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Biochemical Pharmacology 67 (2004) 1647–1656
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Inhibition of osteoclast differentiation and bone resorption by tanshinone IIA isolated from *Salvia miltiorrhiza* Bunge

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Received 6 August 2003; accepted 17 December 2003

Abstract

Osteoclasts, multinuclear cells specialized for bone resorption, differentiate from the monocyte/macrophage lineage of hematopoietic cells. Intervention in osteoclast differentiation is considered an effective therapeutic approach to the treatment of bone diseases involving osteoclasts. In this study, we found that tanshinone IIA, originating from *Salvia miltiorrhiza* Bunge, inhibited the differentiation of osteoclasts. Addition of tanshinone IIA to the osteoclast precursor culture caused a significant decrease in the level of calcitonin receptor, c-Src, and integrin β3 mRNA, which are normally upregulated during the osteoclast differentiation dependent on RANKL (receptor activator of nuclear factor kappa B ligand). RANKL activated the ERK, Akt, and NF-κB signal transduction pathways in osteoclast precursor cells, and tanshinone IIA suppressed this activation. Tanshinone IIA also inhibited the bone resorptive activity of differentiated osteoclasts, which was accompanied with the disruption of the actin ring. Thus, tanshinone IIA has the potential to ameliorate bone-resorption diseases in vivo by reducing both the number and activity of osteoclasts.

Keywords: Osteoclast; Tanshinone IIA; *Salvia miltiorrhiza* Bunge; RANKL; Akt; NF-κB

1. Introduction

Adult bone is in a dynamic state, continually undergoing a process termed remodeling through the coordinated actions of bone resorption and synthesis [1]. An imbalance in bone remodeling leads to adult skeletal defects including osteoporosis, Paget disease, and osteolysis associated with periodontal diseases and multiple myeloma. These diseases are characterized by the excessive activity of osteoclasts, cells that are primarily responsible for bone resorption. Osteoclasts originate from hematopoietic precursor cells of the phagocyte lineage and differentiate into multinucleated cells by the fusion of mononuclear progenitors [2]. A significant breakthrough in the understanding of osteoclast differentiation occurred when an osteoclast differentiation factor derived from osteoblasts/stromal cells was found. It is the receptor activator of nuclear factor κB (RANK) ligand (RANKL), which is a key osteoclastogenic molecule that directly binds to its cognate receptor, RANK, on osteoclast precursor cells [3–5]. RANKL is required to induce the expression of genes that typify differentiated osteoclasts, including those encoding calcitonin receptor, c-Src, and integrin β3 [6–8].

Upon RANKL binding, RANK expressed in osteoclast precursors recruits tumor necrosis factor receptor (TNFR)-associated factors (TRAFs), which are adaptor signaling molecules [9–12]. TRAFs induce a strong activation of NF-κB. Also, PI 3-kinase/Akt, p38, and ERK signaling pathways are involved in osteoclast differentiation through participation in RANKL signaling [13,14]. Blocking this differentiation-related signaling pathway has been considered as a therapeutic approach in the treatment of adult

Abbreviations: RANKL, receptor activator of nuclear factor kappa B ligand; TRAP, tartrate-resistant acid phosphatase; ERK, extracellular signal-regulated kinase; NF-κB, nuclear factor kappa B; M-CSF, macrophage colony stimulating factor

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doi:10.1016/j.bcp.2003.12.031

skeletal diseases. Wortmannin, a fungal metabolite, is a potent and selective inhibitor of PI 3-kinase, and inhibits osteoclastic bone resorption in vitro at nanomolar concentrations [15]. Tyrosine kinase inhibitors such as genistein and herbimycin have inhibitory effects on osteoclastic activity and bone loss in ovariectomized rats [16]. The Src family kinase inhibitor, CGP77675, has been reported to suppress bone resorption in vitro and in animal models of hypercalcemia and osteoporosis [17].

To find natural compounds that may inhibit osteoclastogenesis, we screened herb extracts on an osteoblast/bone marrow coculture system. During the process of this screening, we found that tanshinone IIA inhibited osteoclast differentiation and bone resorption. Tanshinone IIA originated from *Salvia miltiorrhiza* Bunge, which belongs to the family of Labiatae. This plant has been traditionally used as an oriental medical herb and is reported to be efficacious for coronary heart diseases [18]. Besides its protective effects on cardiovascular system, it induced apoptosis in several tumor cell lines through the activation of the caspase pathway [19,20]. The molecular mechanisms for the various effects of tanshinone IIA are unknown.

In this study, we found that tanshinone IIA inhibits osteoclast differentiation, fusion, actin ring formation, and bone resorption. Tanshinone IIA profoundly reduced the induction of osteoclast differentiation-related genes including calcitonin receptor, c-Src, and integrin β3. In addition, tanshinone IIA blocked the RANK signaling pathways of Akt, ERK, and NF-κB. These inhibitory effects of tanshinone IIA might result from suppression of osteoclast differentiation-related genes including calcitonin receptor, c-Src, and integrin β3, and tanshinone IIA blocks Akt, ERK, and NF-κB activation induced by RANKL in osteoclast precursors.

2. Materials and methods

2.1. Isolation of tanshinone IIA

Tanshen, the roots of *S. miltiorrhiza* (Labiatae) was purchased from a local herb drug market in Gwangju, Korea, and was authenticated by the Department of Pharmacognosy, Chosun University. Voucher specimens were deposited in the Herbarium of the College of Pharmacy, Chosun University (853-16). Tanshen (600 g) was extracted with methanol at room temperature, and then partitioned by methylene chloride, ethyl acetate and *n*-butanol in turn. The methylene chloride fraction (5.7 g) was subjected to column chromatography over a silica gel eluting with a gradient system of hexane:ethyl acetate (10:1 → 8:1 → 5:1 → 2:1 → 1:1 → 1:2, methanol only). Fractions were combined based on their TLC pattern to yield a subfraction designated as D1–D11. Subfraction D2 was further purified by repeated column chromatography over a silica gel to afford tanshinone IIA. The physical and chemical data including UV, IR, ^{1}H NMR, ^{13}C NMR, HSQC, HMBC, and HR-MS of tanshinone IIA were identical with those previously reported. The purity of tanshinone IIA used was more than 99%.

2.2. Osteoclast culture

Osteoclast differentiation was induced by coculturing mouse bone marrow cells and calvarial osteoblasts. Primary osteoblasts were obtained by growing calvarial cells from ICR newborn mice for 1 day in α-MEM/10% FBS. Bone marrow cells were obtained by flushing tibiae from 6- to 7-week-old ICR mice with α-minimum essential medium (α-MEM; Gibco BRL) containing 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml macrophage-colony stimulating factor (M-CSF; Peprotech EC). Then 1×10^7 to 2×10^7 bone marrow cells and 1×10^6 osteoblasts were seeded on a well of a 48-well plate and incubated for 6–7 days in the presence of 10^{-8} M VtD_3 and 10^{-6}M PGE_2. Alternatively, osteoclasts were generated by culturing bone marrow-derived macrophages in the presence of M-CSF and RANKL. Bone marrow cells were cultured for 24 h in α-MEM/10% FBS. Nonadherent cells were collected and cultured for 3 days in the presence of 30 ng/ml M-CSF. Cells were harvested and re-seeded at 1×10^6 cells/ml in α-MEM/10% FBS, and cultured in the presence of 30 ng/ml M-CSF and 50 ng/ml RANKL (Peprotech EC) for 7 days. Osteoclasts were stained for tartrate-resistant acid phosphatase (TRAP) with the leukocyte acid phosphatase kit (Sigma, St. Louis, MO).

2.3. Fusion assay

Bone marrow-derived macrophages prepared as above were suspended at 1×10^6 cells/ml in α-MEM/10% FBS, and cultured in the presence of 30 ng/ml M-CSF and 50 ng/ml RANKL for 3 days. These cells were pretreated with tanshinone IIA for 1 h and then incubated with 30 ng/ml M-CSF and 100 ng/ml RANKL for 12 h. Cells were stained for TRAP, and TRAP-stained cells were incubated with Giemsa solution for 5 min to stain nuclei, followed by washing with 1% sodium carbonate. The fusion index was calculated as the percentage of nuclei contained in multinucleated (nuclei number $\geq$5) TRAP-positive cells among the nuclei contained in all TRAP-positive cells.

2.4. Actin ring formation

To obtain mature osteoclasts, 1×10^7 to 2×10^7 bone marrow cells and 1×10^6 osteoblasts were seeded on a 90-mm culture dish and cultured for 6–7 days in the presence of 10^{-8} M VtD_3 and 10^{-6} M PGE_2. Cells were detached by treating with 0.2% collagenase (Wako Chemicals) at 37 °C for 10 min, replated on 60-mm dishes, and cultured for another day. The dishes were then treated with 0.1%

collagenase at 37 °C for 15 min and intensely pipetted to remove osteoblasts. The remaining cells were considered enriched mature osteoclasts. Purified osteoclasts were starved with α-MEM containing 0.5% FBS and pretreated without or with the indicated concentrations of tanshinone IIA for 15 min and then treated with 100 ng/ml RANKL for 30 min. Cells were fixed and stained with rhodamine phalloidin.

2.5. Resorption pit assay

Mature osteoclasts were obtained from a 6-day coculture of bone marrow cells and calvarial osteoblasts. Crude osteoclasts were replated on dentine slices that had been placed in 96-well plates and treated with tanshinone IIA for 20 h. After the incubation, attached cells were completely removed from the plate by abrasion with a cotton tip, and resorption pits were visualized by staining with hematoxylin solution (Sigma). Photographs were taken under a light microscope at 40× magnification, and total areas of resorption pits were analyzed by the Image Pro-Plus program version 4.0 (Media Cybernetics).

2.6. RT–PCR analysis

Total RNA was prepared using TRI reagent, and 2 μg was reverse-transcribed with SuperScriptII reverse transcriptase (Gibco BRL). Ten percent of the reverse-transcribed cDNA was amplified by PCR. The sequences of primers were designed for mouse genes: integrin β3, 5′-TGACTCGGACTGGACTGGCTA-3′ and 5′-CACTCAGGCTCTTCCACCACA-3′; TRAF6, 5′-GCTCAAACGGACCATTCGGA-3′ and 5′-GGGATTGTGGGTCGCTGAAA-3′; carbonic anhydrase II, 5′-CTCTCAGGACAATGCAGTGCTGA-3′ and 5′-ATCCAGGTCACACATTCCAGCA-3′; calcitonin receptor, 5′-ACCGACGAGCAACGCCTACGC-3′ and 5′-GCCTTCACAGCCTTCAGGTAC-3′; c-Src, 5′-CCAGGCTGAGGAGTGGTACT-3′ and 5′-CAGCTTGCGGATCTTGTAGT-3′; RANK, 5′-CACAGACAAATGCAAACCTTG-3′ and 5′-GTGTTCTGGAACCTATCTTCCTCC-3′; GAPDH, 5′-ACTTTGTCAAGCTCATTTCC-3′ and 5′-TGCAGCGAACTTTATTGATG-3′. PCR consisted of 22–25 cycles of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C. The number of cycles for each gene was decided to be in the range of linear amplification through an optimization experiment. The PCR product were separated on 1.2–2.0% agarose gels and stained with ethidium bromide.

2.7. Western blotting

Cells were lysed in a buffer containing 20 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors. The lysates (30–40 μg) of cellular proteins were separated by 10% SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). After blocking with 5% skim milk, the membrane was probed with anti-phospho Akt, ERK, JNK, p38 and anti-I-κB (Cell Signaling Technology). The same membranes were stripped and reprobed with anti-Akt, ERK, JNK, p38, and actin (Cell Signaling Technology).

2.8. Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described [21]. Cells were lysed in a hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) containing 0.6% NP-40 and microfuged at 3000 × *g* for 15 min. The pellet was lysed in 15 μl of a high salt buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) for 20 min on ice. After adding 75 μl of storage buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT), samples were agitated for 10 s by vortexing, and then microfuged at 12,000 × *g* for 20 min. Nuclear extracts (10 μg) were incubated with approximately 20,000 cpm of ^{32}P-labeled NF-κB binding site oligomer 5′-AGTTGAGGGGACTTTCCCAGGC-3′ or ^{32}P-labeled AP-1 binding site oligomer 5′-CGCTTGATGACTCAGCCGGAA-3′ (Santa Cruz Biotechnology) for 30 min at 20 °C. For supershift experiments, an antibody was added and incubation was continued for another 30 min. Fifty-fold excess unlabeled probe was added in the negative control experiments. The DNA-bound NF-κB and AP-1 proteins were subjected to 4–5% polyacrylamide gel electrophoresis followed by autoradiography.

2.9. Statistical analysis

Each experiment was performed three to five times, and all quantitative data are presented as mean ± S.D. Statistical differences were analyzed by Students' *t*-test.

3. Results

3.1. Inhibitory effects of tanshinone IIA on osteoclast differentiation

We screened natural products derived from plant extracts to identify compounds that inhibit osteoclast differentiation in cocultures of mouse bone marrow cells and osteoblasts. We found a compound, tanshinone IIA, isolated from *S. miltiorrhiza* Bunge, which effectively reduced the formation of TRAP-positive multinuclear osteoclasts (Fig. 1). The addition of tanshinone IIA during the coculture significantly reduced osteoclastogenesis in a dose-dependent manner (data not shown). Tanshinone IIA had some inhibitory effect on RANKL expression in osteoblasts (data not shown). To exclude this indirect effect, we used the culture condition under which osteo-

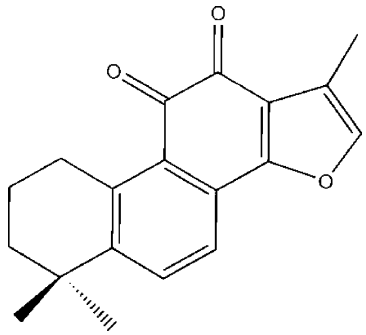

Fig. 1. Chemical structure of tanshinone IIA.

clasts are differentiated from bone marrow-derived macrophages in the presence of M-CSF plus RANKL without supporting osteoblasts. In this culture, the inhibitory effect of tanshinone on osteoclastogenesis was also observed (Fig. 2A). Tanshinone IIA inhibited the osteoclast differentiation by 100, 93.62 and 79.70% at 20, 10, and 5 μg/ml, respectively (Fig. 2B). These inhibitory effects of tanshinone IIA might be due to cytotoxicity or reduced growth of osteoclast precursors. This possibility was excluded when the effect of tanshinone IIA on the proliferation of the precursor cells was evaluated using MTT assays (Fig. 2C). No cytotoxicity of tanshinone IIA was further supported by the reversibility of the inhibitory effect on osteoclastogenesis when tanshinone IIA was removed and osteoclastogenic culture was continued (data not shown).

3.2. Inhibition of osteoclast fusion by tanshinone IIA

To carry out bone resorption, mononuclear perfusion osteoclasts (pOCs) fuse each other to form mature multinucleated osteoclasts [2]. We examined whether tanshinone IIA has any effect on osteoclast fusion. Mononuclear TRAP-positive pOCs were obtained by culturing bone marrow cells for 3 days in the presence of 30 ng/ml M-CSF and 50 ng/ml RANKL. Tanshinone IIA inhibited the fusion of pOCs into osteoclast-like cells (OCLs) in a dose-dependent manner (Fig. 3). When the extent of cell fusion was calculated, tanshinone IIA (20 μg/ml) decreased the fusion index by 2.86-fold (Fig. 3B). This result suggests that tanshinone IIA can modulate the RANKL-dependent osteoclast maturation by decreasing cell fusion.

3.3. Blockade of actin ring formation in osteoclasts by tanshinone IIA

To resorb bone, osteoclasts must form a sealing zone by generating a ring structure made of F-actin. We investigated whether tanshinone IIA affects the formation of actin rings. Staining F-actin with rhodamine-phalloidin showed a ring structure in mature osteoclasts purified from the coculture (Fig. 4A). When osteoclasts were treated with RANKL, the actin ring became more dense and smooth, showing clear margins (Fig. 4B). However, tanshinone IIA

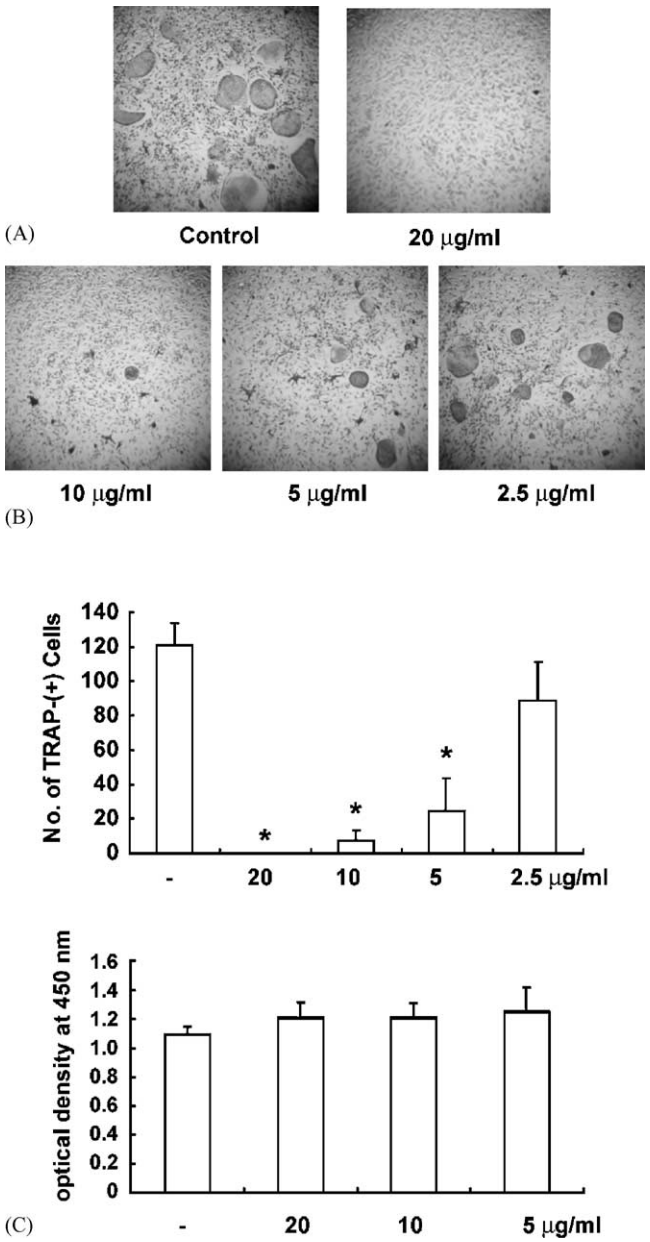

Fig. 2. Effects of tanshinone IIA on osteoclast differentiation. (A) Bone marrow-derived osteoclast precursor cells were cultured in the presence of M-CSF and RANKL for 7 days. The indicated concentration of tanshinone IIA was added to the culture. Cells were fixed and stained for TRAP. (B) TRAP-positive multinuclear cells containing three or more nuclei were counted as osteoclasts. Data from three experiments are presented as mean ± S.D. $^{*}P < 0.05$, significant differences from the control. (C) Bone marrow-derived osteoclast precursor cells were cultured in the presence of M-CSF, RANKL, and tanshinone IIA for 3 days. The MTT assay was performed and the absorbance was read at 450 nm.

treatment abolished the effect of RANKL on the ring structure in a dose-dependent manner (Fig. 4C–F). The ring was loose and fuzzy in the presence of tanshinone IIA. Addition of tanshinone IIA immediately before the phalloidine stain did not have any effect, indicating that the effect of tanshinone IIA on actin ring was not due to an interference with the staining itself by this compound (data not shown). These results indicate that tanshinone IIA

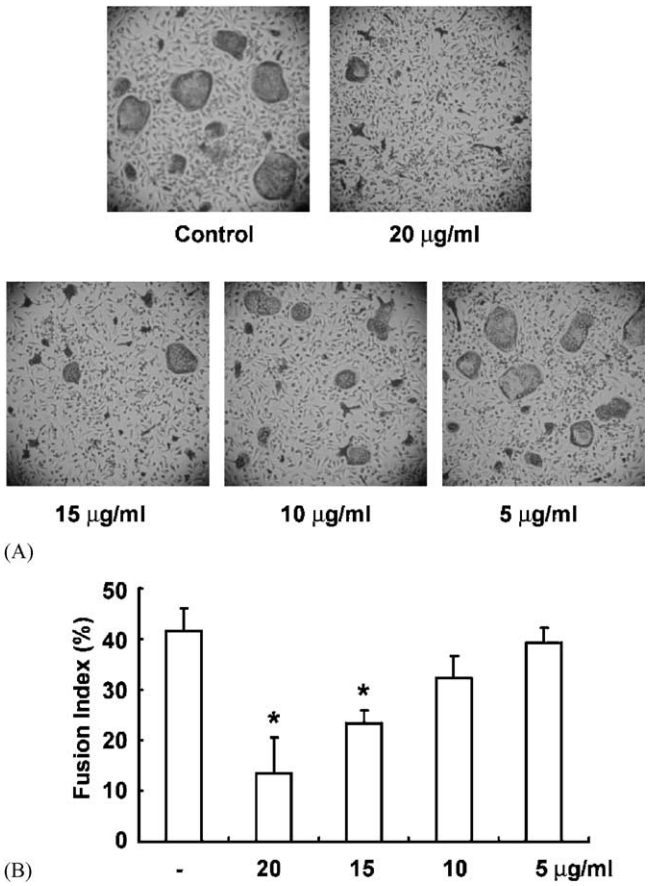

Fig. 3. Inhibition of osteoclast fusion by tanshinone IIA. (A) The TRAP-positive mononuclear cells were pretreated with tanshinone IIA for 1 h and then incubated with 30 ng/ml M-CSF and 100 ng/ml RANKL for 12 h. Cells were stained for TRAP and TRAP-stained cells were incubated with Giemsa solution for 5 min to stain nuclei followed by washing with 1% sodium carbonate. (B) The fusion index was calculated as the percentage of nuclei contained in multinucleated (nuclei number ≥5) TRAP-positive cells among the nuclei contained in all TRAP-positive cells. Data from three experiments are presented as mean ± S.D. $^{*}P < 0.05$, significant differences from the control.

affects cytoskeletal organization, which is necessary for bone resorption by osteoclasts.

3.4. Inhibition of bone-resorbing activity of osteoclasts by tanshinone IIA

Differentiated multinuclear osteoclasts undergo a morphological and functional polarization, and begin to resorb mineralized bone surface. We next assayed whether tanshinone IIA affects the bone resorption activity of mature osteoclasts. Purified mature osteoclasts derived from cocultures were placed on dentine slices and cultured in the presence or absence of various concentrations of tanshinone IIA for 24 h. In the presence of tanshinone IIA, bone resorption was inhibited in a dose-dependent manner (Fig. 5). This result shows that tanshinone IIA not only inhibits the osteoclast differentiation and maturation, but also decreases the bone resorption activity of mature osteoclasts.

3.5. Effects of tanshinone IIA on osteoclast differentiation-related genes

To gain an insight into the effect of tanshinone IIA on osteoclast differentiation, we investigated the expression levels of genes induced during osteoclastogenesis. Osteoclast precursors isolated from bone marrow macrophages were cultured with M-CSF and RANKL in the presence or absence of tanshinone IIA. Total RNA was obtained at 1, 3, and 6 days, and changes in gene expression were assessed by RT–PCR. The calcitonin receptor, c-Src, and integrin β3 genes were induced during the whole differentiation period (Fig. 6, panels 2, 4, and 5). Addition of tanshinone IIA to the culture strongly impeded the expression of calcitonin receptor, c-Src, and integrin β3 (Fig. 6, panels 2, 4, and 5; lanes 4–6). The expression of carbonic anhydrase II was only moderately increased, and the mRNA levels of TRAF6 and RANK showed little change during differentiation (Fig. 6, panels 1, 3, and 6). Therefore, tanshinone IIA has a prominent effect on the regulation of some genes that are induced during osteoclast differentiation.

3.6. Suppression of RANK signaling to Akt, ERK, and NF-κB in tanshinone IIA-treated osteoclast precursors

Previously, we provided evidence for the involvement of PI 3-kinase/Akt, ERK, p38, and JNK in osteoclast differentiation [14,22]. Consequently, the Akt, ERK, p38, and JNK signaling pathways have emerged as therapeutic targets in a variety of bone-resorbing diseases. We next determined whether tanshinone IIA affects the signaling pathways involving these kinases. When osteoclast precursors were stimulated with RANKL, the phosphorylation of Akt, ERK, JNK, and p38 increased (Fig. 7). Tanshinone IIA treatment blocked the phosphorylation of Akt and ERK induced by RANKL (Fig. 7, panels 1 and 3). On the other hand, tanshinone IIA did not affect the phosphorylation of JNK, and greatly increased the phosphorylation of p38 (Fig. 7, panels 5 and 7).

Activation of the NF-κB pathway is a prerequisite for osteoclast differentiation [23]. To activate NF-κB, I-κB is first phosphorylated, then degraded. When osteoclast precursors were stimulated with RANKL, degradation of I-κB occurred within 5–15 min after treatment (Fig. 8A, lanes 2 and 3). Addition of tanshinone IIA to purified osteoclast precursors prevented the I-κB degradation induced by RANKL (Fig. 8A, lanes 6 and 7). When assessed by EMSA, the activation of NF-κB was observed in osteoclast precursors stimulated with RANKL for 15 min (Fig. 8B, lane 3). Treatment of the cells with tanshinone IIA inhibited the RANKL-induced NF-κB activation (Fig. 8B, lane 3 versus lane 7). The specificity of NF-κB binding was verified by the competition experiment with unlabeled probe and the supershift assay with NF-κB antibodies (Fig. 8C). To examine whether the inhibitory effects on NF-κB binding activity is specific of treatment

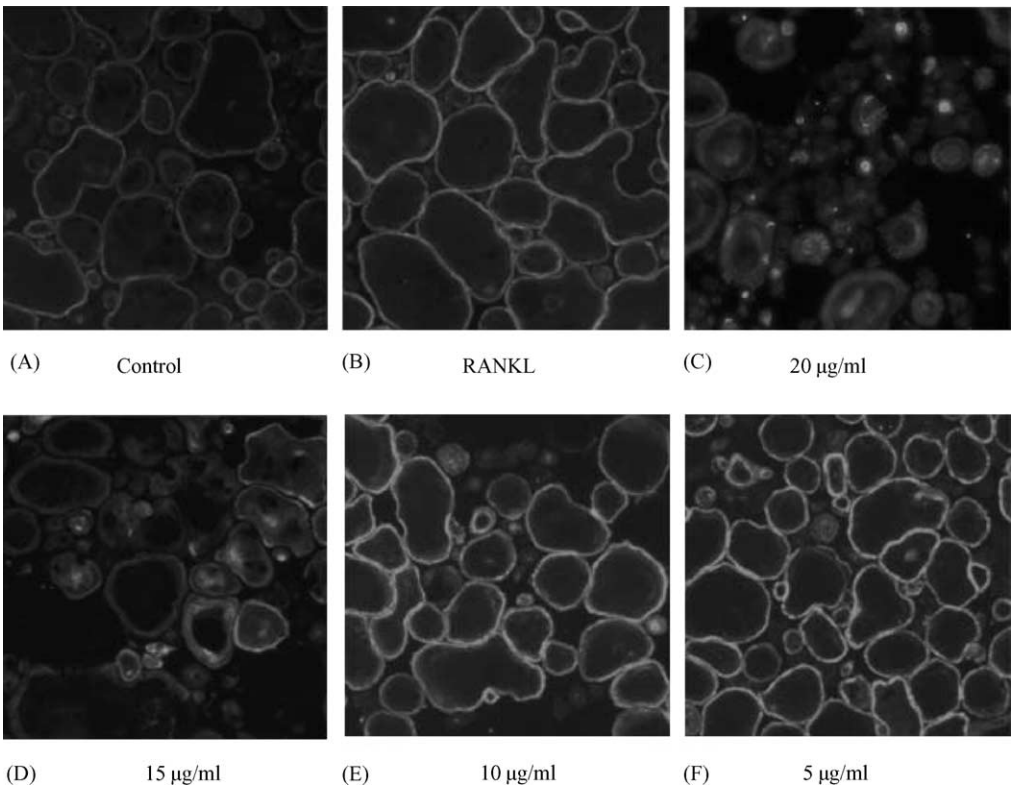

Fig. 4. (A–F) Inhibition of actin ring formation by tanshinone IIA. Mature osteoclasts from coculture were replated on glass cover slips. After osteoblasts were removed, purified osteoclasts were deprived of serum in medium containing 0.5% FBS for 1 h. Cells were pretreated with the indicated concentrations of tanshinone IIA for 15 min and incubated with 100 ng/ml RANKL for 30 min. Cells were fixed and stained with rhodamine phalloidin.

with tanshinone IIA, effects of tanshinone IIA on AP-1 binding was assessed. As shown in Fig 8D, tanshinone IIA did not affect the AP-1 binding activity stimulated by RANKL. Taken together, tanshinone IIA disrupted multiple signaling pathways that have been implicated in osteoclast differentiation.

4. Discussion

Bone mass in adults decreases with age because of the imbalance between the rate of bone formation and resorption. Osteoclasts are the only cell type capable of resorbing mineralized bone. Bone resorption is influenced by various factors that govern osteoclast number and activity. We conducted a random screening for compounds that reduce the generation or activation of osteoclasts and thus may have therapeutic applications for bone-resorbing diseases such as osteoporosis, Paget disease, and periodontal disease. In this study, we report a novel inhibitor of osteoclast differentiation, fusion, and resorption. This compound, tanshinone IIA, inhibits both the differentiation of precursor cells to TRAP-positive multinucleated osteoclasts and also the bone resorption activity of differentiated osteoclasts (Figs. 2 and 5).

The differentiation process of osteoclasts from the progenitor cells of hematopoietic origin has not yet been precisely defined. However, it is generally accepted that two critical steps occur in osteoclastogenesis: commitment of the progenitor cells to osteoclast precursor cells and fusion of the TRAP-positive mononuclear cells to form multinucleated osteoclasts [24]. During the differentiation process, the expression of several genes is specifically stimulated. These include TRAP, V-ATPase, carbonic anhydrase II, c-Src, calcitonin receptor, and integrin β3. We investigated whether tanshinone IIA might modulate the expression of some of these genes. Tanshinone IIA affected different genes to different extents (Fig. 6). In general, genes that are induced to a greater extent during differentiation were more prominently inhibited by tanshinone IIA. Tanshinone IIA treatment also suppressed the fusion of mononuclear osteoclast precursors into multinucleated functional osteoclasts (Fig. 3). The inhibition of gene induction and cell fusion by tanshinone IIA may account for its negative effect on the differentiation of bone marrow precursor cells to osteoclasts.

To resorb the bone, mature osteoclasts form an actin ring, which corresponds exactly to clear zones in bone-resorbing osteoclasts [25]. This unique cytoskeletal organization is thought to be a functional marker of activated osteoclasts [26,27]. Therefore, finding drugs that disturb the integrity of the actin ring could be a useful approach to therapy to slow bone resorption. Tanshinone IIA showed disruption of the actin ring formation induced by RANKL (Fig. 4). We checked whether the effect of tanshinone IIA was reversible or not in actin ring formation of mature

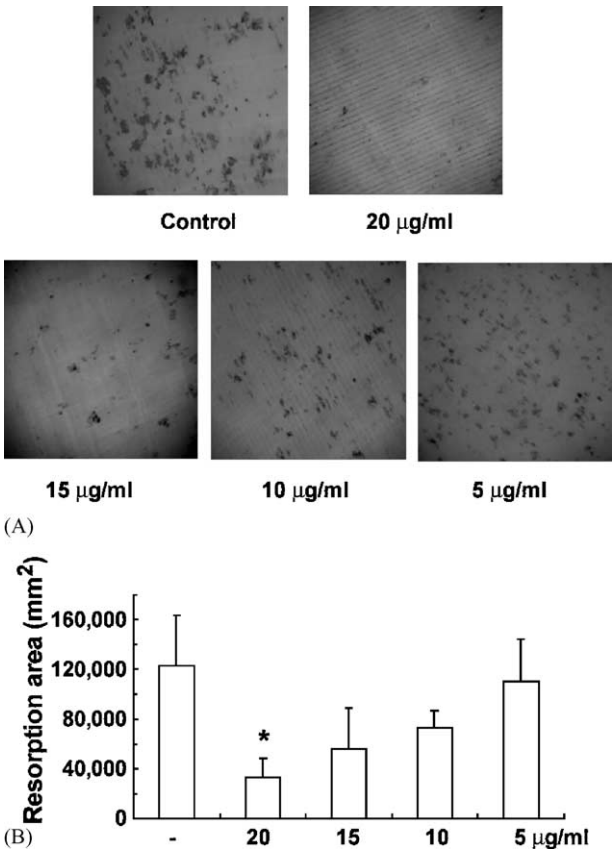

Fig. 5. Inhibition of bone resorption activity of osteoclasts by tanshinone IIA. Mature osteoclasts obtained from co-cultures of bone marrow cells and calvarial cells were replated on dentine slices and cultured for 24 h in the presence of the indicated concentrations of tanshinone IIA. (A) Resorption pits on dentine slices were visualized by staining with hematoxylin. (B) Resorption pit areas were analyzed by image analysis. Data from three experiments are presented as mean ± S.D. * $P < 0.05$, significant differences from the control.

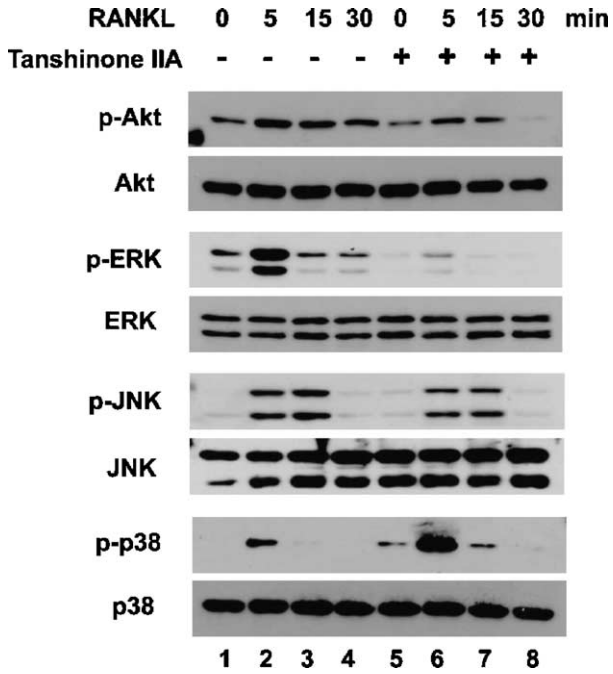

Fig. 7. Inhibition of tanshinone IIA on Akt and ERK activation induced by RANKL in osteoclast precursors. Bone marrow macrophages were deprived of serum in medium containing 0.1% FBS for 5 h, pretreated with 20 μg/ml of tanshinone IIA for 30 min, and then stimulated with 100 ng/ml RANKL for the indicated times. Cell lysates were prepared and subjected to Western blotting with the indicated antibodies.

osteoclasts. Once actin ring was destroyed by treatment with tanshinone IIA, actin ring could not be recovered upon removal of tanshinone IIA and subsequent addition of RANKL (data not shown). The actin ring disruption would lead to the inactivation of osteoclasts. Indeed, tanshinone IIA significantly reduced bone resorption by

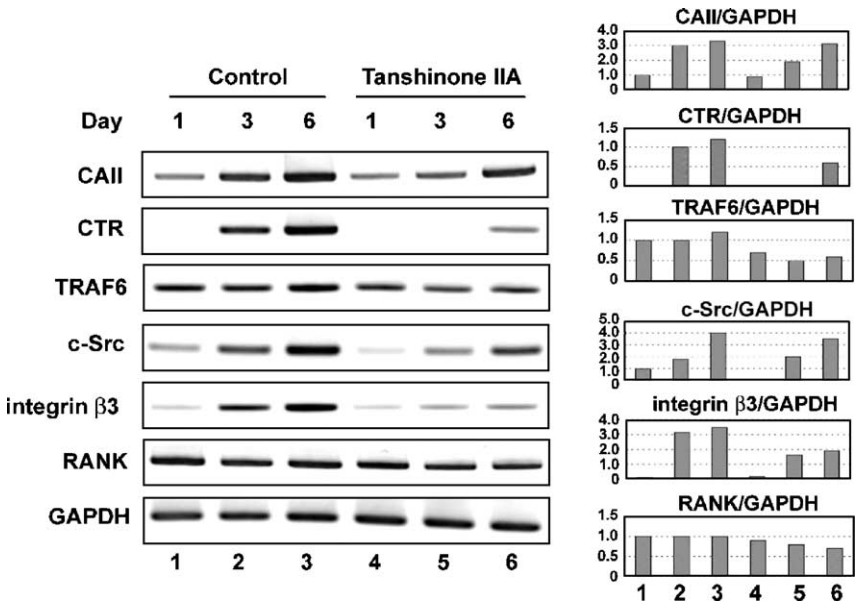

Fig. 6. Effects of tanshinone IIA on RANKL-induced gene expression during osteoclast formation from osteoclast precursor cells. Osteoclast precursors isolated from bone marrow cells were cultured with 30 ng/ml M-CSF and 100 ng/ml of RANKL in the presence or absence of 20 μg/ml tanshinone IIA. Total RNA was obtained at 1, 3, and 6 days. The mRNA expression levels of the indicated genes were determined by RT–PCR. Photographs of ethidium bromide stained gels (left panel) and the results of densitometric analysis (right panel) are shown.

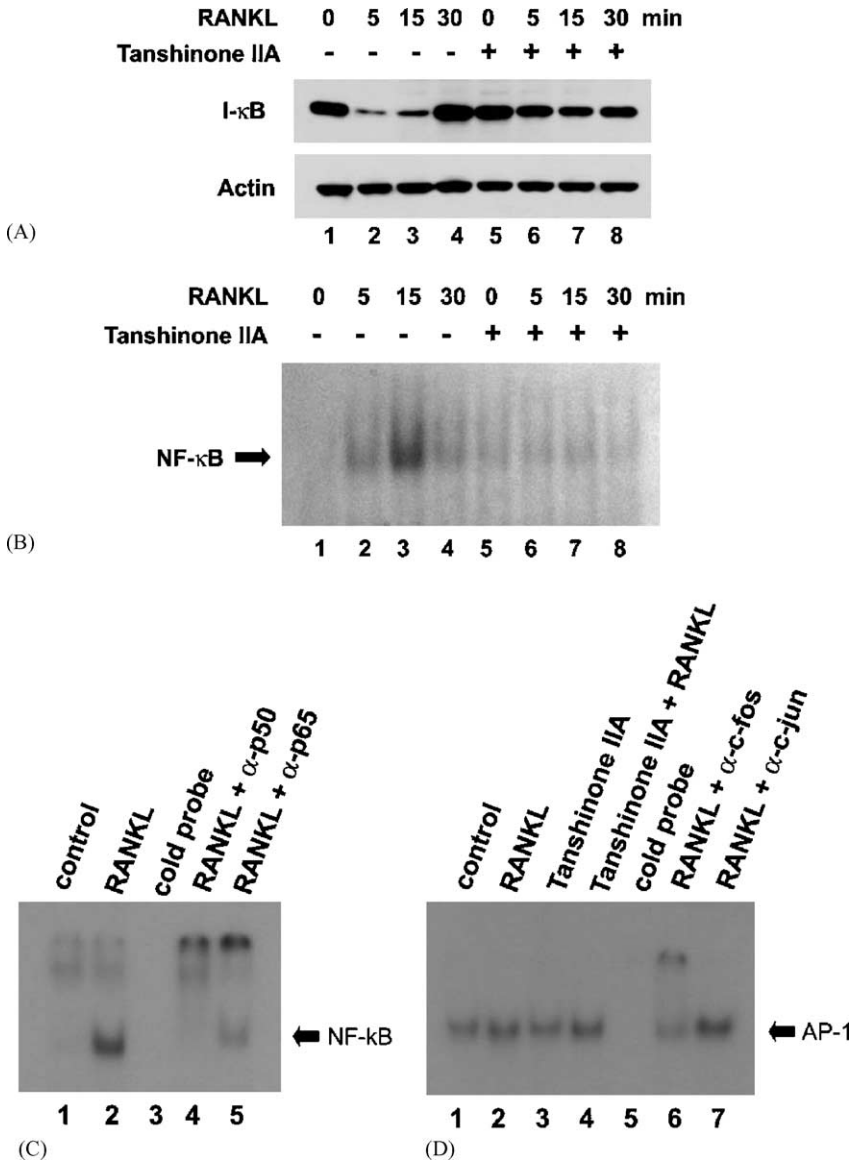

Fig. 8. Inhibition of tanshinone IIA on NF-κB activation induced by RANKL in osteoclast precursors. (A) Bone marrow-derived osteoclast precursors were deprived of serum in medium containing 0.1% FBS for 5 h, pretreated with 20 μg/ml tanshinone IIA for 30 min, and then stimulated with 100 ng/ml RANKL for the indicated times. Cells were lysed and analyzed by Western blotting with anti-I-κB (top). The same membrane was stripped and reprobed with anti-actin (bottom). (B) Cells were pretreated with 20 μg/ml tanshinone IIA for 30 min and then stimulated with 100 ng/ml RANKL for the indicated times. Cells were harvested and nuclear extracts were prepared. NF-κB activity was measured by EMSA with 10 μg of nuclear extracts. (C) Cells were pretreated with 20 μg/ml tanshinone IIA for 30 min and stimulated with 100 ng/ml RANKL for 15 min. NF-κB activity was measured by EMSA with 10 μg of nuclear extracts. For supershift assays, the indicated antibody was added. (D) Cells were treated with 20 μg/ml tanshinone IIA for 30 min prior to 15 min stimulation with 100 ng/ml RANKL. Ten micrograms of nuclear extracts were used for AP-1 binding activity by EMSA.

mature osteoclasts in vitro (Fig. 5). Tanshinone IIA may reduce bone loss in vivo both by inhibiting the differentiation and fusion of osteoclast precursors and also by disrupting the cytoskeletal architecture of multinucleated mature osteoclasts.

As a member of tumor necrosis factor receptor (TNFR) superfamily, RANK shares many signaling pathways with TNFR and other related receptors. Stimulation of RANK results in the activation of the PI 3-kinase/Akt, NF-κB, and MAPK signaling pathways. In RANKL-driven differentiation of bone marrow precursors into osteoclasts, PI 3-kinase and Akt are activated through Src family kinases [12]. In our previous report, the ERK pathway was also shown to be important in osteoclast differentiation [14]. Tanshinone IIA suppressed the activation of Akt and ERK in response to RANKL (Fig. 7). We have previously reported that RANKL stimulation leads to the activation of activator protein-1 (AP-1) transcription factor through the JNK activation pathway [11,22]. Recently, JNK activation was shown to be required for efficient osteoclast differentiation from bone marrow monocytes, and to protect bone marrow monocytes from RANKL-induced apoptosis during osteoclast differentiation [28]. However, we found that tanshinone IIA did not affect RANKL-stimulated JNK

activation (Fig. 7). Interestingly, tanshinone IIA significantly increased the activation of p38 in response to RANKL (Fig. 7). The p33 MAPK pathway has been implicated in apoptosis induced by a novel TNF-α-like cytokine and lysophosphatidylcholine in endothelial cells [29,30]. It is possible that the activation of p38 by tanshinone IIA stimulates apoptosis of osteoclast precursor cells and thereby contributes to the suppression of differentiation of osteoclasts. The activation of NF-κB has an essential role in osteoclast differentiation and function [23,31]. Tanshinone IIA treatment blocked the degradation of I-κB and decreased the activation of NF-κB induced by RANKL (Fig. 8). As tanshinone IIA inhibited Akt, ERK and NF-κB activation, it was possible that this compound affected cell survival. However, we could not detect any effect on osteoclast survival by tanshinone IIA (data not shown). Taken together, the effects of tanshinone IIA on suppression of osteoclast differentiation may result from the disturbance in the signal transduction by RANK for the activation of Akt, NF-κB and MAPKs.

In summary, tanshinone IIA, a compound identified from the medicinal herb—*S. miltiorrhiza*, suppressed osteoclast differentiation and bone resorption. Also, it inhibited the fusion of osteoclast precursors during differentiation. Therefore, tanshinone IIA might be a candidate for drug development for the treatment of bone-resorbing diseases such as osteoporosis, bone-erosive rheumatoid arthritis, and advanced periodontal disease.

Acknowledgments

The authors acknowledge the financial support of grants from the Ministry of Science and Technology, Korea and the Korea Science and Engineering Foundation through the Research Center for Proteineous Materials, and the National Research Laboratory. H.-H. Kim was supported in part by the New Faculty Grant (2003) from Seoul National University.

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