(2S)-2′-Methoxykurarinone Inhibits Osteoclastogenesis and Bone Resorption through Down-Regulation of RANKL Signaling

Ju-Young Kim, a Jung Young Kim, b Jeong Joong Kim, c,d Jaemin Oh, a,c,d Youn-Chul Kim,* e and Myeung Su Lee* a,c,f

a Imaging Science-based Lung and Bone Diseases Research Center, Wonkwang University; b Department of Anatomy, School of Medicine, Wonkwang University; c Institute for Skeletal Disease, Wonkwang University; d Standardized Material Bank for New Botanical Drugs, College of Pharmacy, Wonkwang University; e Division of Rheumatology, Department of Internal Medicine, Wonkwang University; Iksan, Jeonbuk 570–749, Republic of Korea; and f Department of Oriental Medicine, Daejeon University; Daejeon 300–716, Republic of Korea.

Received September 4, 2013; accepted October 26, 2013

(2S)-2′-Methoxykurarinone (MK), a compound isolated from the roots of Sophora flavescens, has various physiological properties, such as anti-inflammatory, antipyretic, antidiabetic, and antineoplastic effects. However, the effect of S. flavescens-derived MK on osteoclastogenesis remains unknown. Therefore, we examined the effect and mechanism of action of MK on receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclast differentiation and bone resorption. MK inhibited osteoclast differentiation in bone marrow cell–osteoblast cocultures but did not affect the RANKL-to-osteoprotegerin ratio induced by osteoclastogenic factors in osteoblasts. MK also inhibited RANKL-induced osteoclast differentiation from bone marrow macrophages in a dose-dependent manner, without cytotoxicity. Pretreatment with MK significantly suppressed the Akt, p38, c-Jun N terminal kinase (JNK), c-Fos, and nuclear factor of activated T cells cl (NFATc1) pathways and inhibited the bone-resorbing activity of mature osteoclasts. These results collectively suggest that MK inhibits osteoclast differentiation and bone resorption through RANKL-induced mitogen-activated protein kinases (MAPKs) and c-Fos-NFATc1 signaling pathways.

Key words (2S)-2′-methoxykurarinone; bone resorption; osteoclast; osteoporosis; receptor activator of nuclear factor-κB ligand (RANKL)

Osteoclasts, which originate from hematopoietic cells of the monocyte macrophage lineage, play a crucial role in bone remodeling.1,2 The bone loss in many important skeletal disorders, such as osteoporosis, rheumatoid arthritis, and bone metastases, occurs mainly because of increased osteoclast activity.3,4 Bone formation is regulated by osteoblasts, which also express the receptor activator of nuclear factor kappa-B ligand (RANKL), which has been identified as a member of the tumor necrosis factor (TNF) superfamily and regulates osteoclast differentiation through cell-to-cell contact.3,4 Osteoblasts and stromal cells also produce a soluble decoy receptor for RANKL, osteoprotegerin (OPG), which inhibits osteoclast formation by interrupting the interaction between RANKL and RANK, resulting in an increase in bone density and bone volume.2,3 Several osteoclastogenic factors, such as 1,25-dihydroxyvitamin D3 (VitD3) and proinflammatory cytokines, can stimulate osteoclast formation by up-regulating the ratio of RANKL to OPG in osteoblasts or stromal cells.1,2 Thus, osteoclast formation is regulated directly or indirectly by environmental cells in many conditions.

The binding of RANKL to its receptor RANK causes receptor trimerization and leads to the recruitment of several adaptor molecules, such as tumor necrosis factor receptor-associated factor 6 (TRAF6), which activates multiple downstream signaling pathways, including those of Akt, p38, c-Jun N-terminal protein kinase (JNK), and nuclear factor kappa-B (NF-κB).5–7 Consequently, RANKL-RANK-TRAF6-mediated signaling induces transcription factors, including c-Fos and nuclear factor of activated T cells cl (NFATc1), that are required for osteoclast differentiation and activation.8–10 NFATc1, a critical transcription factor downstream of c-Fos, is thought to be a master transcription factor for osteoclastogenesis, which regulates the expression of osteoclastogenic genes, such as tartrate-resistant acid phosphatase (TRAP) and osteoclast-associated receptor (OSCAR).3,4 Several compounds derived from natural products have been recently reported to possess inhibitory effects on osteoclast differentiation and function.9–11 (2S)-2′-Methoxykurarinone (MK), used in our experiments, is a compound isolated from the root of Sophora flavescens Aiton (Family Leguminosae). The root of S. flavescens is an herbal medicine, used as a diuretic and for the treatment of jaundice, leucorrhea, carbuncles, pyogenic infections of the skin, scabies, enteritis, and dysentery. Recently, biological and pharmacological studies have revealed anti-inflammatory,12 and antitumor,13 antioxidant14 for the crude extracts or isolated constituents of S. flavescens. In particular, MK, the main component of S. flavescens, was reported that had the antioxidant activity, it results in the down-regulation of glutamate-induced neurotoxicity via the induction of heme oxygenase-1 (HO-1).15 However, the other pharmacological effects of MK remains unknown; in particular, the effect of MK on osteoclastogenesis and bone resorption has not yet been well defined.

In this study, we investigated the effects of MK and the molecular mechanisms of its action on osteoclast differentiation and resorption in vitro.

MATERIALS AND METHODS

Reagents and Materials MK was kindly provided by Dr. Youn-Chul Kim (Department of Pharmacology, Wonkwang University, Iksan, Korea). The structure is shown in Fig. 1.

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: yckim@wku.ac.kr; ckhlms@wku.ac.kr © 2014 The Pharmaceutical Society of Japan
Recombinant soluble human RANKL, human macrophage-colony stimulating factor (M-CSF), and mouse interleukin (IL)-1α were purchased from PeproTech EC (London, U.K.). 1,25-Dihydroxyvitamin D₃ and prostaglandin E₂ (PGE₂) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Penicillin/streptomycin antibiotics, α-minimum essential medium (α-MEM), and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, U.S.A.). Anti-c-Jun N-terminal kinase, anti-phospho-JNK, anti-Akt, anti-phospho-Akt, anti-p38 mitogen-activated protein kinase (MAPK), anti-phospho-p38 MAPK, anti-inhibitory xB (IxB), and anti-phospho IxB antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, U.S.A.). Anti-c-Fos, anti-NFATc1, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All other chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

**Coculture of Bone Marrow Cells (BMCs) and Osteoblasts** BMCs were obtained by flushing the femurs and tibias with α-MEM containing 1% antibiotics, and then, red blood cells were removed with red blood cell lysis buffer. The cells were suspended in α-MEM complete medium containing 10% FBS and 1% antibiotics and were used as BMCs. Calvarial osteoblasts were isolated from mouse calvarial bones by using 0.1% collagenase and 0.2% dispase digestion. All mouse experiments in this study were performed in accordance with the animal experiment guidelines of the Institute Committee of Wonkwang University. Calvarial osteoblasts and BMCs were seeded in 48-well plates and then cocultured for 6 d with IL-1α (20 ng/mL) in the presence or absence of MK.

**Organ Culture** Calvarial bones were obtained from 3-d-old ICR mice. The bones were placed in 24-well plates in α-MEM complete medium containing 10% FBS and 1% antibiotics, and then cultured for 7 d with or without IL-1α (20 ng/mL) in the presence or absence of MK (20 µM). Calvariae were fixed with 3.7% formalin, permeabilized with 0.1% Triton X-100, and stained with TRAP (Sigma).

**In Vitro Osteoclast Formation Assay** BMCs were obtained from the femurs and tibias of 5-week-old ICR mice and then incubated overnight in α-MEM complete medium containing 10% FBS and 1% antibiotics, on 10-cm culture dishes in the presence of M-CSF (10 ng/mL). Non-adherent BMCs were transferred to 10-cm Petri dishes and cultured in the presence of M-CSF (30 ng/mL) for 3 d. Adherent cells were used as bone marrow macrophages (BMMs), as osteoclast precursors, after the non-adherent cells had been washed out. To generate osteoclasts, BMMs (3.5×10⁴ cells/well) were cultured with M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 4 d in 48-well plates, with or without MK. At the end of the culture period, the cells were fixed in 3.7% formalin for 15 min, permeabilized with 0.1% Triton X-100 for 15 min, and then stained with TRAP. TRAP-positive multinucleated cells with >5 nuclei were counted as osteoclasts.

**Cell Viability Assay** The XTT assay was performed to examine the effect of MK on the viability of BMMs. The BMMs (1×10⁴ cells/well) were cultured with various concentrations of MK for 3 d, in the presence of M-CSF (30 ng/mL), on 96-well plates. Then, 50 µL of the XTT solution was added to each well and incubation was carried out for 4 h. The plate was read at 450 nm using an ELISA reader (Molecular Devices, CA, U.S.A.).

**Bone Resorption Assay** BMCs (1×10⁷ cells) and primary osteoblasts (1×10⁶ cells) were seeded on collagen gel-coated...
Fig. 2. Effects of MK on IL-1-Induced Osteoclastogenesis in Coculture

(A) Mouse bone marrow cells and primary osteoblasts were cocultured in the presence of IL-1 (20 ng/mL), with or without MK, for 6 d. After culturing, the generated osteoclasts were detected by TRAP staining, and TRAP-positive multinucleated cells were counted as osteoclasts. (B) Calvarial bones were treated for 7 d with or without IL-1 in the presence or absence of MK (20 µM). Cultured calvariae were stained with TRAP solution. The bones were photographed with a digital camera. (C) Primary osteoblasts were pretreated with MK (20 µM) or DMSO for 12 h and then stimulated with IL-1 for 24 h. The expression of mRNA for RANKL and OPG was analyzed by the quantitative real-time RT-PCR. ***p<0.001 versus the control (DMSO).

Fig. 3. Effects of MK on RANKL-Induced Osteoclast Differentiation in BMMs

(A) BMMs were cultured with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in the presence or absence of MK at the indicated concentrations. After 4 d, the cells were fixed and stained with TRAP solution. (B) TRAP-positive multinucleated cells contacting more than 5 nuclei were counted as osteoclasts. (C) BMMs were cultured with MK for 3 d at the indicated concentration in the presence of M-CSF. Cell viability was determined by the XTT assay. **p<0.01, ***p<0.001 versus the control (DMSO).
10-cm culture dishes and cultured for 6 d in the presence of VitD₃ (10⁻⁸ M) and PGE₂ (10⁻⁶ M). Cells were detached by treatment with 0.2% collagenase at 37°C for 10 min, after which they were replated on hydroxyapatite-coated plates (osteo assay plate; Corning, NY, U.S.A.) and incubated with or without MK (20 μM) for 1 h. This was followed by a further culture in the presence of RANKL (100 ng/mL). After 24 h, the cells were removed, and total resorption pits were photographed and analyzed using Image-Pro Plus version 4.0 (Media Cybernetics, Silver Spring, MD, U.S.A.).

Statistical Analyses Experiments were conducted separately at least 3 times, and all data are presented as the mean±standard deviation (S.D.). All statistical analyses were performed using SPSS (Korean version 14.0). Student’s t-test was used to compare the parameters between 2 groups, and the ANOVA test, followed by the Tukey post-hoc test, was used to compare the parameters among 3 groups. A value of p<0.05 was considered statistically significant.

RESULTS

MK, Suppresses Osteoclast Formation in Bone Marrow Cell–Osteoblast Cocultures We first examined whether MK suppresses osteoblast-supported osteoclast differentiation. The stimulation of IL-1 for 6 d led to the formation of TRAP-positive multinucleated osteoclasts in the cocultures, and, under these conditions, treatment with MK significantly inhibited osteoclast formation (Fig. 2A). Furthermore, the osteoclast formation in calvarial bone cultures treated with IL-1 was strongly inhibited in the presence of MK (Fig. 2B). In this culture system, osteoblasts support osteoclastogenesis by increasing the ratio of RANKL to OPG. Thus, we assessed the expression of RANKL and OPG in osteoblasts by using real-time quantitative RT-PCR. IL-1 increased the RANKL expression and decreased the OPG expression in the osteoblasts at 24 h. Pretreatment with MK did not alter the expression of RANKL or OPG (Fig. 2C).

Fig. 4. MK Suppressed the Expression of c-Fos and NFATc1, and RANKL-Induced Early Signaling

(A) BMMs were pretreated with or without MK (20 μM) for 1 h prior to RANKL (100 ng/mL) stimulation at the indicated time. The mRNA expression of the indicated genes was analyzed by real-time RT-PCR. ***p<0.001 versus the control (DMSO) at 0 h; *p<0.05, ***p<0.001 versus the control (DMSO) at the indicated time. (B) MK inhibited the protein expressions of c-Fos and NFATc1 induced by RANKL. BMMs were pretreated with or without MK for 1 h and were treated with RANKL for the indicated times. (C) BMMs were pretreated with or without MK (20 μM) for 1 h prior to RANKL stimulation at the indicated time. Lysates were analyzed by Western blot analysis with the indicated antibodies.
MK Inhibits Osteoclastogenesis via Direct Action on Osteoclast Precursors

We next examined the effects of MK on RANKL-induced osteoclast formation from osteoclast precursor (BMMs). When BMMs were incubated with M-CSF and RANKL for 4d, numerous TRAP-positive multinucleated osteoclasts were generated. Pretreatment with MK suppressed the osteoclast formation in a dose-dependent manner (Figs. 3A, B). The inhibitory effect of MK in osteoclastogenesis was not attributed to cellular toxicity or cell proliferation (Fig. 3C).

MK Inhibits RANKL-Induced c-Fos and NFATc1 Expressions

To determine the molecular mechanisms of the effect of MK in osteoclastogenesis, we examined the effect of MK on the expression of key transcription factors, such as c-Fos and NFATc1. The expressions of c-Fos and NFATc1 were up-regulated in BMMs by RANKL stimulation. Pretreatment with MK effectively inhibited the RANKL-induced mRNA expression of c-Fos or NFATc1 at 12 or 48h, respectively (Fig. 4A), and also suppressed the RANKL-induced c-Fos and NFATc1 protein expressions (Fig. 4B). In addition, the RANKL-induced expressions of OSCAR and TRAP, 2 other markers of osteoclastogenesis, were significantly reduced in the presence of MK (Fig. 4A).

MK Inhibits the RANKL-Induced Phosphorylation of Akt, p38, and JNK in BMMs

To further investigate the molecular mechanisms underlying the inhibitory effect of MK on RANKL-induced osteoclast formation, we examined the effects of MK on RANKL-induced early signaling pathways, including those of Akt, p38, JNK, and NF-κB. Phosphorylation of these signaling molecules was observed at 5min after RANKL treatment in BMMs. Among these pathways, the activations of Akt, p38, and JNK by RANKL were all significantly inhibited by MK, except for the phosphorylation and degradation of IκB, which was apparently unaffected (Fig. 4C).

MK Inhibits the Bone-Resorbing Activity of Osteoclasts

We also investigated the effects of MK on the bone resorption activity of mature osteoclasts. Mature osteoclasts from cocultures were replated on hydroxyapatite-coated plates and cultured in the presence or absence of MK. As shown in Fig. 5A, MK reduced the RANKL-induced bone-resorbing activity. It did not affect RANKL-induced survival of mature osteoclasts (Fig. 5B).

DISCUSSION

The balance between bone formation and bone resorption is regulated by a network of cytokines that include M-CSF, RANKL, IL-1, IL-6, and TNF-α, which are synthesized by osteoblasts and stromal cells and modulate osteoclast differentiation and activity by both direct effects on its precursors and indirect effects via osteoblasts. In particular, IL-1 is thought to be a critical mediator of the pathological bone destruction induced by estrogen deficiency. The blocking of IL-1 signaling can reduce bone loss and cartilage degradation in animal models of rheumatoid arthritis. In the present study, we found that MK prevented osteoclast differentiation from BMCs and primary osteoblast cocultures, as well as organ cultures induced by IL-1, without affecting the expressions of RANKL and OPG (Fig. 2). We also found that MK inhibited RANKL-induced formation of osteoclasts from osteoclast precursors in the absence of osteoblasts. The number of TRAP-positive multinuclear cells from this culture was decreased in a dose-dependent manner after treatment with MK (Fig. 3), which was similar to the observations in the co-culture.
cultures. These results suggest that MK suppresses osteoclast differentiation by directly acting on osteoclast precursors. However, it has recently been found that mineralized matrix-embedded chondrocytes and osteocytes are critical sources of RANKL, which controls osteoclastic resorption in adult bone remodeling. Thus, further studies on the effect of MK on RANKL expression in these cells might provide better understanding of its inhibitory effect.

The RANKL receptor RANK lacks intrinsic enzymatic activity in its intracellular domain, but it transduces signaling by recruiting adapters such as TRAF6, and further leads to the activation of several signaling cascades. Signaling molecules, such as Akt, p38, JNK, and NF-xB, have been reported to be activated by RANKL stimulation and the associated osteoclastogenesis. Akt has been shown to regulate osteoclast survival and differentiation, p38 is important at the early stage of osteoclast generation, regulating the microphthalmia-associated transcription factor, whereas dominant-negative JNK prevents RANKL-induced osteoclastogenesis. NF-xB is an important signal mediator for inflammatory and immune reactions, and is a major transcription factor for RANKL-activated osteoclastogenesis. MK significantly inhibited the phosphorylation of Akt, p38, and JNK, and did not affect the phosphorylation and degradation of IxB in the present study (Fig. 4C). The activation of signaling molecules induces transcription factors such as c-Fos and NFATc1. Each factor acts at different stages of osteoclast differentiation, and a lack of c-Fos and NFATc1 interferes with osteoclastogenesis. Futhermore, NFATc1 is a master regulator of osteoclastogenesis, which autoamplifies and results in the expression of osteoclast-specific genes such as OSCAR and TRAP. Our data suggest that suppression of the RANKL-induced activation of c-Fos and NFATc1 by MK correlates with osteoclastogenesis inhibition (Figs. 4A, B). Moreover, the mRNA levels of major osteoclast markers such as OSCAR and TRAP were inhibited by MK (Fig. 4A).

Osteoclastic bone resorption requires the activation of mature osteoclasts, which includes the polarization of the cell membrane to bone and the secretion of protons and lytic enzymes into a sealed resorption vacuole. Several drugs targeting osteoclast activation, including nitrogen-containing bishphosphonates, Src inhibitors, and cathepsin K inhibitors, are currently available or under clinical investigation for treatment of osteoporosis. The present study demonstrated for the first time that MK decreases the bone-resorbing activity of mature osteoclasts, albeit the underlying mechanism remains to be elucidated. Further study of the underlying mechanism is likely to provide a molecular basis for the development of new drugs for the treatment of bone diseases with excessive bone resorption.

In conclusion, MK efficiently prevents the RANKL-induced osteoclastogenesis of BMMS as well as mature osteoclastic bone resorption. The therapeutic effect of MK is associated with a down-regulation of Akt, p38, and JNK, as well as of c-Fos and NFATc1, leading to the lowered expressions of TRAP and OSCAR. Hence, MK may be of use in the treatment of osteoclast-related disorders, including osteoporosis.

Acknowledgment This study was supported by a Grant of the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI12C0110).

REFERENCES


