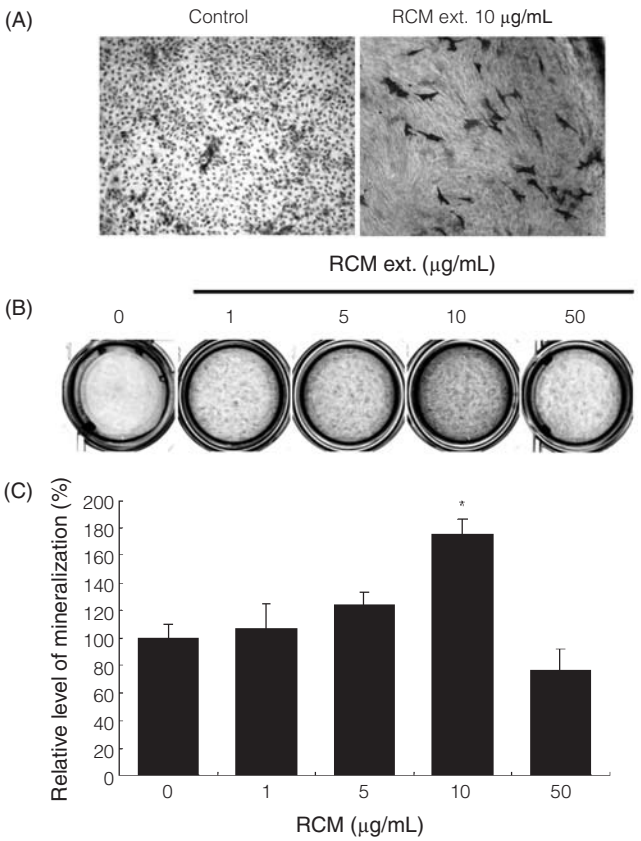

Figure 3. Effect of RCM extract on MC3T3-E1. Cells were cultured with α-MEM containing 10% FBS, 50 μg/mL ascorbic acid, and 10 mM β-glycerol phosphate. (A) Alkaline phosphatase of extracellular matrix by MC3T3-E1 cells. ALP staining assay was carried out with treatment of cells with vehicle control and 10 μg/mL RCM extract. After 12 days, cells were fixed and stained. (B) RCM-induced bone nodules formation in MC3T3-E1 cells. Alizarin Red staining of mineralized nodules in MC3T3-E1 cells treated with 0, 1, 5, 10, 50 μg/mL of RCM extract. Cell treatment was similar to that of ALP staining. (C) Calcium salt deposition was determined as peak nodule formation ratio by absorbance spectra. AR-stained cell was dissolved with 10% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) for 15 min. The AR concentration was determined by measuring the absorbance at 562 nm. Each bar represents means ± SD. *$p < 0.05$ vs. control.

IV. *Effect of RCM on Osteocalcin Secretion*

We further investigated whether RCM plays a role in the secretion of osteocalcin. MC3T3-E1 cells were treated with RCM (1-10 μg/mL) extract and the content of osteocalcin in the medium was measured (Figure 4). The level of osteocalcin was significantly increased at 5 and 10 μg/mL of RCM extract in MC3T3-E1 cell cultures. These results indicated that RCM stimulates osteocalcin synthesis in osteoblasts.

V. *Expression of mRNA in Osteoblast*

The expression of osteoblast differentiation genes in various concentration of RCM was also studied. After 30 cycles of PCR, products of the expected sizes were observed for ALP, OCN, Col-I, OPN and GAPDH (Figure 5). Also, 5 and 10 μg/mL of RCM induced the expression of differentiation gene in osteoblast cell.

DISCUSSION

This report shows that RCM significantly induce cell proliferation and induce osteoblastic differentiation of MC3T3-E1 cells at concentration of 1-10 μg/mL. RCM can promote cell growth, ALP activity, osteocalcin secretion, protein expression and gene expression in osteoblastic MC3T3-E1 cells *in vitro*.

The preosteoblast MC3T3-E1 cell line is an excellent cell differentiation model that simulates the events of early osteoblastogenesis[24]. Cells were stimulated by growth and differentiation factors in the culture medium undergo series of developmental processes that include proliferation of undifferentiated precursors of osteoblasts, which subsequently differentiate into post-mitotic osteoblasts capable of expressing the osteogenic phenotype. During the proliferative phase, these cells undergo DNA synthesis and cell division resulting in rapid increase in cell number until the cultures become confluent. At this juncture, proliferation is down regulated and increased expression of the osteogenic phenotype is observed indicating the presence of mature osteoblasts. Osteoblasts produce alkaline phosphatase, process procollagen to collagen, and deposit extracellular matrix proteins (e.g. osteopotin, bone sialoprotein and osteocalcin) on the substrate, which is subsequently mineralized[25].

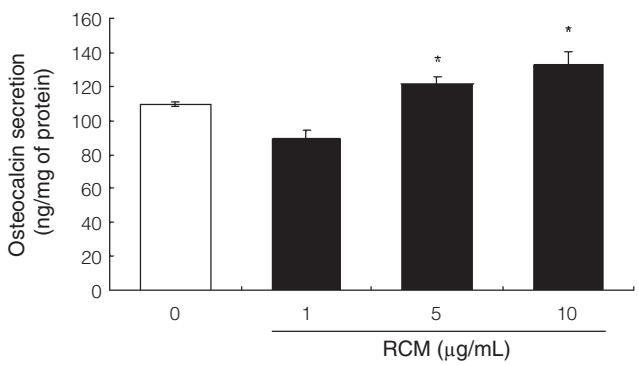

Figure 4. Effect of RCM on the osteocalcin secretion of MC3T3-E1 cells. The cells were treated with various concentrations of RCM (1-10 μg/mL). Osteocalcin secretion was determined with conditioned medium. MC3T3-E1 cells were seeded at 70~80% density in 96-well plate and grown for 12 days in α-MEM containing 10% FBS, vitamin C (50 ng/mL), and β-glycerophosphate (10 mM). Cells were treated with the indicated concentrations of RCM extract. Data shown are mean ± SD., expressed as a percentage of control. *$p < 0.05$ vs. control.

Rubus coreanus Miquel, is used as a folk medicine and as a diuretic. Yet, no data are available as to the formation of bone by this folk medicine. In several studies in which the components of RCM were examined, it was found to contain abundant level of inorganic phosphate, iron, calcium, organic acids, vitamin C and flavonoids. Among the flavonoids, kampferol, quercetin, sanguiin H-5, ellagic acid and 3-O-β-D-glucuronide, etc. have been reported[26]. These flavonoids have multiple beneficial biological activities owing to their antioxidant, anti-inflammatory and estrogenic effects[27,28]. Estrogen exerts bone anabolic effects by promoting key osteoblast functions and inhibiting osteoclastogenesis[29]. Estrogen actions are predominantly mediated by its high affinity to nuclear receptors and many of the flavonoids are considered phytoestrogens owing to their ability to bind and signal via estrogen receptors[30]. In the context of bone health, recent reports indicate that the isoflavonoids genistein and daidzein significantly prevent bone loss in ovariectomized rats[31]. Similar effects were observed with the flavonol, quercetin and kaempferol[32]. To investigate the effect of RCM on bone metabolism, we employed cell culture system. As a results, RCM enhanced the osteocalcin secretion and mRNA expression. The level of osteocalcin was significantly increased at 5 or 10 μg/mL of RCM extract in MC3T3-E1 cell cultures. Horcajada-Molteni *et al*.[32] reported that the effect of quercetin on bone metabolism in ovariectomized rats. They suggested that quercetin increased not only the osteoblastic activity but also osteocalcin concentration in plasma. Osteocalcin is a bone-specific protein and serves as a marker for osteoblastic acivity. It comprises about 15% of the noncollagenous protein components of bone[33]. While the function of this protein in bone is uncertain, its conserved nature among species and its appearance early in the process of mineralization suggest a fundamental role for osteocalcin in the attainment and maintenance of the bone mineral matrix, as well as in bone remodeling[34,35].

On the other hand, Korean medicines, which have been developed over some 3000 year[36]s, are known to have low toxicity over long term usage[37]. RCM, one of the traditional Korean medicines, increased the ALP activity significantly. ALP is the most widely recognized

Journal of Food and Drug Analysis, Vol. 16, No. 1, 2008

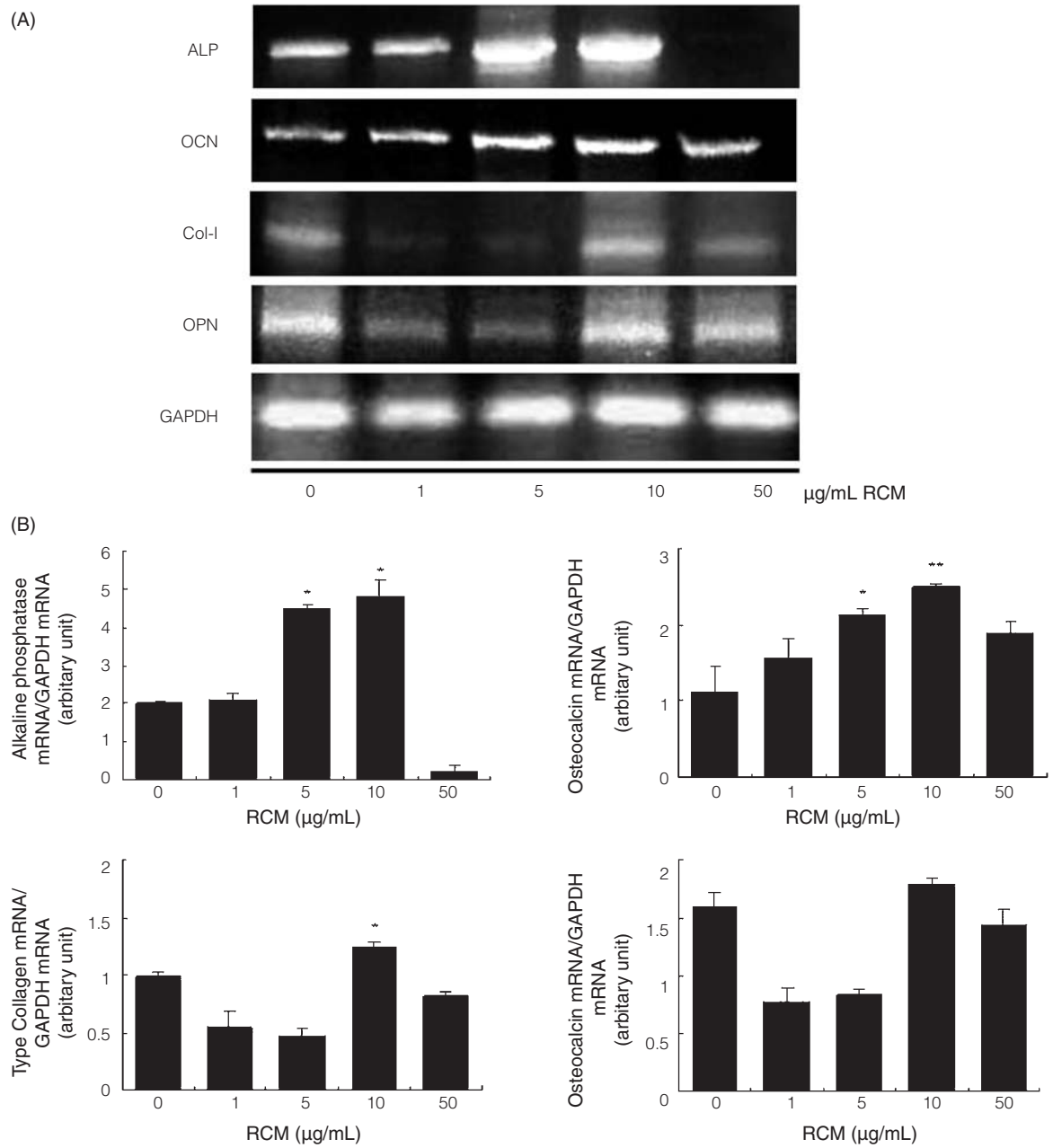

Figure 5. Effect of RCM on the osteoblast differentiation gene expression. (A) The gene expression examined for ALP, OCN (Osteocalcin), Col-I (Type I Collagen), OPN (Osteopontin), GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) by RT-PCR. Cells were treated with the indicated concentrations of RCM in α-MEM containing 10% FBS, vitamin C (50 ng/mL), and β-glycerophosphate (10 mM) for 12 days. Expression of mRNA levels was detected by PCR amplification of cDNA fragment from reverse-transcribed total RNA prepared from mouse osteoblastic MC3T3-E1 cells. GAPDH was used as an internal control. (B) The values were normalized to GAPDH mRNA level of each sample. Each bar represents means ± SD. $*p < 0.05$ vs. control.

biochemical marker for osteoblastic activity. Although its precise mechanism of action is poorly understood, this enzyme is believed to play a role in bone mineralization. Therefore, we screened the effects of various Korean traditional medicine extract (0-100 μg/mL) on the ALP activity of osteoblastic MC3T3-E1 cells (data not shown). Among them, RCM (1-10 μg/mL) is the most effective in inducing the ALP activity.

Moreover, we have examined mineralization formation by the Alizarin-Red staining method. Bone nodule formation is an important marker of the differentiation of osteoblasts. Alizarin is a plant dye with a specifically high affinity for calcium. This dye stains the mineralized matrix of cells, and mineralization is proportional to the level of staining[38,39]. Consistent with ALP activity result, increased mineralization/bone nodule formation

was observed upon the treatment with RCM. Thus, RCM seems to stimulate the proliferation and differentiation of osteoblasts. In addition, RCM (10 μg/mL) accelerated mRNA and protein expression of ALP, osteocalcin, Type I collagen and osteopontin.

In conclusion, these results suggested that RCM is effective for bone formation in bone cells and could be useful for preventing osteoporosis. Although the study had limitations and our findings are preliminary, continued and advanced study on the alterations in gene expression of bone cells and on compounds of RCM will provide a basis for understanding the observed bone cell responses to various pharmacological interventions.

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