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Jolkinolide B inhibits RANKL-induced osteoclastogenesis by suppressing the activation NF-κB and MAPK signaling pathways

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ABSTRACT

Osteoclasts together with osteoblasts play pivotal roles in bone remodeling. The unique function and ability of osteoclasts to resorb bone makes them critical in both normal bone homeostasis and pathologic bone diseases such as osteoporosis and rheumatoid arthritis. Thus, new compounds that may inhibit osteoclastogenesis and osteoclast function may be of great value in the treatment of osteoclast-related diseases. In the present study, we examined the effect of jolkinolide B (JB), isolated from the root of *Euphorbia fischeriana* Steud on receptor activator of NF-κB ligand (RANKL)-induced osteoclast formation. We found that JB inhibited RANKL-induced osteoclast differentiation from bone marrow macrophages (BMMs) without cytotoxicity. Furthermore, the expression of osteoclastic marker genes, such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (CtsK), and calcitonin receptor (CTR), was significantly inhibited. JB inhibited RANKL-induced activation of NF-κB by suppressing RANKL-mediated IκBα degradation. Moreover, JB inhibited RANKL-induced phosphorylation of mitogen-activated protein kinases (p38, JNK, and ERK). This study thus identifies JB as an inhibitor of osteoclast formation and provides evidence that JB might be an alternative medicine for preventing and treating osteolysis.

1. Introduction

Bone remodeling is a physiological process that involves the delicate balance between osteoblastic bone formation and osteoclastic bone resorption. Osteoclasts are derived from hematopoietic stem cells, which are unique multinucleated cells responsible for bone resorption [1,2]. Excessive osteoclast-induced bone resorption leads to an imbalance in bone remodeling and causes bone lytic diseases such as osteoporosis, rheumatoid arthritis, and cancer metastasis to the bone [3].

Many factors are involved in osteoclast differentiation. The receptor activator of NF-κB ligand (RANKL) is a tumor necrosis factor (TNF) family member, which plays a critical role in regulating the differentiation and activation of osteoclasts. RANKL-induced activation of RANK on osteoclast progenitor cells leads to the stimulation of TNF receptor-associated factors and the subsequent activation of several signaling cascades, including nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPK), activating protein 1 (AP-1), and nuclear factor of activated T cells (NFATc1), resulting in the formation of multinucleated bone-resorbing osteoclasts [1,4].

Jolkinolide B, a typical ent-abietane-type diterpenoid, has been proven to exhibit significant inhibitory effects against several tumor lines, including prostate LNCaP cancer cell lines [5]. It is also known to induce apoptosis in human ovarian carcinoma SKOV-3 cells and leukemic U937 cells [6]. Moreover, *in vitro* studies have indicated that jolkinolide B inhibits cell proliferation and induces apoptosis of human breast cancer cell lines [7,8]. Molecular analysis demonstrated that jolkinolide B induces the apoptosis of cancer cells through the phosphoinositol-3-kinase (PI3K)/Akt signaling transduction pathway, suggesting that it may have therapeutic applications in the treatment of breast cancer. Moreover, JB possesses anti-inflammatory actions in macrophages [9].

In this study, we aimed to clarify the effect of JB on RANKL-induced osteoclast formation in bone marrow macrophages (BMMs). Herein, we report the mechanism by which RANKL-induced osteoclast formation is inhibited by JB.

2. Materials and methods

2.1. Media and reagents

Jolkinolide B was purchased from Dalian Meilun Biology Technology Co., Ltd. (Dalian, Liaoning, China). Dulbecco's modified

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Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY, USA). NF-κB (anti-IκBα) and MAPKs (anti-ERK1/2, anti-JNK, and anti-p38) mouse or rabbit antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technology (Japan). Soluble mouse recombinant macrophage colony-stimulating factor (M-CSF) and RANKL were purchased from Peprotech (USA). Tartrate-resistant acid phosphatase (TRAP) staining solution was from Sigma–Aldrich (St Louis, MO, USA).

2.2. Cell culture

BMMs were prepared as previously described [10,11]. Briefly, cells were extracted from the femur of a 4-week-old C57/BL6 mouse and incubated in complete cell culture media with 20 ng/mL M-CSF. In order to deplete residual stromal cells, the culture plates were washed before the medium was replaced. Cells that adhered to the bottom of the dish were classified as BMMs. The complete cell culture medium used for routine subculturing was DMEM, supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL). The cells were maintained at subconfluence in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C and were subcultured every 3 days.

2.3. Cell viability assay

Cell viability was measured using a cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). BMMs were seeded on 96-well plates and cultured as described above for 24 h. The medium was then replaced with medium containing JB at various concentrations. After incubation for 48 h at 37 °C in a 95% air and 5% CO_2 atmosphere incubator, CCK8 solution was added accordingly. The plates were incubated for 4 h, after which absorbance was measured at 450 nm using a microplate reader.

2.4. TRAP activity and osteoclastogenesis assay

For the TRAP activity assay, BMMs were plated in 48-well plate at 2×10^4 cells/well. RANKL (100 ng/mL) and M-CSF (20 ng/mL)

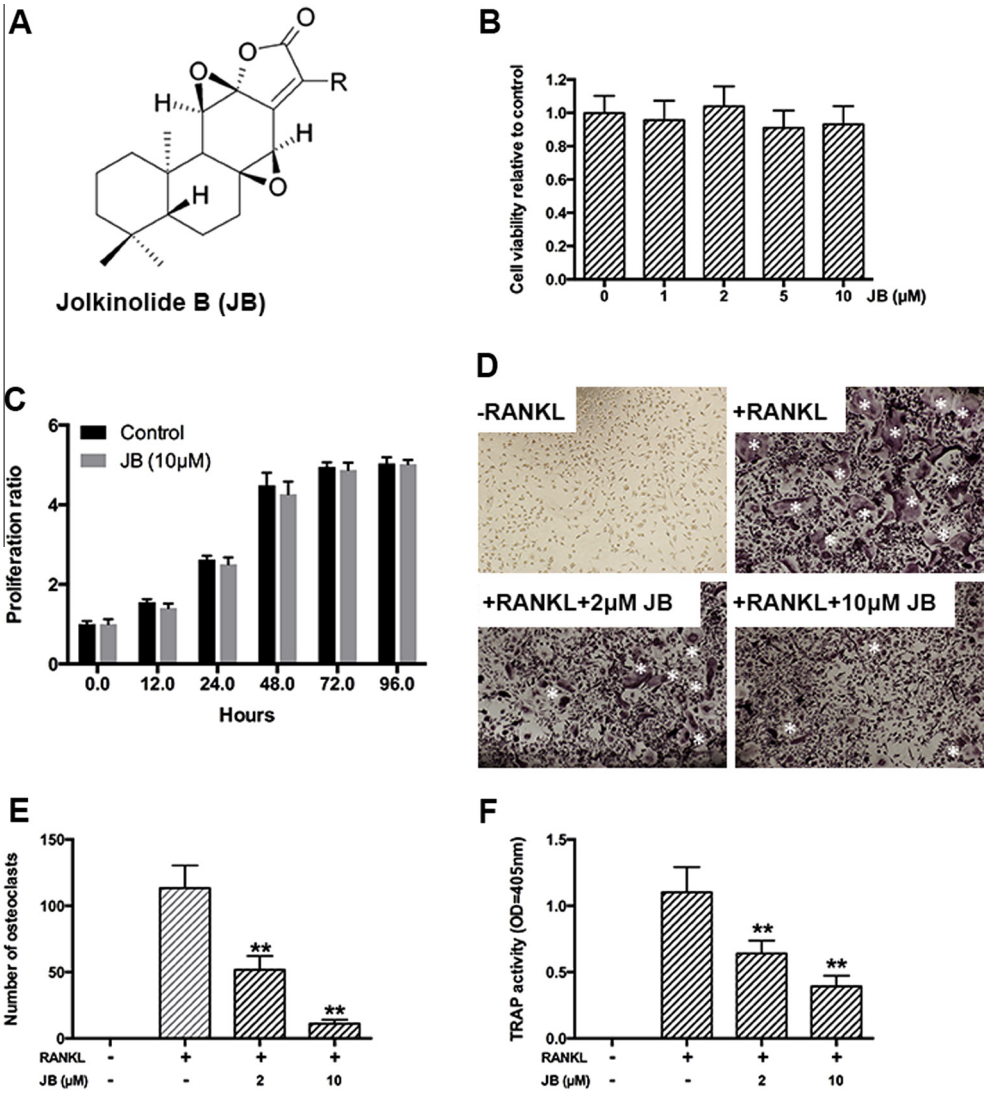

Fig. 1. JB inhibited RANKL-induced osteoclast differentiation in a dose-dependent manner without cytotoxicity. (A) Structure of JB. (B) BMMs were treated with various concentrations of JB for 48 h, and cell viability was measured by using the CCK8 assay. (C) BMMs were treated with 10 μM JB for the indicated times, and cell viability was measured by using the CCK8 assay. (D) BMMs were stimulated with or without RANKL (100 ng/mL) and M-CSF (20 ng/mL) in the presence of JB or vehicle control for 7 days. TRAP staining was carried out, and the cells were observed by light microscopy. (E) Multinucleated osteoclasts were counted. (F) BMMs were treated as in (D), and TRAP activity was quantitatively measured. The results are the mean ± SD and represent three independent experiments. $^{**}p < 0.01$ versus controls.

with JB were then added to the cultured cells and incubated for 3 days. The cell culture medium from this treatment was subsequently used to measure TRAP activity using a TRAP assay kit with an ELISA plate reader at 450 nm. In another osteoclastogenesis assay, which evaluated the effect of JB on osteoclast differentiation, BMMs cells were plated at 1×10^4 cells/well in a 96-well plate in the presence of RANKL (100 ng/mL) and M-CSF (20 ng/mL) with various concentrations of JB for 7 days. Cells were then fixed in 4% formalin for 30 min followed by staining for TRAP according to the manufacturer's instructions (Sigma–Aldrich). After incubation at 37 °C for 1 h, cells were washed 3 times with PBS. TRAP-positive cells with 3 or more nuclei were classified as osteoclasts.

2.5. Bone resorption assay

For the bone resorption assay, BMMs at 8×10^3 cells/well were seeded on bone slices in 96-well plates and stimulated with M-CSF (20 ng/mL) and RANKL (100 ng/mL). Four days later, pre-osteoclasts were treated with various concentrations of JB for another four days. Bone slices were then fixed with 2.5% glutaraldehyde and imaged using a scanning electron microscope (SEM; FEI Quanta 250). Pit areas were quantified and analyzed. The experiments were repeated at least three times.

2.6. Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated from cell culture by using TriZol reagent (Invitrogen), and cDNA was synthesized from 0.2 μg RNA by using RevertAid™ Reverse Transcriptase (Fermentas). Real-time PCR was performed using Maxima™ SYBR Green qPCR Master Mix (Fermentas). The detector was programmed with the following conditions: 40 cycles of 5 s denaturation at 95 °C and 34 s amplification at 60 °C. All reactions were run in triplicate and were normalized to the housekeeping gene β-actin. The specific primers used to amplify genes are as follows: β-actin: forward, 5′-TCTGCTGGAA GGTGGACAGT-3′ and reverse, 5′-CCTCTATGCCAACACAGTGC-3′; TRAP: forward, 5′-CTGGAGTGCACGATGCCAGCGACA-3′ and reverse, 5′-TCCGTGCTCGGCGATGGACCAGA-3′; cathepsin K: forward, 5′-CTTCCAATACGTGCAGCAGA-3′ and reverse, 5′-TCTTCAGGGCTTT CTCGTTC-3′; CTR: forward, 5′-TGCAGACAACTCTTGGTTGG-3′ and reverse, 5′-TCGGTTTCTTCTCCTCTGGA-3′; NFATc1: forward, 5′-CCGTTGCTTCCAGAAAATAACA-3′ and reverse, 5′-TGTGGGATGT-GAACTCGGAA-3′.

2.7. Western blot

Cells treated for the indicated days with various concentrations of JB were washed with cold PBS and lysed with RIPA buffer plus PMSF. Cell lysates were centrifuged at 12,000 rpm for 10 min, and supernatants were collected as samples. Protein (30 μg) was separated on 10% SDS–PAGE and transferred to PVDF membranes. The membranes were blocked with 5% skim milk in TBST containing 0.05% Tween-20 and probed successively with mouse anti IκB, phospho-IκBα, phospho-JNK, JNK, phospho-p38, p38, phospho-ERK, or ERK overnight at 4 °C. Horseradish peroxidase-conjugated rabbit anti-mouse IgG antibodies were used as secondary antibodies for 1 h at room temperature. The signals were detected by exposure in an Odyssey infrared imaging system (LI-COR).

2.8. Luciferase reporter gene activity assay

The effects of JB on RANKL-induced NF-κB activation were measured using RAW264.7 cells that had been stably transfected with an NF-κB luciferase reporter construct, as previously described [12,13]. Briefly, cells were seeded into 48-well plates and maintained in cell culture media for 24 h. Cells were then pretreated with or without the indicated concentrations of JB for 1 h, followed by addition of RANKL (1000 ng/mL) for 8 h. Luciferase activity was measured using the Promega Luciferase Assay System (Promega, Madison, WI, USA). Similarly, the effect of JB on RANKL-induced AP-1-dependent luciferase reporter assays was determined as described previously [12,13].

2.9. Statistical analysis

All values are presented as the mean ± standard deviation (SD) of the values obtained from three or more experiments. Statistical

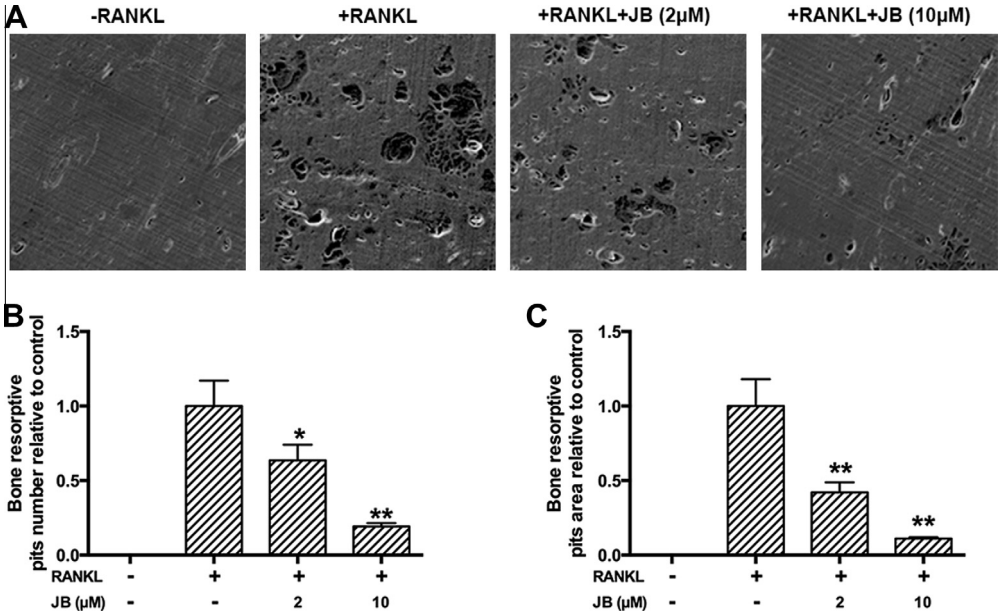

Fig. 2. JB inhibits RANKL-induced bone resorption. (A) BMMs were plated on bone slices and cultured with or without M-CSF (20 ng/mL) and RANKL (100 ng/mL). Four days later, cells were cultured in the presence of the indicated concentrations of JB for another four days. Bone resorption pits on bone slices were then examined by scanning electron microscopy. (B) The number of resorption pits and (C) the bone resorptive pit areas formed by osteoclasts were analyzed and processed using the Image pro-plus system. Resorptive areas and pit number are expressed as means ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus controls.

significances were determined by Student's *t*-test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Effects of JB on cell viability

BMMs were treated with various concentrations of JB for 48 h, and cell viability was assessed using a CCK8 assay. JB (10 μM) (Fig. 1A) had no cytotoxic effects on cells, as compared to the control cells that received no treatment (Fig. 1B). Furthermore, 10 μM JB was chosen for subsequent cytotoxic analysis for longer periods. As shown in Fig. 1C, during the whole culture period of four days, JB had no inhibitory effect on BMM proliferation.

3.2. Effect of JB on osteoclast differentiation in RANKL-stimulated BMMs

To examine the effect of JB on osteoclast differentiation, various concentrations of JB were applied to mouse BMMs undergoing osteoclast differentiation in response to a mixture of RANKL (100 ng/mL) and M-CSF (20 ng/mL). Characterization of osteoclast-like cells was monitored by formation of multinucleated giant cells and by measuring the activity of TRAP, a marker enzyme of osteoclasts. The BMMs treated with RANKL and M-CSF plus 10 μM JB showed a dramatic reduction in TRAP activity down to 21.2% ($p < 0.01$) compared to the RANKL and M-CSF-treated positive controls (Fig. 1D and E). In correlation with TRAP activity, the number of $TRAP^+$ osteoclasts was also reduced by JB (Fig. 1D and F). These results demonstrate that JB effectively suppresses both the number of multinucleated $TRAP^+$ cells and the activity of TRAP in different osteoclast differentiation systems, and suggest that JB may be potent inhibitor of osteoclast differentiation.

3.3. Effect of JB on osteoclastic bone resorption in vitro

We next investigated whether JB could inhibit osteoclastic bone resorption *in vitro*. Osteoclast precursors were plated on bone slices and treated with the indicated concentrations of JB. As shown in Fig. 2A, the JB-treated group substantially reduced both bone resorption area and pit number. Osteoclastic bone resorption was almost completely abrogated after treatment with 10 μM JB (Fig. 2). Collectively, these findings suggested that JB impaired osteoclast bone resorption *in vitro*.

3.4. Effect of JB on expression of osteoclastic marker gene expression in RANKL-stimulated BMMs

To further elucidate the role of JB in osteoclast differentiation, we examined its effect on the expression of osteoclastic marker

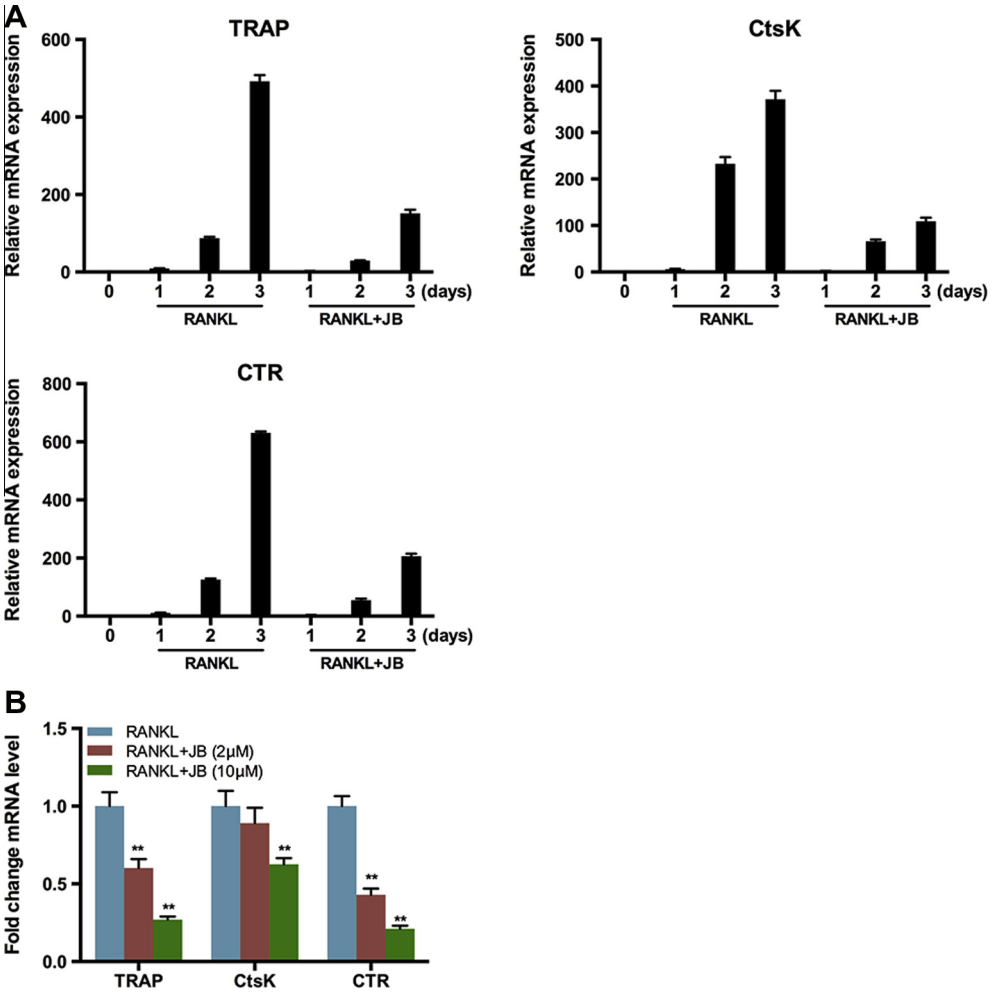

Fig. 3. JB suppresses RANKL-induced osteoclast specific gene expression. (A) BMMs were cultured with or without M-CSF (20 ng/mL), RANKL (100 ng/mL) and 10 μM JB for 0, 1, 2 or 3 days. RANKL-induced gene expression was detected by real-time PCR assay. (B) BMMs were cultured with M-CSF (20 ng/mL), RANKL (100 ng/mL) and indicated concentrations of JB. RANKL-induced gene expression was detected by real-time PCR assay. Expression levels were normalized relative to the expression of β-actin. The results are expressed as means ± SD. $^{**}p < 0.01$ versus controls.

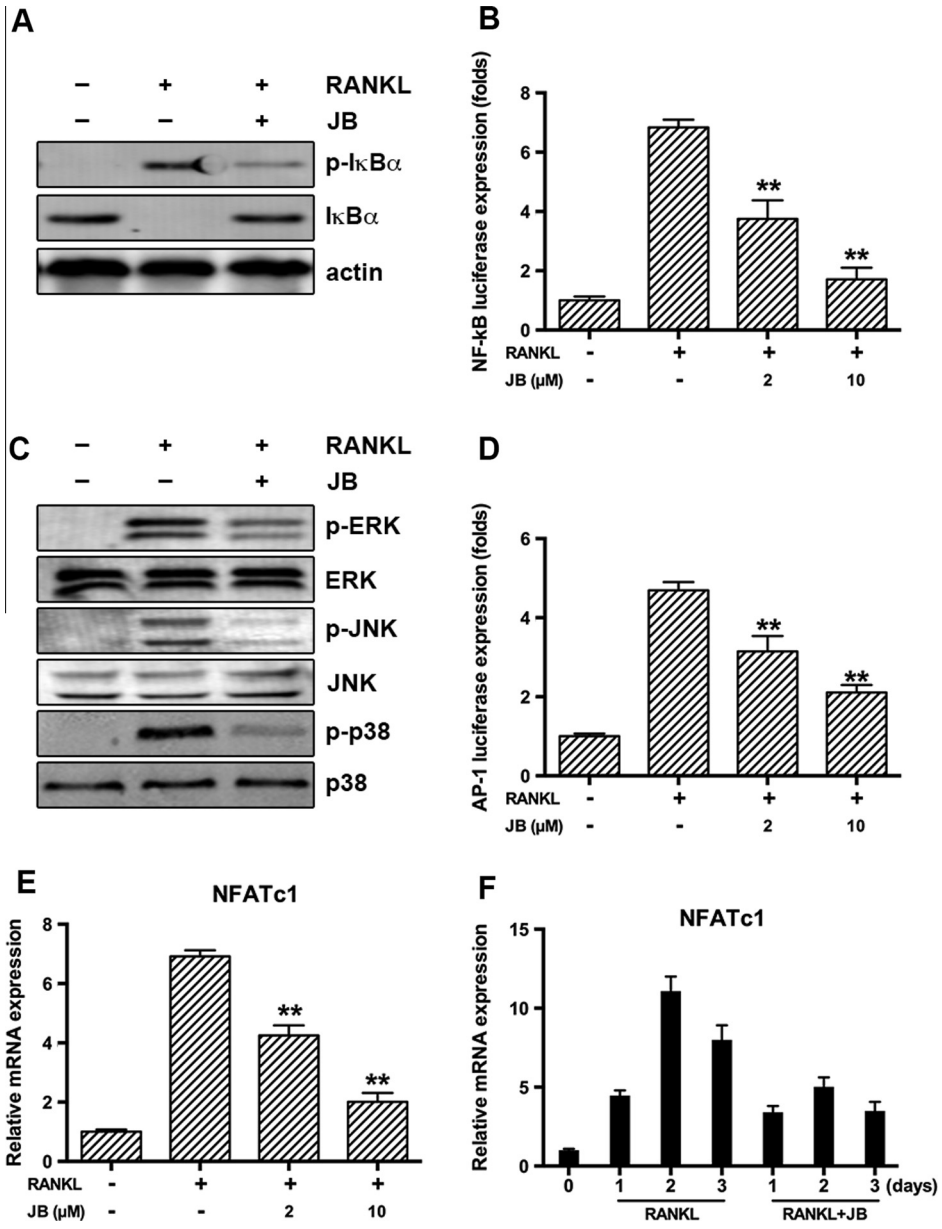

Fig. 4. JB blocks RANKL-induced activation of NF-κB and MAPK/AP-1 signaling pathways. (A) JB suppresses RANKL-induced IκBα phosphorylation and degradation. RAW264.7 cells were incubated with or without JB (10 μM) for 4 h and then treated with or without RANKL (100 ng/mL) for 10 min. Cell lysates were prepared for immunoblotting with antibodies as indicated. (B) JB inhibits RANKL-induced NF-κB-dependent luciferase reporter gene expression. After transfection of the NF-κB-luciferase reporter gene in RAW264.7 cells, the cells were incubated with the indicated concentrations of JB for 24 h and then stimulated with 100 ng/mL of RANKL for another 24 h. Cell supernatants were collected and assayed for luciferase activity as described in the Section 2. (C) JB inhibits the phosphorylation of MAPKs (JNK, p38, and ERK) induced by RANKL. RAW264.7 cells were incubated with or without JB (10 μM) for 4 h and then treated with or without RANKL (100 ng/mL) for 10 min. Cell lysates were prepared for immunoblotting with antibodies as indicated. (D) JB inhibits the transcriptional activity of AP-1 induced by RANKL. After transfection of RAW264.7 cells with the AP-1 luciferase reporter gene, the cells were incubated with the indicated concentrations of JB for 24 h and then incubated with 100 ng/mL of RANKL for another 24 h. Cell supernatants were collected and assayed for luciferase activity as described in the Section 2. (E) JB inhibits RANKL-induced NFATc1 expression in a dose-dependent manner. BMMs were cultured with M-CSF (20 ng/mL) and RANKL (100 ng/mL) at indicated concentrations of JB. RANKL-induced gene expression was detected by real-time PCR. Expression levels were normalized relative to the expression of β-actin. (F) JB inhibits RANKL-induced NFATc1 expression in a time-dependent manner. BMMs were cultured with or without M-CSF (20 ng/mL), RANKL (100 ng/mL) and 10 μM JB for 0, 1, 2 or 3 days. RANKL-induced gene expression was detected by real-time PCR assay. Expression levels were normalized relative to the expression of β-actin. The results are expressed as means ± SD. $^{**}p < 0.01$ versus controls.

genes during osteoclastogenesis. Osteoclastic markers, including TRAP, cathepsin K (CtsK), and calcitonin receptor (CTR), were significantly upregulated upon treatment with RANKL. However, the upregulation of osteoclastic marker genes was attenuated by addition of JB for the whole culture period (Fig. 3A). Moreover, JB suppressed osteoclast-specific gene expression in a dose-dependent manner (Fig. 3B). Collectively, these data support the inhibition of osteoclast formation by JB.

3.5. Effect of JB on NF-κB activation

The activation of NF-κB is critical for RANKL-induced osteoclastogenesis [14]. NF-κB is inactive in the cytosol because it is bound to IκB, and becomes active after IκB has been phosphorylated and subsequently degraded [15]. Thus, we investigated whether JB inhibits phosphorylation and degradation of IκB. Accordingly, BMMs were pretreated for 4 h with JB, and IκBα

protein levels were determined after 10 min of further exposure to RANKL (100 ng/mL). JB was found to significantly suppress RANKL-induced phosphorylation and degradation of IκBα (Fig. 4A). Furthermore, the inhibitory effects of JB on NF-κB activation were further supported by luciferase assays. The transcriptional activity of NF-κB increased dramatically following treatment with RANKL. However, NF-κB activity was inhibited by JB in a concentration-dependent manner (Fig. 4B).

3.6. Effects of JB on activation of MAPKs/AP-1

Besides the NF-κB signaling pathway, activation of the MAPK/AP-1 pathway plays a pivotal role in osteoclastogenesis [12,16]. To evaluate the effects of JB on MAPKs following the stimulation of RANKL in BMMs, we examined the phosphorylation of p38, JNK, and ERK by Western blot analysis. Our results showed that JB significantly inhibited RANKL-induced phosphorylation of p38, JNK and ERK, while the amount of total ERK, p38, and JNK was unaffected by RANKL and/or JB treatment (Fig. 4C). These results indicate that JB can inhibit RANKL-induced activation of MAPKs in osteoclasts.

Furthermore, by using AP-1 luciferase reporter gene assays, we also examined whether JB could suppress AP-1 activity. Similar to the NF-κB luciferase reporter gene assay, the activation of AP-1 transcriptional activity in RAW264.7 cells sharply increased when exposed to RANKL, whereas JB suppressed AP-1 luciferase activity in a dose-dependent manner (Fig. 4D), suggesting that JB can inhibit RANKL-induced activation of MAPK/AP-1.

3.7. Effects of JB on RANKL-induced NFATc1 expression

NFATc1 is a well-known master regulator of osteoclastogenesis and osteoclast function [17]. NF-κB and MAPK/AP-1 pathways are the two main activators that can regulate the NFATc1 promoter and NFATc1 expression [12,18]. NF-κB induces the initial induction of NFATc1, the expression of which is auto-amplified by NFATc1 binding to its own promoter in cooperation with c-Fos [19,20]. To determine whether JB regulates the expression of NFATc1 by inhibiting the NF-κB and MAPK/AP-1 signaling pathways, we examined the expression of NFATc1 at the mRNA level. NFATc1 was increased when cells were exposed to RANKL, whereas JB abrogated the RANKL-induced increases in NFATc1 at the mRNA level in a dose-dependent manner (Fig. 4E), suggesting that JB can suppress RANKL-induced NFATc1 expression. To further confirm that JB inhibited NFATc1 expression, we examined various time points and demonstrated that JB suppressed NFATc1 expression throughout the experimental period (Fig. 4F). Taken together, our results indicate that JB suppresses RANKL-induced NFATc1 expression in osteoclastogenesis.

4. Discussion

Excessive bone resorption plays a central role in pathologic bone diseases [21]. As such, inhibiting osteoclast activity should be a valuable treatment for osteoclast-related diseases. In this study, we determined the effect of JB on the formation of osteoclasts from BMMs. Osteoclasts are generated from BMMs by the activation of RANKL [22,23]. The results of our study show that JB can inhibit RANKL-induced formation of osteoclasts from precursor cells without cytotoxicity.

Osteoclasts are derived from monocyte–macrophage lineages [24]. Terminal differentiation of osteoclasts is characterized by the expression of mature phenotypic markers, such as the expression of TRAP, CTR, and CtsK, as well as morphological conversion into large multinucleated cells and the capability to form resorption lacunae on the bone [25–28]. Our study showed that JB reduced RANKL-induced expression of TRAP, CTR, and CtsK genes in a dose-dependent manner.

Activation of the NF-κB pathway is a key factor in RANKL-induced osteoclast differentiation [17]. The classical NF-κB signaling pathway involves activation of the IκB kinase complex, which phosphorylates IκBα and targets it for ubiquitin-dependent degradation [18]. Our results showed that JB inhibited cytoplasmic degradation of IκBα, resulting in reduced levels of NF-κB transactivation. These results indicated that inhibition of the NF-κB-dependent pathway is one of the mechanisms involved in the anti-osteoclastogenic effect of JB.

The MAPKs (JNK, ERK and p38) have been reported to be activated by RANKL stimulation and to be associated with osteoclastogenesis [1,29]. p38 is important in the early stages of osteoclast generation because it regulates the microphthalmia-associated transcription factor [30], while dominant-negative JNK prevents RANKL-induced osteoclastogenesis [31]. Meanwhile, ERK is known to induce c-Fos for osteoclastogenesis [32], and inhibition of ERK has been shown to decrease osteoclast formation [33]. In this study, we evaluated the effects of JB on the activation of these MAPKs and found that JB dose-dependently inhibited the phosphorylation of MAPKs. These results demonstrate that phosphorylation of MAPKs may contribute to the anti-osteoclastogenic effect of JB in RANKL-stimulated BMMs.

In summary, the present study demonstrated that JB inhibited osteoclastogenesis from macrophages and bone resorption *in vitro*. JB also reduced the RANKL-induced expression of osteoclastic marker genes. In addition, JB attenuated RANKL-induced ERK, p38, JNK, and NF-κB activation. Although additional experiments are needed to confirm the efficacy of JB in treating disease conditions *in vivo*, our results indicate that JB has potential as a therapy for disorders associated with bone loss.

Conflicts of interest

The authors have no conflicts of interest to declare.

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References

[1] W.J. Boyle, W.S. Simonet, D.L. Lacey, Osteoclast differentiation and activation, Nature 423 (2003) 337–342.
[2] G. Karsenty, E.F. Wagner, Reaching a genetic and molecular understanding of skeletal development, Dev. Cell 2 (2002) 389–406.
[3] Y. Tanaka, S. Nakayamada, Y. Okada, Osteoblasts and osteoclasts in bone remodeling and inflammation, Curr. Drug Targets Inflamm. Allergy 4 (2005) 325–328.
[4] S.L. Teitelbaum, F.P. Ross, Genetic regulation of osteoclast development and function, Nat. Rev. Genet. 4 (2003) 638–649.
[5] W. Liu, J. Ho, G. Qin, C.-T. Che, Jolkinolide B induces neuroendocrine differentiation of human prostate LNCaP cancer cell line, Biochem. Pharmacol. 63 (2002) 951–957.
[6] J.-H. Wang, Y.-J. Zhou, X. Bai, P. He, Jolkinolide B from *Euphorbia fischeriana* Steud induces apoptosis in human leukemic U937 cells through PI3K/Akt and XIAP pathways, Mol. Cells 32 (2011) 451–457.
[7] Y. Lin, H. Cui, H. Xu, L. Yue, H. Xu, L. Jiang, J. Liu, Jolkinolide B induces apoptosis in MDA-MB-231 cells through inhibition of the PI3K/Akt signaling pathway, Oncol. Rep. 27 (2012) 1976–1980.
[8] H.-Y. Xu, Z.-W. Chen, J.-C. Hou, F.-X. Du, J.-C. Liu, Jolkinolide B induces apoptosis in MCF-7 cells through inhibition of the PI3K/Akt/mTOR signaling pathway, Oncol. Rep. 29 (2013) 212–218.
[9] T. Uto, G.-W. Qin, O. Morinaga, Y. Shoyama, 17-Hydroxy-jolkinolide B, a diterpenoid from *Euphorbia fischeriana*, inhibits inflammatory mediators but activates heme oxygenase-1 expression in lipopolysaccharide-stimulated murine macrophages, Int. Immunopharmacol. 12 (2012) 101–109.
[10] M. Kogawa, K. Hisatake, G.J. Atkins, D.M. Findlay, Y. Enoki, T. Sato, P.C. Gray, Y. Kanesaki-Yatsuka, P.H. Anderson, S. Wada, The paired-box homeodomain

transcription factor Pax6 binds to the upstream region of the trap gene promoter and suppresses receptor activator of NF-κB ligand (RANKL)-induced osteoclast differentiation, J. Biol. Chem. 288 (2013) 31299–31312.
[11] W. Zou, T. Izawa, T. Zhu, J. Chappel, K. Otero, S.J. Monkley, D.R. Critchley, B.G. Petrich, A. Morozov, M.H. Ginsberg, Talin1 and Rap1 are critical for osteoclast function, Mol. Cell. Biol. 33 (2013) 830–844.
[12] F. Ikeda, R. Nishimura, T. Matsubara, S. Tanaka, J.-I. Inoue, S.V. Reddy, K. Hata, K. Yamashita, T. Hiraga, T. Watanabe, Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation, J. Clin. Invest. 114 (2004) 475–484.
[13] T.J. Weber, L.M. Markillie, Regulation of activator protein-1 by 8-iso-prostaglandin E2 in a thromboxane A2 receptor-dependent and-independent manner, Mol. Pharmacol. 63 (2003) 1075–1081.
[14] N. Soysa, N. Alles, NF-κB functions in osteoclasts, Biochem. Biophys. Res. Commun. 378 (2009) 1–5.
[15] K.M. Ghosh Sankar, Missing pieces in the NF-kappaB puzzle, Cell 109 (2002) 81–96.
[16] A.E. Grigoriadis, Z.-Q. Wang, M.G. Cecchini, W. Hofstetter, R. Felix, H.A. Fleisch, E.F. Wagner, C-Fos: a key regulator of osteoclast–macrophage lineage determination and bone remodeling, Science 266 (1994) 443–448.
[17] Q. Zhao, X. Wang, Y. Liu, A. He, R. Jia, NFATc1: functions in osteoclasts, Int. J. Biochem. Cell Biol. 42 (2010) 576–579.
[18] M. Asagiri, H. Takayanagi, The molecular understanding of osteoclast differentiation, Bone 40 (2007) 251–264.
[19] M. Asagiri, K. Sato, T. Usami, S. Ochi, H. Nishina, H. Yoshida, I. Morita, E.F. Wagner, T.W. Mak, E. Serfling, Autoamplification of NFATc1 expression determines its essential role in bone homeostasis, J. Exp. Med. 202 (2005) 1261–1269.
[20] H. Takayanagi, Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems, Nat. Rev. Immunol. 7 (2007) 292–304.
[21] S.R. Cummings, L.J. Melton, Epidemiology and outcomes of osteoporotic fractures, The Lancet 359 (2002) 1761–1767.
[22] T. Suda, N. Takahashi, N. Udagawa, E. Jimi, M.T. Gillespie, T.J. Martin, Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families, Endocr. Rev. 20 (1999) 345–357.
[23] S.L. Teitelbaum, RANKing c-Jun in osteoclast development, J. Clin. Invest. 114 (2004) 463–465.
[24] S.G.-K. Mohamed, E. Sugiyama, K. Shinoda, H. Taki, H. Hounoki, H.O. Abdel-Aziz, M. Maruyama, M. Kobayashi, H. Ogawa, T. Miyahara, Interleukin-10 inhibits RANKL-mediated expression of NFATc1 in part via suppression of c-Fos and c-Jun in RAW264. 7 cells and mouse bone marrow cells, Bone 41 (2007) 592–602.
[25] O. Anusaksathien, C. Laplace, X. Li, Y. Ren, L. Peng, S.R. Goldring, D.L. Galson, Tissue-specific and ubiquitous promoters direct the expression of alternatively spliced transcripts from the calcitonin receptor gene, J. Biol. Chem. 276 (2001) 22663–22674.
[26] G. Motyckova, K. Weilbaecher, M. Horstmann, D. Rieman, D. Fisher, D. Fisher, Linking osteopetrosis and pycnodysostosis: regulation of cathepsin K expression by the microphthalmia transcription factor family, Proc. Nat. Acad. Sci. 98 (2001) 5798–5803.
[27] S. Reddy, J. Hundley, J. Windle, O. Alcantara, R. Linn, R. Leach, D. Boldt, G. Roodman, Characterization of the mouse tartrate-resistant acid phosphatase (trap) gene promoter, J. Bone Miner. Res. 10 (1995) 601–606.
[28] H.J. Choi, Y.R. Park, M. Nepal, B.-Y. Choi, N.-P. Cho, S.H. Choi, S.R. Heo, H.S. Kim, M.-S. Yang, Y. Soh, Inhibition of osteoclastogenic differentiation by Ikarisoside A in RAW 264.7 cells via JNK and NF-κB signaling pathways, Eur. J. Pharmacol. 636 (2010) 28–35.
[29] Z.H. Lee, H.-H. Kim, Signal transduction by receptor activator of nuclear factor kappa B in osteoclasts, Biochem. Biophys. Res. Commun. 305 (2003) 211–214.
[30] M. Matsumoto, T. Sudo, T. Saito, H. Osada, M. Tsujimoto, Involvement of p38 mitogen-activated protein kinase signaling pathway in osteoclastogenesis mediated by receptor activator of NF-κB ligand (RANKL), J. Biol. Chem. 275 (2000) 31155–31161.
[31] F. Ikeda, R. Nishimura, T. Matsubara, S. Tanaka, J. Inoue, S.V. Reddy, K. Hata, K. Yamashita, T. Hiraga, T. Watanabe, Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation, J. Clin. Invest. 114 (2004) 475–484.
[32] P. Monje, J. Hernández-Losa, R.J. Lyons, M.D. Castellone, J.S. Gutkind, Regulation of the transcriptional activity of c-Fos by ERK A novel role for the prolyl isomerase PIN1, J. Biol. Chem. 280 (2005) 35081–35084.
[33] E. Ang, Q. Liu, M. Qi, H.G. Liu, X. Yang, H. Chen, M.H. Zheng, J. Xu, Mangiferin attenuates osteoclastogenesis, bone resorption, and RANKL-induced activation of NF-κB and ERK, J. Cell. Biochem. 112 (2011) 89–97.