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Osteoblastogenesis and Osteoprotection Enhanced by Flavonolignan Silibinin in Osteoblasts and Osteoclasts

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ABSTRACT

Bone-remodeling imbalance induced by decreased osteoblastogenesis and increased bone resorption is known to cause skeletal diseases such as osteoporosis. Silibinin is the major active constituent of silymarin, the mixture of flavonolignans extracted from blessed milk thistle (*Silybum marianum*). Numerous studies suggest that silibinin is a powerful antioxidant and has anti-hepatotoxic properties and anti-cancer effects against carcinoma cells. This study investigated that silibinin had bone-forming and osteoprotective effects in in vitro cell systems of murine osteoblastic MC3T3-E1 cells and RAW 264.7 murine macrophages. MC3T3-E1 cells were incubated in osteogenic media in the presence of 1–20 μM silibinin up to 15 days. Silibinin accelerated cell proliferation and promoted matrix mineralization by enhancing bone nodule formation by calcium deposits. In addition, silibinin furthered the induction of osteoblastogenic biomarkers of alkaline phosphatase, collagen type 1, connective tissue growth factor, and bone morphogenetic protein-2. Differentiated MC3T3-E1 cells enhanced secretion of receptor activator of nuclear factor-κB ligand (RANKL) essential for osteoclastogenesis, which was reversed by silibinin. On the other hand, RAW 264.7 cells were pre-incubated with 1–20 μM silibinin for 5 days in the presence of RANKL. Non-toxic silibinin markedly attenuated RANK transcription and intracellular adhesion molecule-1 expression elevated by RANKL, thereby suppressing the differentiation of macrophages to multi-nucleated osteoclasts. It was also found that silibinin retarded tartrate-resistant acid phosphatase and cathepsin K induction and matrix metalloproteinase-9 activity elevated by RANKL through disturbing TRAF6-c-Src signaling pathways. These results demonstrate that silibinin was a potential therapeutic agent promoting bone-forming osteoblastogenesis and encumbering osteoclastic bone resorption. J. Cell. Biochem. 113: 247–259, 2012.

KEY WORDS: BONE RESORPTION; MATRIX MINERALIZATION; OSTEOBLAST; OSTEOCLAST; SILIBININ

Bone remodeling entails the elimination of mineralized bone by osteoclasts, followed by the formation of bone matrix by osteoblasts subsequently becoming mineralized [Tanaka et al., 2005; Henriksen et al., 2009]. The constant process of bone remodeling consists of four consecutive stages [Hadjidakis and Androulakis, 2006]: bone resorption, during which osteoclasts gnaw old bone; reversal, in which mononuclear cells appear on the bone surface; bone formation, in which osteoblasts completely replace resorbed bone with new bone; and bone mineralization, during which osteocytes are embedded within the bone matrix. Regulation of osteoclastogenesis by osteoclasts usually present at the bone surface is essentially mediated via osteoprotegerin (OPG)/receptor activator of nuclear factor-κB ligand (RANKL)/RANK system [Khosla, 2001]. Pre-osteoblastic/stromal cells produce RNAKL for osteoclastogenesis and also generate OPG, a decoy receptor for RANKL, which inhibits osteoclast differentiation and function by interrupting the interaction between RANKL and RANK. Therefore, through the OPG/RANKL/RANK system the processes of bone resorption and

Abbreviations used: ALP, alkaline phosphatase; BMP, bone morphogenetic protein; CTGF, connective tissue growth factor; ECM, extracellular matrix; ICAM-1, intracellular cell adhesion molecule-1; MMP-9, matrix metalloproteinase-9; MT-1 MMP, membrane type-1 matrix metalloproteinase; RANKL, receptor activator of nuclear factor-κB ligand; c-Src, cellular-sarcoma; TRAF, tumor necrosis factor receptor-associated factor; TRAP, tartrate-resistant acid phosphatase.

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formation are tightly coupled allowing a wave of bone formation to follow each cycle of bone resorption, maintaining skeletal integrity [Khosla, 2001; Hadjidakis and Androulakis, 2006]. If any, a disruption of the OPG/RANKL/RANK system prevents bone resorption and osteoclastogenesis.

Osteogenesis is a strictly regulated developmental process, in which numerous hormones and growth factors activate osteoblast-specific signaling proteins and transcription factors required for osteoblast differentiation [Qi et al., 2003; Franceschi et al., 2007]. During differentiation osteoblasts produce a matrix of osteoid, which is composed mainly of collagen type 1 with expression of alkaline phosphatase (ALP). Osteoblasts are responsible for the mineralization of extracellular matrix (ECM), the terminal step of osteoblast differentiation [Franceschi et al., 2007; Huang et al., 2007]. In addition, bone morphogenetic proteins (BMP) are required for normal postnatal bone formation and osteoblast differentiation. Several natural compounds have been shown to promote osteoblast differentiation and bone-forming activity via a BMP signaling pathway [Lo et al., 2010; Trzeciakiewicz et al., 2010]. However, the underlying mechanism(s) behind the positive effects of natural compounds on osteogenesis remain to be elucidated. One possible mechanism involves redox signaling in osteoblast differentiation [Rached et al., 2010; Imhoff and Hansen, 2011]. Natural compounds as antioxidants influencing intracellular redox status can be positive regulators of bone formation by regulating redox-sensitive elements that are involved in the differentiation signaling [Lo et al., 2010; Trzeciakiewicz et al., 2010].

It was also shown that redox status diminishes during osteoclast differentiation, regulating RANKL-induced osteoclastogenesis [Huh et al., 2006]. Through the mechanisms of osteoclastogenesis and activation of bone resorption, RANK signaling pathway providing the molecular basis for developing therapeutics to treat osteoporosis and other diseases of bone loss [Wada et al., 2006]. RANKL induces TRAF6 activation, which leads to stimulation of nuclear factor for activated T cells (NFAT) c1 responsible for osteoclast differentiation. The targeted disruption of c-Src tyrosine kinase, downstream of TRAF6, impairs bone resorbing activity of osteoclasts [Miyazaki et al., 2006]. Epigallocatechin-3-gallate has an anti-osteoclastogenic effect by inhibiting RANKL-induced the activation of JNK/c-Jun and nuclear factor κB (NF-κB) pathways [Lee et al., 2010]. Kaempferol exerts profound anti-osteoclastogenic effects by antagonizing TNF receptor family action on bone cells by disrupting production of osteoclastogenic cytokines from osteoblasts and attenuating osteoclast precursor cell differentiation [Pang et al., 2006]. Dried plum polyphenols inhibit osteoclastogenesis by down-regulating NFATc1 and inflammatory mediators [Bu et al., 2008]. It is deemed that the inhibition of osteoclastogenesis by these compounds may be partially attributed to their anti-inflammatory and antioxidant properties. Accordingly, natural compounds and dietary components with antioxidant and anti-inflammatory activity may optimize bone health and stimulate bone formation.

Silibinin (Fig. 1A), also known as silybin, is the major active constituent of the natural compound silymarin, the isomeric mixture of flavonolignans extracted from milk thistle (*Silybum marianum*) consisting of silibinin A and B, isosilibinin A and B, silicristin, and silidianin. Silibinin is known to be an effective

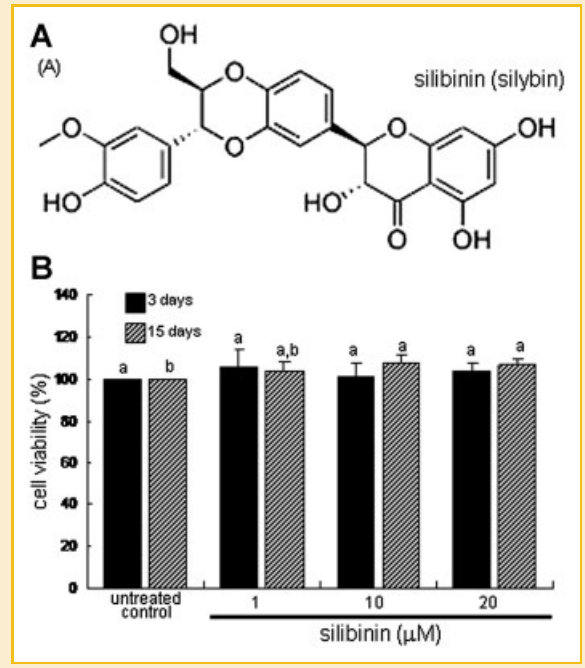

Fig. 1. Chemical structure of silibinin (A) and cell viability of MC3T3-E1 cells (B). Osteoblastic MC3T3-E1 cells were cultured for 3 or 15 days in the presence of 1–20 μM silibinin (B). Cell toxicity was measured by MTT assay. Values are means ± SEM (n = 5) and expressed as percent cell survival relative to untreated cells (cell viability = 100%). Values in bar graphs that do not share the same letter group indicate significant difference at $P < 0.05$.

bioactive antioxidant, conserving glutathione in live cells while stabilizing the cell membranes against oxidative attack [Ha et al., 2010]. Both in vitro cell and in vivo animal studies demonstrate that silibinin has hepatoprotective activity that protects cells against toxic compounds [Jayaraj et al., 2007; Al-Anati et al., 2009]. In addition, silibinin possesses anti-cancer effects against various human carcinoma cells [Sharma et al., 2003; Hogan et al., 2007; Mokhtari et al., 2008]. However, there is little information available on the actions of silibinin in bone-remodeling process. It was shown that silibinin had the potential to inhibit osteoclast differentiation by attenuating the downstream signaling cascades associated with RANKL and TNF-α [Kim et al., 2009].

This study attempted to evaluate both the promoting effect of silibinin on osteoblastogenesis of MC3T3-E1 cells and its inhibitory effects on osteoclastogenesis of RAW264.7 macrophages. Murine MC3T3-E1 cells were cultured in osteogenic media for 15 days to differentiate to mature osteoblasts, and murine RAW 264.7 macrophages were cultured in RANKL-supplemented media for 5 days to differentiate to multi-nucleated osteoclasts. The stimulating effects of silibinin on induction of osteoblastogenic markers of ALP, collagen type 1, connective tissue growth factor (CTGF), and BMP-2 and on matrix mineralization were examined in murine osteoblastic MC3T3-E1 cells. In addition, the dampening effects of silibinin on tartrate-resistant acid phosphatase (TRAP) activity, induction of osteoclastogenic markers of ICAM-1, MMP-9, and cathepsin K, and on bone resorption signaling of TRAF6 and

c-Src tyrosine kinase were examined in murine RAW 264.7 macrophages.

MATERIALS AND METHODS

MATERIALS

Fetal bovine serum (FBS), penicillin–streptomycin, trypsin–EDTA were purchased from Lonza (Walkersville, MD). 3-(4,5-Dimethylthiazol-yl)-diphenyl tetrazoliumbromide (MTT) was provided by DUCHEFA Biochemie (Haarlem, The Netherlands). Minimum essential medium alpha medium (α-MEM), Dulbecco's modified Eagle's media (DMEM), Alizarin Red S, RANKL, and silibinin were supplied by Sigma–Aldrich Chemicals (St. Louis, MO), as were all other reagents, unless specifically stated elsewhere. Antibodies of mouse CTGF and BMP-2 were provided by AbCam (Cambridge, UK). Antibodies of mouse collagen type 1, ICAM-1 and cathepsin K, TRAF6, and c-Src were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and donkey anti-goat IgG were obtained from Jackson ImmunoReserach Laboratories (West Grove, PA). Reverse transcriptase and Taq DNA polymerase were purchased from Promega (Madison, WI).

Silibinin was dissolved in dimethyl sulfoxide (DMSO) for live culture with cells; a final culture concentration of DMSO was $<0.5\%$.

MC3T3-E1 CELL CULTURE AND OSTEOBLAST DIFFERENTIATION

MC3T3-E1 cells (mouse calvaria origin) were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. MC3T3-E1 was cultured in α-MEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For the osteoblast differentiation [Kwun et al., 2010], MC3T3-E1 cells were seeded on 24-well plates and grown to ≈90% confluence, and then the culture medium was changed to a fresh normal osteogenic medium containing 10 mM β-glycerolphosphate and 50 μg/ml ascorbic acid in the presence of silibinin to initiate matrix mineralization. Cell culture medium was changed every 3 days for 15 days.

Cell proliferation and toxicity was determined using a colorimetric assay based on the uptake of MTT by viable cells [Kang et al., 2009]. After exposure to various concentrations of silibinin for 15 days, a solution of 1 mg/ml MTT was added to cells and incubated at 37°C to allow cleavage of the tetrazolium ring by mitochondrial dehydrogenase, resulting in the formation of blue formazan crystals. After 3 h, the purple formazan product was dissolved in 0.5 ml of isoprophanol with gentle shaking. Absorbance of formazan dye was measured at $\lambda = 570$ nm with background subtraction using $\lambda = 690$ nm.

MEASUREMENT OF ECM CALCIUM DEPOSITS

Staining with Alizarin red S is used to visualize bone nodule formation and calcium deposition of osteoblasts cultured in vitro. Since the dye forms a complex with ECM calcium by chelation, heavy reddish staining of Alizarin red S is proportional to area of mineralized ECM [Hale et al., 2000]. At harvest, MC3T3-E1 cells were fixed with 70% ethanol for 1 h and stained with 40 mM Alizarin Red S (pH 4.2) for 10 min. Alizarin red S staining for calcium precipitation were visualized under light microscopy.

To quantify bound dye, the stain was solubilized in 10 mM sodium phosphate buffer (pH 7.0) with 1 ml 10% (w/v) cetylpyridinum chloride. Absorbance of the solubilized stain was subsequently measured at $\lambda = 562$ nm by spectrophotometer.

MEASUREMENT OF ALP ACTIVITY

ALP activity of osteoblastic MC3T3-E1 cells was measured by a modified colorimetric enzyme assay [Akcakaya et al., 2007]. After culture protocols, cells were lysed with 0.2% Triton X-100 and the lysates were centrifuged at 14,000*g* for 10 min at 4°C. Lysate aliquots were incubated with 0.5 M Tris–HCl (pH 9.9) containing 6 mM *p*-nitrophenyl phosphate and 1 mM $MgCl_2$ at 37°C for 1 h. The protein contents of lysates were determined by the Lowry method. ALP activity was expressed as nmol *p*-nitrophenol (PNP) produced/min/mg protein. Absorbance was measured at $\lambda = 405$ nm and compared with PNP standards.

WESTERN BLOT ANALYSIS

Western blot analysis was carried out using culture medium and cell lysates prepared from cultured osteoblastic MC3T3-E1 cells and macrophage RAW264.7 cells. Equal volumes of culture supernatants and equal amounts of cell lysate proteins were electrophoresed on 6–12% SDS–PAGE gels and transferred onto a nitrocellulose membrane. Non-specific binding was blocked by soaking membranes in a TBS buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20] containing 3% bovine serum albumin or 5% non-fat milk for 3 h. The membranes were incubated with a primary antibody to mouse collagen type 1, mouse CTGF, mouse BMP-2, mouse ICAM, mouse cathepsin K, mouse TRAF6, and mouse c-Src. The membranes were then incubated with a secondary antibody, a goat anti-rabbit IgG, goat anti-mouse IgG, or donkey anti-goat IgG conjugated to horseradish peroxidase. The protein levels on gels were determined by using Supersignal West Pico Chemiluminescence detection reagents (Pierce Biotechnology, Rockford, IL), and Konica X-ray film (Konica, Tokyo, Japan). Incubation with monoclonal mouse β-actin antibody was conducted for comparative control.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

To determine the RANKL secretion from MC3T3-E1 cells, culture media were assayed by using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

RAW 264.7 CELL CULTURE AND OSTEOCLAST DIFFERENTIATION

Murine macrophage RAW264.7 cells were cultured in DMEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. For the osteoclast differentiation [Kwun et al., 2010], cells were seeded on 24-well plates and grown to ≈90% confluence in α-MEM containing 50 ng/ml RANKL for 5 days. Cell culture media were changed every 2 days for 5 days. RAW264.7 cell proliferation and silibinin cytotoxicity were also determined using MTT assay.

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

Total RNA was isolated from RAW264.7 cells using a commercially available Trizol reagent kit (Invitrogen, Carlsbad, CA). The RNA (5 μg) was reversibly transcribed with 200 U of reverse transcriptase and 0.5 mg/ml oligo-$(dT)_{15}$ primer (Bioneer, Daejeon, Korea). The expressions of the mRNA transcripts of RANK (forward primer: 5′-AGATGTGGTCTGCAGCTCTTCCAT-3′, reverse primer: 5′-ACACACTTGTTGCTGACTGGAGGT-3′, 350 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, forward primer: 5′-AACTTTGGCATTGTGGAAGGG-3′, reverse primer: 5′-GACACATTGGGGGTAGGAACAC-3′, 224 bp) were evaluated by RT-PCR. The housekeeping gene GAPDH was used for internal normalization. The PCR was performed in 25 μl of 10 mM Tris–HCl (pH 9.0), 25 mM $MgCl_2$, 10 mM dNTP, 5 U of Taq DNA polymerase, and 10 μM of each primer and started with 5 min denaturation at 95°C followed by 28 PCR cycles. Each cycle consisted of 30 s at 95°C, 30 s at 60°C, and 45 s at 72°C, and the final extension was for 10 min at 72°C. After thermocycling and electrophoresis of the PCR products (10 μl) on 1% agarose gel containing 0.5 mg/ml ethidium bromide, the bands were visualized using a TFX-20 M model-UV transilluminator (Vilber-Lourmat, Marne-la-Vallée, France) and gel photographs were obtained. The absence of contaminants was routinely checked by the RT-PCR assay of negative control samples without a primer addition.

IMMUNOCYTOCHEMICAL ANALYSIS

Raw 264.7 cells grown on glass slides were washed with phosphate buffered saline containing 0.2% Tween 20, fixed with 4% ice-cold formaldehyde for 20 min and made permeable with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. For blocking any non-specific binding, cells were incubated for 1 h with 20% FBS. After being washed, fixed cells were incubated overnight with polyclonal rabbit anti-mouse ICAM at 4°C. FITC-conjugated anti-rabbit IgG was added as a secondary antibody. Fluorescent images were taken with an Axiomager Optical fluorescence microscope (Carl Zeiss, Germany).

MEASUREMENTS OF TRAP ACTIVITY

After silibinin protocol, RAW 264.7 cells were fixed with 4% formalin solution for 10 min and stained using leukocyte acid phosphatase kit (Sigma Chemicals), according to the manufacturer's instructions. TRAP-positive multinucleated cells were visualized and under light microscopy.

For measuring the TRAP activity, cells were fixed with 4% formalin solution for 10 min and 95% ethanol for 1 min. Subsequently, the dried cells were incubated in 10 mM citrate buffer (pH 4.6) containing 10 mM sodium tratrate and 5 mM *p*-nitrophenylphosphate. After incubation for 1 h, the reaction mixtures were transferred to new well plates containing an equal volume of 0.1 N NaOH. Absorbance was measured at $\lambda = 405$ nm by spectrophotometer, and the TRAP activity was expressed as percent of that of untreated control.

BONE RESORPTION ASSAY

The osteoclast bone resorption assay was performed by using a commercially available bone resorption assay kit (CosMo Bio, Tokyo, Japan). Raw 264.7 cells were suspended in phenol red-free α-MEM containing 10% FBS and plated at a density of 1×10^4 cells/well in the absence and presence of 50 ng/ml RANKL and 1–20 μM silibinin. After 5 days culture, conditioned media was collected and incubated with resorption assay buffer in a 96-well plate. The fluorescence intensity was measured at an excitation wavelength of 485 nm and emission wavelength of 535 nm. To measure the pit areas, the cells were washed in 6% NaOCl to remove cells. The resorbed areas on the plate were visualized under light microscopy.

GELATIN ZYMOGRAPHY

Cells were starved for 24 h in DMEM containing 0.1% BSA instead of 15% FBS for the analysis of gelatin zymography. After 3 days culture in α-MEM containing 10% FBS and 24 h starvation, culture media were collected and gelatin zymography for MMP-9 was performed. Culture supernatants were subject to electrophoresis on 8% SDS–PAGE (300 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 0.03% bromophenol blue) co-polymerized with 1% gelatin as the substrate. After electrophoresis was complete, the gel was incubated for 1 h at 37°C in a 2.5% Triton X-100 solution, washed in 50 mM Tris–HCl buffer (pH 7.5) for 30 min, and incubated for 20 h in incubation buffer containing 200 mM NaCl, 10 mM $CaCl_2$, and 0.05% Brij-35. The gel was stained with 0.1% Coomassie Brilliant Blue G-250, 2% acetic acid, and 45% methanol, and then destained in a solution with 30% methanol and 10% acetic acid. Gelatinolytic activity was detected as unstained bands against the background of Coomassie blue-stained gelatin. The active form of MMP-9 was identified as bands at 90 KDa in relation to the relative mobility of marker proteins.

STATISTICAL ANALYSES

The data are presented as means ± SEM. Statistical analyses were carried out employing Statistical Analysis Systems statistical software package (SAS Institute, Cary, NC). Significance was determined by one-way ANOVA, followed by Duncan range test for multiple comparisons. Differences were considered significant at $P < 0.05$.

RESULTS

PROLIFERATION AND ECM MINERALIZATION BY SILIBININ IN DIFFERENTIATED OSTEOBLASTS

MC3T3-E1 cell proliferation was minimally increased by 1–20 μM silibinin during 15-day differentiation in osteogenic medium (Fig. 1B), suggesting that silibinin is not cytotoxic in stimulating osteoblast differentiation.

In undifferentiated MC3T3-E1 cells no calcium deposit was observed, whereas there was a substantial staining of Alizarin red S in cells differentiated for 15 days in osteogenic medium(Fig. 2A). This indicates that bone nodule formation by calcium deposits was markedly enhanced. However, treating MC3T3-E1 cells with 1–20 μM silibinin further augmented calcium deposits in a

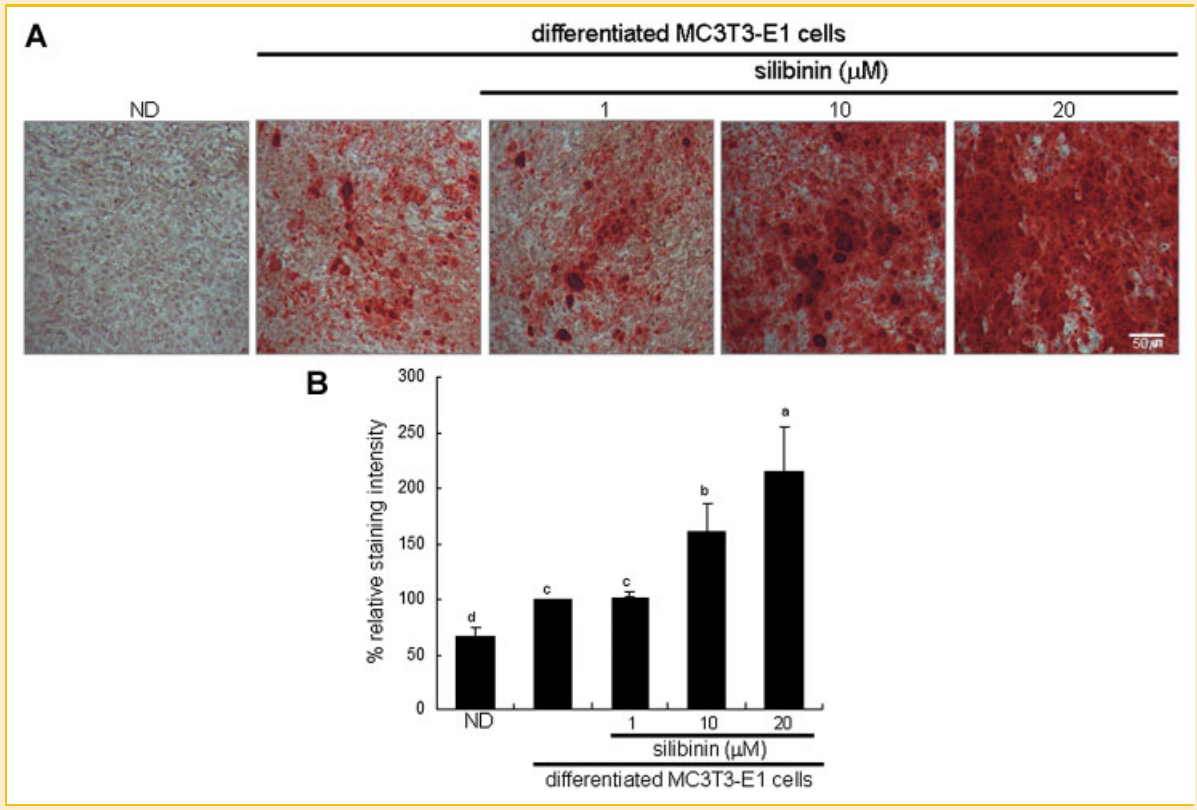

Fig. 2. Enhancement of calcium precipitation (matrix mineralization) in osteoblastic MC3T3-E1 cells by silibin. Extracellular matrix (ECM) calcium deposits (bone nodule formation) for matrix mineralization were measured by Alizarin red S staining. MC3T3-E1 osteoblasts were cultured in 24-well plates for 15 days in the presence of 1–20 μM silibinin. Microphotographs were representative cells after 15 days of growth are shown. Dark reddish staining of Alizarin red S is proportional to the area of mineralized ECM. The intensity of Alizarin red S staining was quantified at $\lambda = 562$ nm. The bar graphs (means ± SEM, n = 5) represent quantitative results obtained from spectrophotometer. Values (means ± SEM, n = 5) not sharing a letter indicate significant different at $P < 0.05$.

dose-dependent manner. The quantitative intensity of Alizarin Red S staining was consistent with its image patterns (Fig. 2A,B).

ENHANCEMENT OF BONE-FORMING BIOMARKERS OF MC3T3-E1 CELLS BY SILIBININ

ALP and collagen type 1 are early and mid-stage biomarkers for matrix maturation of osteoblasts [Komori, 2008]. This study revealed that the cellular ALP activity increased during 6-day differentiation in normal osteogenic medium (Fig. 3A), which was positively influenced by treatment with ≥10 μM silibinin. Similarly, Western blotting analysis showed that the treatment with silibinin in osteogenic medium dose-dependently increased collagen type 1 secretion (Fig. 3B). Since collagen type 1 is a major extracellular collagenous protein for organic matrix formation in bone [Clarke, 2008], it is deemed that silibinin promoted ECM accumulation (Fig. 3B).

Furthermore, osteoblast-derived CTGF plays an important role in the osteoblastogenesis possibly by inhibiting NFAT transactivation [Smerdel-Ramoya et al., 2008]. This study evaluated whether silibinin enhanced the CTGF expression during differentiation. Treating silibinin notably elevated CTGF expression in differentiated osteoblasts, which may play a role in the activation of bone formation (Fig. 3C). Protein expression of BMP, another osteoblast differentiation marker [Chen et al., 2004], was markedly increased at the very-early stage of differentiation (data not shown). The BMP-2 induction was further enhanced in ≥10 μM silibinin-treated osteoblasts cultured in osteogenic medium for 1 day (Fig. 3D).

BLOCKADE OF RANKL-INDUCED OSTEOCLAST DIFFERENTIATION BY SILIBININ

Signaling by RANKL is essential for terminal differentiation of monocytes/macrophages into osteoclasts [Takayanagi, 2007]. This study examined a possible link between the osteoblast and the osteoclast precursors, thereby facilitating the interaction between RANKL and its receptor RANK for osteoclast differentiation. It was found that silibinin disturbed RANKL secretion enhanced during the early stage of osteoblast differentiation (Fig. 4A). It should be noted that there was no detectable secretion of RANKL in undifferentiated cells. Thus, RANKL played a role in coupling osteoblastogenesis and osteoclastogenesis. Submicromolar silibinin was not toxic to RAW 264.7 cells and instead the proliferation of murine macrophages occurred due to the silibinin treatment during 2-day differentiation (Fig. 4B). When RAW 264.7 cells were exposed to 50 ng/ml RANKL, the RANK transcription of osteoclasts was fostered. Non-toxic silibinin attenuated the up-regulated RANK transcription (Fig. 4C).

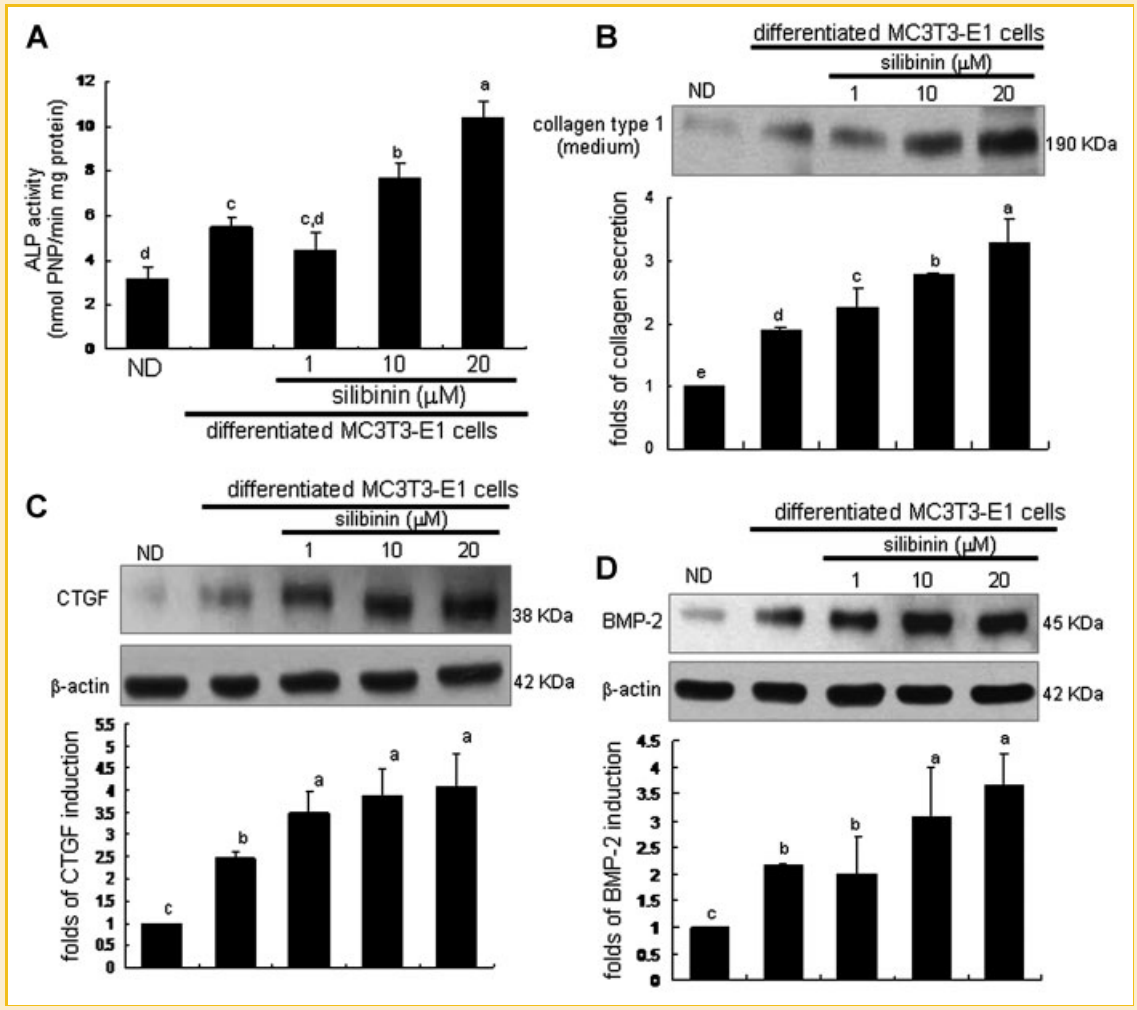

Fig. 3. Up-regulation of alkaline phosphatase activity (ALP, A) and collagen type 1 (B) by silibininn. MC3T3-E1 cells were cultured for 6 days in presence of 1–20 μM silibinin. The ALP enzyme activity in media (secreted) was expressed as nmol PNP/min/mg protein (A). Absorbance was measured at $\lambda = 405$ nm and compared with *p*-nitrophenol (PNP) standard (mean ± SEM, n = 4). Values in bar graphs not sharing a letter indicate significant different at $P < 0.05$. Collagen type 1 secretion was examined by Western blot analysis with a primary antibody against collagen type 1 (B). For the measurement of connective tissue growth factor (CTGF) and bone morphogenetic protein (BMP)-2, MC3T3-E1 cells were cultured for 1 day (BMP-2) or 6 days (CTGF) in presence of 1–20 μM silibinin (C,D). Cell extracts were subject to Western blot analysis with a primary antibody against BMP-2. Representative blot data were obtained from three independent experiments and β-actin protein was used as an internal control. The bar graphs (mean ± SEM, n = 3) in the bottom panels represent quantitative results obtained from a densitometer. Values in bar graphs not sharing a letter indicate significant different at $P < 0.05$.

It has been shown that ICAM-1 plays an active role in the formation and maturation of osteoclasts [Fernandes et al., 2008]. ICAM expression was enhanced on 1st day of 5-day differentiation of RAW 264.7 cells exposed to 50 ng/ml RANKL (Fig. 5A). However, the ICAM induction declined in 1–20 μM silibinin-treated osteoclasts exposed to 50 ng/ml RANKL (Fig. 5B). The immunocytochemical results confirmed the Western blot data showing inhibitory effects of silibinin on ICAM-1 induction. There was no detectable expression of ICAM in undifferentiated cells, while heavy fluorescent staining in 1-day differentiated cells was observed (Fig. 5C). In cells treated with 1–20 μM silibinin, the up-regulated ICAM expression decreased in a dose-dependent manner.

SUPPRESSION OF BONE-RESORPTION BIOMARKERS OF OSTEOCLAST BY SILIBININ

This study attempted to further examine that RANKL differentiated RAW 264.7 macrophages to TRAP-positive multinucleated cells, which was disrupted by silibinin. Mononuclear osteoclast precursor cells and multinucleated osteoclasts are TRAP-positive but RAW 264.7 cells negative for staining [Mbalaviele et al., 1995]. Stimulation with RANKL for 5 days enhanced TRAP activity in differentiated RAW 264.7 cells (Fig. 6A). When cells were incubated with 1–20 μM silibinin, the TRAP activity was notably diminished (Fig. 6B). RANKL treatment augmented bone resorption activity in RAW264.7 cells and silibinin dose-dependently dulled the increased activity (Fig. 6C). Consistently, the number and size of bone

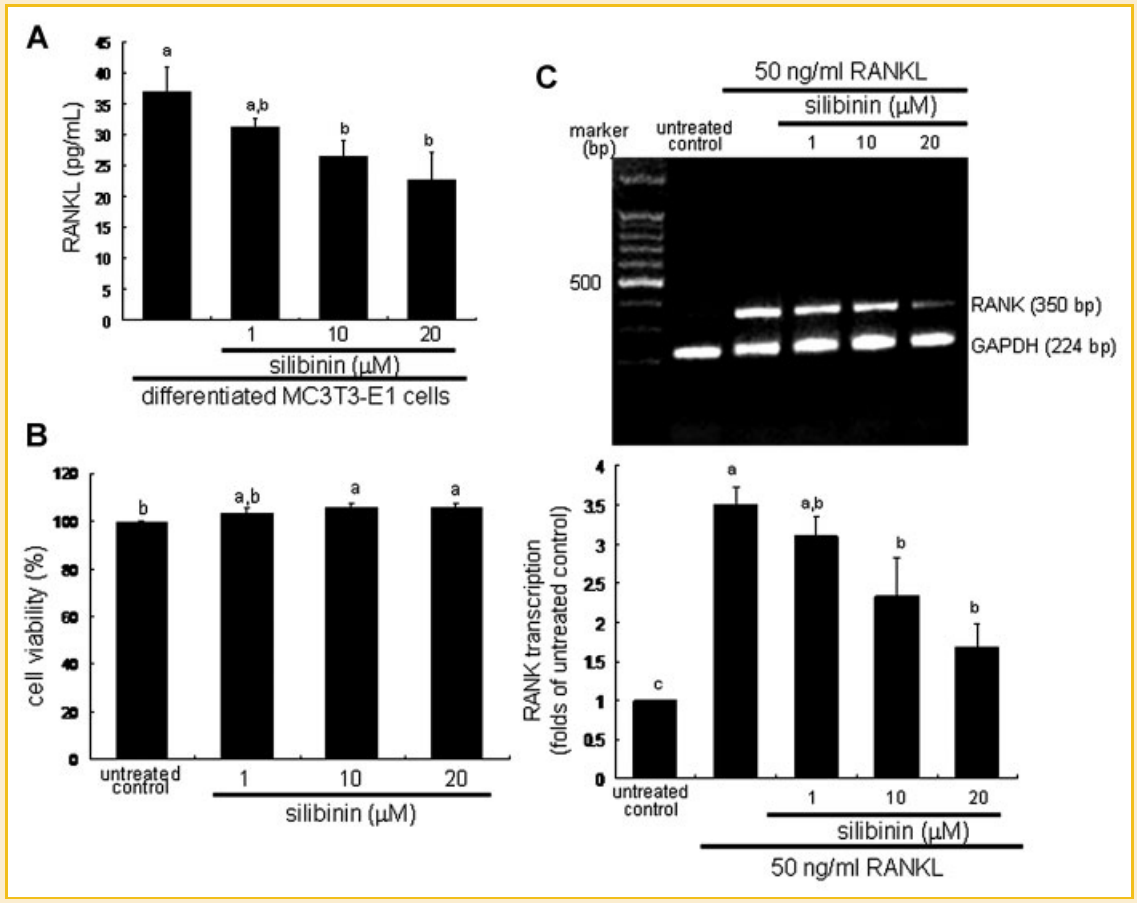

Fig. 4. RANKL secretion of osteoblasts (A), and cell viability (B) and RANK transcription (C) in silibinin-treated osteoclasts. MC3T3-E1 cells were cultured for 3 days in differentiating media containing 1–20 μM silibinin. RANKL secretion from MC3T3-E1 osteoblasts was determined by using a commercial ELISA kit and respective data represent mean ± SEM from three independent experiments (A). RAW 264.7 cells were cultured in DMEM in the absence and presence of silibinin and exposed to 50 ng/ml RANKL for 5 days, and cell viability was measured by MTT assay (B). Values are mean ± SEM (n = 5) and expressed as percent cell survival relative to untreated cells (cell viability = 100%). The RANK mRNA level of osteoclasts was determined by using RT-PCR analysis. GAPDH gene was used as an internal control for the co-amplification with RANK (three separate experiments). The bar graphs (means ± SEM, n = 3) represent quantitative results obtained from a luminometer. Values not sharing a letter are different at $P < 0.05$.

resorption areas increased in RANKL-differentiated cells cultured for 5 days on well plates coated with calcium phosphate which acts as a substrate for the osteoclast. However, silibinin alleviated the resoption ability (Fig. 6D).

The functional osteoclast marker, cathepsin K, is a major protease responsible for the bone resorption [Delaisse et al., 2003]. As expected, cellular cathepsin K expression was elevated during 5 days RANKL-induced osteoclastogenesis (Fig. 7A). The presence of silibinin down-regulated expression and secretion of cathepsin K, suggesting that silibinin was effective in encumbering osteoclast maturation and bone resorption (Fig. 7B). MMP-9 is required for osteoclast maturation, which is requisite for the bone resorption processes mediated by mature osteoclasts [Sundaram et al., 2007]. To clarify whether silibinin modulates RANKL-induced MMP-9 secretion, RAW 264.7 cells were exposed to 50 ng/ml RANKL for 2 days under serum-free conditions. Gelatin zymography data showed that the administration of 50 ng/ml RANKL increased MMP-9 secretion, which was dose-dependently and dramatically ameliorated by silibinin (Fig. 7C,D).

DOWN-REGULATION OF TRAF 6 AND c-Src EXPRESSION BY SILIBININ

RANKL induces activation of the TRAF6 and c-Fos signaling pathways during the differentiation of osteoclast progenitor cells [Wong et al., 1999]. When RAW 264.7 cells were treated with 50 ng/ml RANKL, TRAF 6 expression attained to the peak on 3rd day and gradually subsided afterward (Fig. 8A), and such expression was down-regulated by silibinin supplementation (Fig. 8B). c-Src kinase activity is essential for the regulation of osteoclastic bone resorption [Miyazaki et al., 2006]. Similarly, silibinin inhibited expression of the protein-tyrosine kinase c-Src enhanced by RANKL (Fig. 8C). These results imply that silibinin dampened resorption activity of osteoclasts.

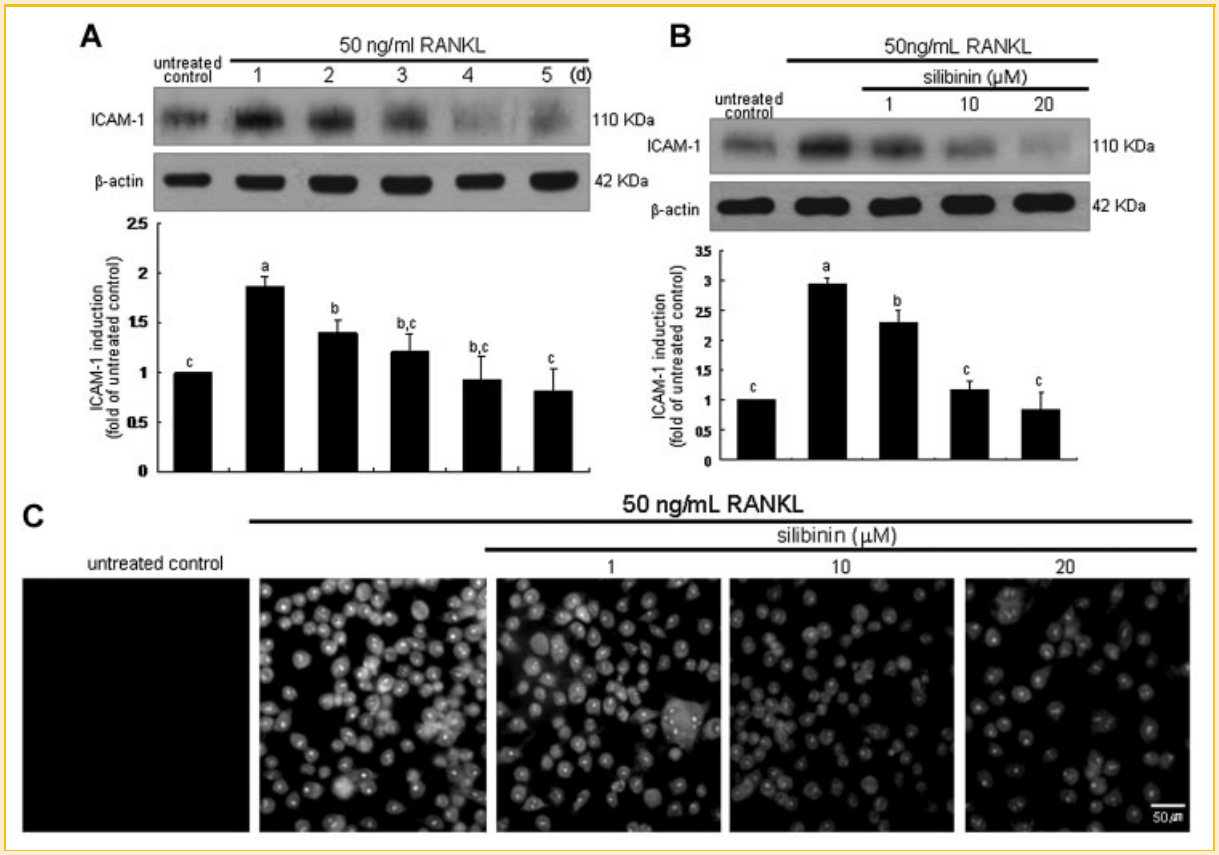

Fig. 5. Time course response (A) and suppression by silibinin (B,C) of RANKL induction of intracellular cell adhesion molecule (ICAM)-1. RAW 264.7 cells were exposed to 50 ng/ml RANKL for 1–5 days in the absence and presence of 1–20 μM silibinin. For Western blot analysis, cells extracts were subject to SDS–PAGE with a primary antibody against ICAM-1. Representative blot data were obtained from three independent experiments, and β-actin protein was used as an internal control. The bar graphs (mean ± SEM, n = 3) in the bottom panels and quantitative results were obtained from a densitometer. Values in bar graphs not sharing a letter indicate significant different at $P < 0.05$. For the immunocytochemical analysis (C), fixed cells were incubated with a primary antibody against ICAM-1. FITC-conjugated anti-rabbit IgG was added as the secondary antibody. Fluorescent microphotographs were taken with a fluorescence microscopy.

DISCUSSION

Six major findings were extracted from this study. (1) Submicromolar silibinin boosted osteoblast proliferation and promoted matrix mineralization possibly with enhancing bone nodule formation by calcium deposits. (2) Silibinin administration furthered the induction of osteoblastogenic proteins of ALP, collagen type 1, CTGF, and BMP-2. (3) Production of RANKL essential for osteoclastogenesis was attenuated in silibinin-treated MC3T3-E1 osteoblasts. (4) Non-toxic silibinin markedly attenuated RANK transcription and ICAM-1 protein expression elevated by RANKL, thereby suppressing the differentiation of macrophages to multinucleated osteoclasts. (5) Silibinin lowered RANKL-stimulated Trap and resorption activity and retarded its cathepsin K induction and gelatinolytic MMP-9 secretion through disturbing TRAF6-c-Src signaling pathways. These observations demonstrate that non-toxic silibinin had bone-forming activity enhancing matrix mineralization and stimulating induction of osteoblastogenic proteins. The results also suggest that silibinin exerted osteoprotective effects blunting osteoclast differentiation and osteoclastic bone resorption through disturbing TRAF6-c-Src signaling pathway. Accordingly, the flavonolignan silibinin was a potential therapeutic agent promoting osteoblastogenesis and encumbering osteoclastogenesis.

Bone remodeling entails the elimination of mineralized bone by osteoclasts, followed by the formation of bone matrix by osteoblasts subsequently becoming mineralized [Tanaka et al., 2005; Henriksen et al., 2009]. Regulation of osteoclastogenesis by osteoclasts present at the bone surface is essentially mediated via OPG/RANKL/RANK system [Hadjidakis and Androulakis, 2006]. Pre-osteoblastic/stromal cells produce RNAKL that binds to RANK on monocytes/macrophages for osteoclastogenesis and also generate OPG, a decoy receptor for RANKL. OPG inhibits osteoclast differentiation and function by interrupting the interaction between RANKL and RANK of osteoclasts. Therefore, the process of bone resorption and formation are tightly coupled allowing a wave of bone formation to follow each cycle of bone resorption [Khosla, 2001; Hadjidakis and Androulakis, 2006]. Accordingly, when RANKL production from osteoblastic cells is blunted, the coupling will be disengaged, thereby preventing bone resorption and osteoclastogenesis. This study found that silibinin suppressed the RANKL production from

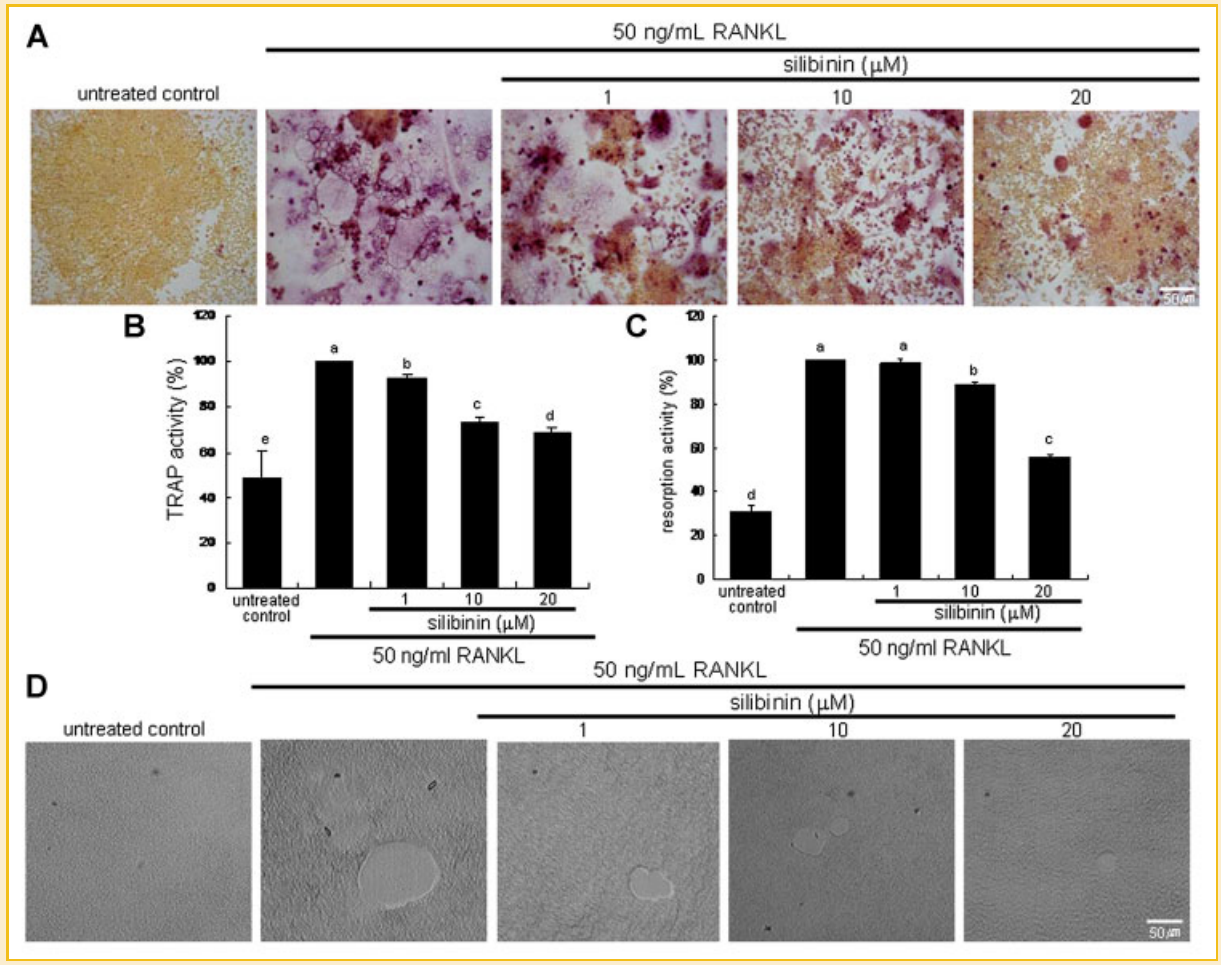

Fig. 6. Inhibition of osteoclast differentiation (A), TRAP activity (B), and bone resorption (C,D) by silibinin. RAW 264.7 cells were exposed to 50 ng/ml RANKL for 5 days in the absence and presence of silibinin. After 5 days culture, cells were fixed and stained using leukocyte acid phosphatase kit, and their TRAP-positive activity was determined. TRAP-positive multinucleated osteoclasts were visualized under light microphotography (A). TRAP activity was measured at $\lambda = 405$ nm (B). The osteoclast bone resorption assay was performed by using a commercially available bone resorption assay kit (C). After 5 days culture, conditioned media was collected and incubated with resorption assay buffer. The fluorescence intensity was spectrophotometrically measured. The bar graphs (mean $\pm$ SEM, $n = 3$) represent quantitative results and values in bar graphs not sharing a letter indicate significant different at $P < 0.05$. The resorbed areas on the plate were visualized under light microscopy (D). Magnification: 200-fold.

MC3T3-E1 osteoblasts, indicating that this compound had a capability to encumber osteoclastogenesis.

Silibinin is the major active constituent of the natural compound silymarin, the isomeric mixture of flavonolignans extracted from milk thistle (*Silybum marianum*) consisting of silibinin A and B, isosilibinin A and B, silicristin, and silidianin. Several animal studies have shown that silymarin and silibinin has hepatoprotective activity against toxins and oxidative attack, in which these compounds act as a bioactive antioxidant [Jayaraj et al., 2007; Al-Anati et al., 2009; Ha et al., 2010]. Silibinin exerts anti-inflammatory and anti-fibrogenic effects on human hepatic stellate cells isolated from human liver [Trappoliere et al., 2009]. However, scant information is available on the actions of silibinin in bone health of healing and regeneration. Silibinin has the potential to inhibit osteoclast differentiation by attenuating the downstream signaling cascades associated with RANKL and TNF-α [Kim et al., 2009]. Consistently, the present study showed that silibinin dampened RANKL-triggered osteoclast differentiation, diminishing the induction of ICAM-1, cathepsin K, and MMP-9, and via the TRAF6-c-Src signaling. Thus, silibinin may exert the potential therapeutic action in monocyte-driven osteoclastogenesis.

Osteogenesis is tightly regulated in the developmental process and osteoblast differentiation requires specific signaling proteins and transcription factors [Qi et al., 2003; Franceschi et al., 2007]. In addition, BMP is required for normal postnatal bone formation and osteoblast differentiation. Several natural compounds have shown to promote osteoblast differentiation and bone-forming activity via BMP signaling pathway [Lo et al., 2010; Trzeciakiewicz et al., 2010]. Silibinin further stimulated BMP-2 expression enhanced during MC3T3-E1 cell differentiation. However, the mechanism(s) underlying the improving effects of natural compounds on osteogenesis remain to be elucidated. Intracellular redox signaling in osteoblast differentiation could influence possible positive regulators of bone formation in differentiation signaling [Rached et al., 2010; Imhoff and Hansen, 2011]. Since silibinin was shown to be a bioactive antioxidant [Jayaraj et al., 2007; Al-Anati et al., 2009; Ha

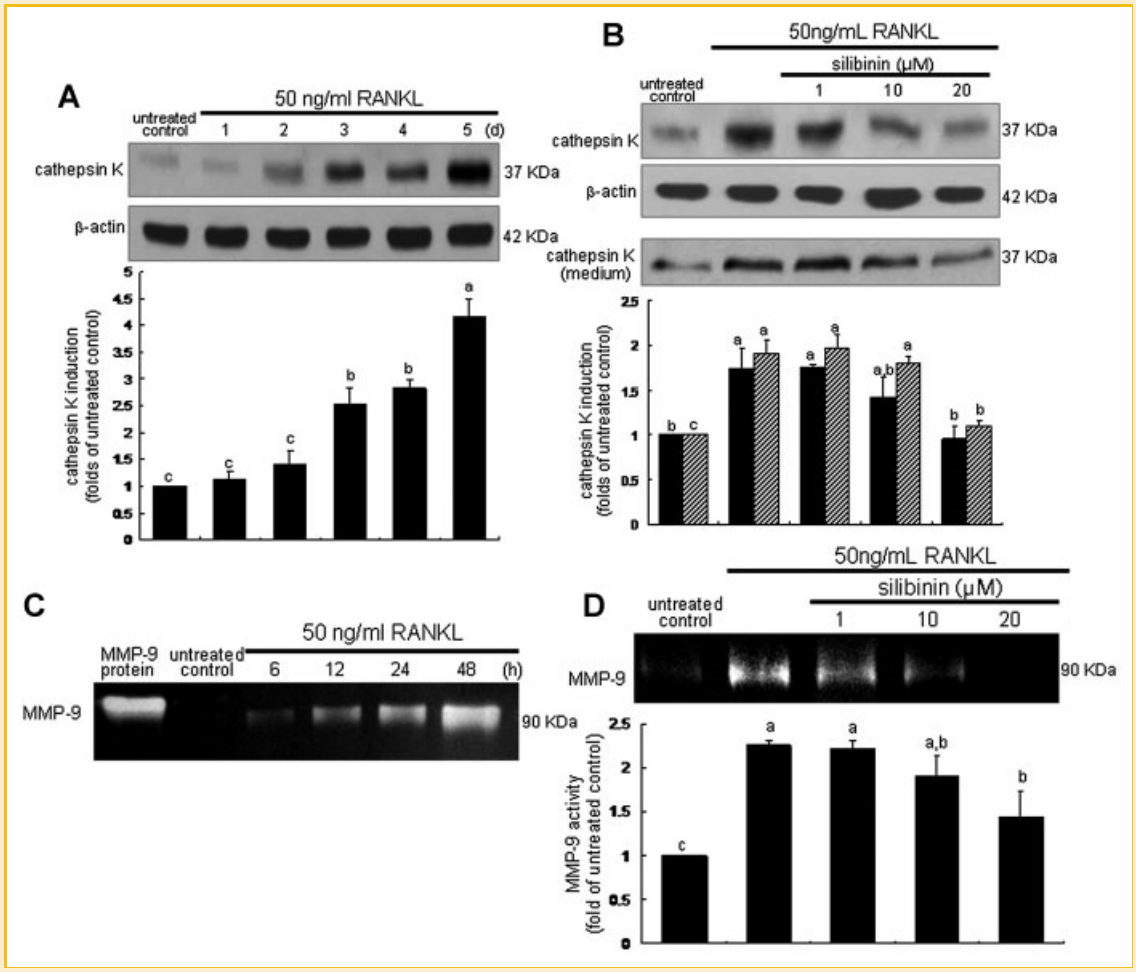

Fig. 7. Western blot data showing time course response of cathepsin K induction by RANKL (A) and its inhibition by silibin (B). RAW 264.7 cells were cultured in DMEM and exposed to 50 ng/ml RANKL for 5 days in the absence and presence of 1–20 μM silibinin. Cell extracts and culture media were subject to SDS–PAGE and Western blot analysis with a primary antibody against cathepsin K. Representative blot data were obtained from three independent experiments, and β-actin protein was used as an internal control. The bar graphs (mean ± SEM, n = 3) in the bottom panels represent quantitative results obtained from a densitometer. Values in bar graphs not sharing a letter indicate significant different at $P < 0.05$. For the gelatin zymography to measure enzyme activity of MMP-9, RAW 264.7 cells were cultured in serum-free DMEM with 50 ng/ml RANKL for differentiation times indicated (C). In another experimental set, RANKL-induced MMP-9 secretion for 48 h was examined in the presence of silibinin (D). Culture media were subject to electrophoresis on 8% SDS–PAGE co-polymerized with 0.1% gelatin as the substrate (three separate experiments). Gelatinolytic activity was detected as unstained against the background of Coomassie blue-stained gelatin. Values in bar graphs not sharing a letter indicate significant different at $P < 0.05$.

et al., 2010], it would modulate cellular redox status, boosting osteogenesis.

During differentiation osteoblasts produce a matrix mainly comprised of collagen type 1with ALP expression and mineralize ECM [Franceschi et al., 2007; Huang et al., 2007]. After termination of bone matrix synthesis, osteoblasts undergo apoptosis or differentiate into either osteocytes or bone-lining cells. This study did not examine the apoptosis or survival during trans-differentiation into osteocytes. It was reported that TGF-β maintained mature osteocyte viability and osteoblast survival during trans-differentiation into osteocytes [Karsdal et al., 2004]. In addition to BMP-2, silibinin increased osteoblasts proliferation, which may enhance TGF-β signaling for the osteoblast trans-differentiation. CTGF is an essential downstream mediator for TGF-β1-induced ECM production in osteoblasts [Arnott et al., 2007]. However, CTGF and TGF-β1 function independently regarding their opposing effects on osteoblast proliferation. In fact, silibinin augmented CTGF expression and collagen type 1 production in differentiated osteoblasts.

Osteoclasts are cells of monocyte–macrophage origin that degrade bone matrix. Redox status diminishes during osteoclast differentiation, regulating RANKL-induced osteoclastogenesis [Huh et al., 2006]. RANKL signaling pathway may be targeted in the molecular basis for developing therapeutics to treat osteoclastogenesis-associated diseases of bone loss [Wada et al., 2006]. Dietary compounds have an anti-osteoclastogenic effect by antagonizing RANKL and TNF receptor family actions on bone [Pang et al., 2006; Lee et al. 2010]. It is deemed that the inhibition of osteoclastogenesis by these compounds may be partially attributed to their anti-inflammatory and antioxidant properties. Silibinin inhibited RANKL-activated osteoclast differentiation, suggesting that its antioxidant and anti-inflammatory activity may blunt bone resorption. RANKL induces activation of TRAF6 and c-fos pathways,

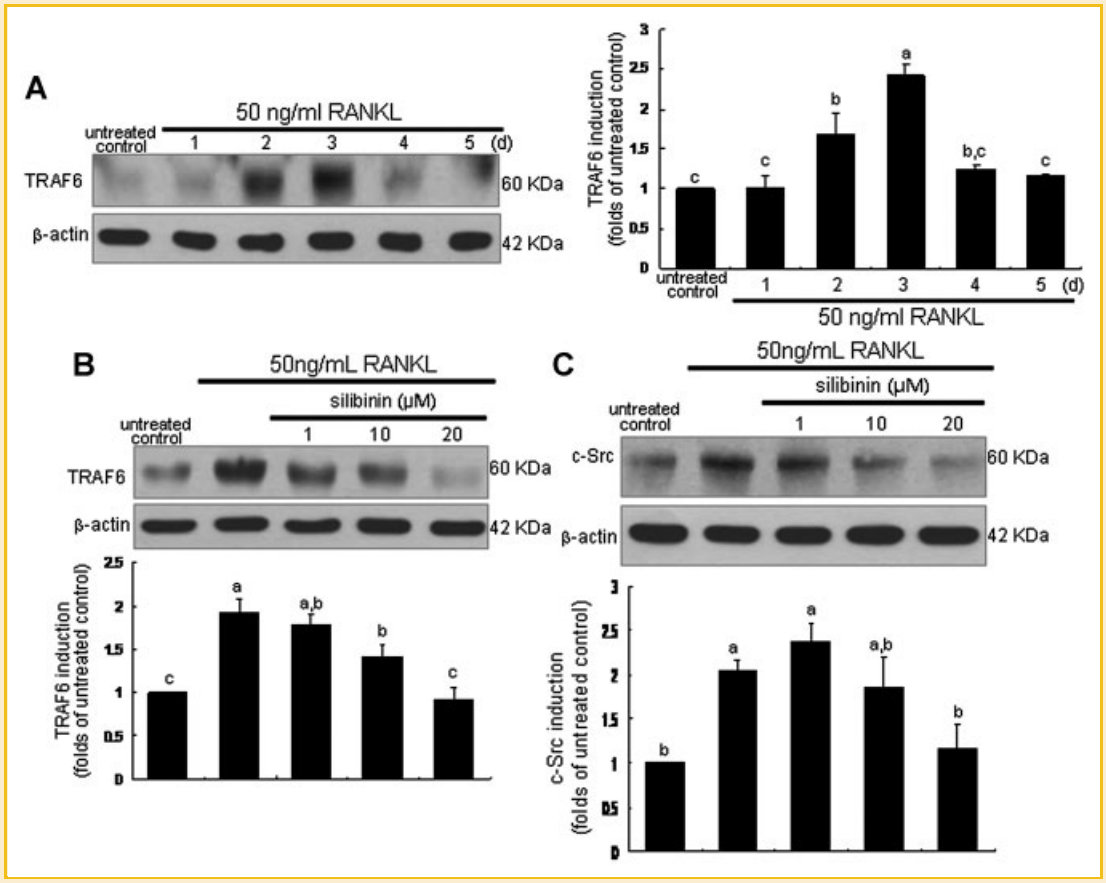

Fig. 8. Time course responses of TRAF6 (A) and inhibitory effects of silibinin on RANKL induction of TRAF6 (B) and c-Src (C). RAW 264.7 cells were cultured in DMEM and exposed to 50 ng/ml RANKL for 5 days. To measure inhibitory activity of TRAF6 and C-Src by silibinin, RAW 264.7 cells were cultured for 3 days in the absence and presence of 1–20 μM silibinin. After cells extracts were subject to SDS–PAGE and Western blot analysis with a primary antibody against TRAF6 and C-Src. Representative blot data were obtained from three independent experiments, and β-actin protein was used as an internal control. The bar graphs (mean ± SEM, n = 3) in the bottom panels and quantitative results were obtained from a densitometer. Values in bar graphs not sharing a letter indicate significant different at $P < 0.05$.

which lead to the auto-amplification of NFATc1, the master transcription factor for osteoclast differentiation. This study found that silibinin inhibited expression of TRAF6 and c-Src in RANKL-treated RAW 264.7 macrophages. The targeted disruption of c-Src as an adaptor and kinase impairs osteoclast bone resorbing activity, causing osteopetrosis [Miyazaki et al., 2006]. Therefore, silibinin failed to differentiate macrphages into osteoclasts in response to RANKL stimulation and to trigger a sustained TRAF6-c-Src-dependent transcriptional program during osteoclast differentiation.

In the current study, silibinin inhibited osteoclastic formation of TRAP-positive multinucleated osteoclasts, and down-modulated the expression of functional osteoclast markers, ICAM-1 and cathepsin K. ICAM-1 provides high-affinity adhesion between the osteoblast and the osteoclast precursor, thereby facilitating the interaction between RANKL and its receptor RANK [Fernandes et al., 2008]. Thus, the silibinin treatment disabled the RANKL–RANK interaction thereby abrogating osteoclast differentiation. It can be speculated that silibinin binds to RANKL or interacts putatively with RANK, thus preventing osteoclastogenesis. However, its precise inhibitory mechanism remains unclear. In addition, silibinin hampered osteoclastogenesis signaling by down-regulated the enzyme activity of RANKL-induced MMP-9. It is shown that MMP-9 enzyme action is engaged in the promotion of RANKL-induced osteoclastogenesis independent of NFATc1 signaling [Franco et al., 2011]. Similarly, the isoflavone genistein down-regulated RANKL production and MMP-9 activity and inhibited osteoclast formation, resulting in the inhibition of osteoclastic bone resorption and prostate cancer bone metastasis [Li et al., 2006].

In summary, the current report demonstrated that silibinin furthered matrix mineralization and osteoblastogenesis by inducing osteogenic biomarkers of ALP and collagen type 1 in differentiated MC3T3-E1 cells. The osteoblastogenic activity of silibinin was mediated by triggering BMP-2-responsive signaling. In addition, silibinin abrogated RANKL-induced osteoclastogenesis and TRAP activity as well as cathepsin K production. The blockade of osteoclastogenesis by silibinin was mediated by counteracting TRAF6-responsive signaling. Accordingly, silibinin works as a BMP modulator and is an osteoprotective agent inhibiting osteoclastic bone resorption. Although silibinin may serve as modulators against osteopetrosis and osteoporosis in vitro, its dietary in vivo role as an osteoprotective agent remains unclear.

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