Risedronate Directly Inhibits Osteoclast Differentiation and Inflammatory Bone Loss

Han Bok Kwak, Jong Yun Kim, Kwang Jin Kim, Min-Kye Choi, Jeong-Joong Kim, Kwang Mee Kim, Yong-II Shin, Myeung Su Lee, Hun Soo Kim, Jeung Woo Kim, Chul Hong Chun, Hae Joong Cho, Gi Youn Hong, Seon Kwan JuHong, Kwon Ha Yoon, Byoung Hyun Park, Ji Myung Bae, Joung-Kyue Han, and Jaemin Oh

Department of Anatomy, School of Medicine, Wonkwang University; Department of Orthopedic Surgery, School of Medicine, Wonkwang University; Department of Physical Medicine and Rehabilitation, School of Medicine, Wonkwang University; Department of Rheumatology, School of Medicine, Wonkwang University; Department of Pathology, School of Medicine, Wonkwang University; Department of Obstetrics and Gynecology, School of Medicine, Wonkwang University; Department of Radiology, School of Medicine, Wonkwang University; Department of Endocrinology, School of Medicine, Wonkwang University; Department of Biomaterials, School of Dentistry, Wonkwang University; Sinyong-dong, Iksan 570–749, Korea; and Department of Physical Education, Chung-Ang University; 221 Hukseok-dong, Dongjak-gu, Seoul 156–756, Korea.

Received December 10, 2008; accepted February 22, 2009; published online April 1, 2009

Risedronate, a nitrogen-containing bisphosphonate, is widely used in the clinical field for the treatment of osteoporosis. Risedronate is known to exert its effects through binding to hydroxyapatite in bone tissue, inhibiting osteoclastic activity, and inducing apoptosis of osteoclasts. The purpose of this study was to determine the effects of risedronate on osteoclast differentiation in vitro and on an inflammatory bone loss model in vivo. Risedronate inhibited osteoclast differentiation in co-culture of bone marrow cells (BMCs) and osteoblasts, and suppressed receptor activator of nuclear factor (NF)-κB ligand (RANKL)-mediated osteoclast differentiation from bone marrow-derived macrophages (BMMs) in a dose-dependent manner without toxicity. Risedronate significantly inhibited expression of c-Fos and nuclear factor of activated T cells (NFAT) c1 induced by RANKL. To examine the effect of risedronate on bone loss in vivo, we used a mouse model of lipopolysaccharide (LPS)-mediated bone loss. Micro-CT analysis of the femurs showed that LPS treatment caused bone loss. However, bone loss was significantly attenuated in mice administered with risedronate. Taken together, we conclude that risedronate exerts beneficial effects on osteoporosis by inhibiting osteoclast differentiation both directly and indirectly. In infectious conditions, the inhibitory effect of risedronate on bone erosion was excellent. Thus risedronate could be a treatment option for osteoporosis caused by inflammatory and infectious conditions.

Key words risedronate; osteoclast; osteoporosis, inflammation

As life expectancy increases, osteoporosis is becoming an important social problem. Fractures caused by osteoporosis are a major cause of morbidity in the elderly and cause significant economic burden. According to the National Health and Nutrition Examination Survey (NHANES) III in the U.S.A., 34—50% of Americans aged >50 years have osteopenia, 17—20% of whom were reported to have osteoporosis.1) In another population-based data model, hip fracture was estimated to cause mortality in 24% of the elderly annually.2) The incidence of vertebral fracture each year was about 760000, 3 times greater than the annual incidence of breast cancer.3) Considering the substantial adverse effects of this disease on the general population, the clinical goal of osteoporosis treatment is prevention of fractures. Among the numerous drugs for osteoporosis, bisphosphonates are the most commonly prescribed and first-line medication in most cases.4) Risedronate is a nitrogen-containing bisphosphonate approved by the FDA for the prevention and treatment of postmenopausal osteoporosis and glucocorticoid-induced osteoporosis. The main mechanism of action of bisphosphonate is inhibition of farnesyl diphosphate synthase in the cholesterol mevalonic acid pathway, which is essential to prenylation of protein in osteoclasts. This ultimately causes a mechanical inhibition of osteoclast adhesion on the margin of bone where absorption takes place, through osteoclast apoptosis. Bisphosphonate is also known to operate indirectly on osteoclasts, thereby suppressing osteoclast differentiation.5) However, the action of bisphosphonates on osteoclast under the stimuli of receptor activator of nuclear factor (NF)-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), which is essential for osteoclast differentiation, is not clearly known. A recent study showed that bisphosphonate suppressed bone erosion in a collagen-induced arthritis model. This model manifests as a chronic inflammatory condition resulting in increased osteoclastic bone resorption, without any reduction of arthritis.5

Osteoclasts are multinucleated cells deriving from hematopoietic precursors of monocyte-macrophage lineage through direct interaction with RANKL present on the surface of osteoblastic cells.7—9) RANKL, identified as a member of the tumor necrosis factor (TNF) superfamily, acts as a key regulator of osteoclasts and induces transcription factors such as NF-κB, c-Fos, and nuclear factor of activated T cells (NFAT) c1, which play an essential role in osteoclast differentiation, fusion, and function.10—12) Bacterial lipopolysaccharide (LPS), produced by Gram-negative bacteria (GNB), is a potent activator of mononuclear cells. LPS induces secretion of TNF and other inflammatory cytokines leading to systemic inflammatory response. Injection of LPS in animals can mimic the condition undergoing bone destruction that occurs under sepsis.13,14) However, the exact mechanism of LPS-induced inflammatory
bone destruction is not fully understood. In the current study, we aimed to demonstrate the direct and indirect effects of risedronate on osteoclast differentiation, stimulated by RANKL and M-CSF, and to demonstrate effects on expression of related transcriptional factors such as c-Fos and NFATc1. We also evaluated any effects of risedronate on bone loss under inflammatory conditions induced by LPS.

**MATERIALS AND METHODS**

**Reagents and Antibodies** 1,α25-Dihydroxyvitamin D3 (VitD3), prostaglandin E2 (PGE2), LPS, and anti-actin antibody were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). Human soluble RANKL and M-CSF were from PeproTech EC (London, U.K.). For cytotoxicity test, XTT assay kit was purchased from Roche Diagnostics (Indianapolis, IN, U.S.A.). Antibodies against c-Fos and NFATc1 were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Risedronate was provided by Sanofi-Aventis Korea (Seoul, Korea). ICR mice were from Dae Han Biolink Co., Ltd. (Chungbuk, Korea).

**Osteoclast Differentiation** Bone marrow cells (BMCs) were isolated from long bones of 6-week-old ICR mouse by flushing with α-minimum essential medium (α-MEM; Welgene, Daegu, Korea) containing antibiotics (Sigma), and further red blood cells (RBC) were removed with RBC lysis buffer (Sigma). The cells were plated on 90-mm culture dishes (Corning, NY, U.S.A.) and incubated for 1 d in α-MEM containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY, U.S.A.) and antibiotics (Gibco BRL) in the presence of 10 ng/ml M-CSF. Nonadherent cells were seeded on 90-mm petri dishes and then cultured for 3 d in the presence of 30 ng/ml M-CSF. Adherent cells were used as bone marrow-derived macrophages (BMMs), namely osteoclast precursors. To generate osteoclasts from osteoclast precursors, BMMs were further cultured for 4 d in the presence of 30 ng/ml M-CSF and 50 ng/ml RANKL with or without risedronate in various concentrations. For osteoclast differentiation from co-culture of BMCs, calvarial osteoblasts were isolated from calvariae of newborn mice using 0.1% collagenase and 0.2% dispase digestion as described previously. Calvarial osteoblasts and BMCs were co-cultured for 5 d with 10−8 M VitD3 and 10−6 M PGE2 in the presence of absence of risedronate.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** Total RNA was isolated from treated cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s protocol. cDNA was synthesized from 1 μg of total RNA using oligo dT, dNTP, dithiothreitol, RNase inhibitor, 1×PCR buffer, and Superscript II reverse transcriptase (Invitrogen). cDNA 1 μl was amplified by PCR using Taq polymerase (Invitrogen). The following primers were used: c-Fos sense, 5′-CTGTGAACACCGCACTCTGGTGC-3′; c-Fos antisense, 5′-CTTCTGGACAGATTGGGCAATCTC-3′; NFATc1 sense, 5′-CAACGCCCTGACCCTTTCAGCAGATT-3′; NFATc1 antisense, 5′-GGCTGCCTTCCCACGATAAG-3′; GAPDH sense, 5′-ACCACAGTCCATGCCATCAC-3′; and GAPDH antisense, 5′-TCCACCACCCCTGGTCTGCTGTA-3′. PCR products were electrophoresed on 1% agarose gels and visualized under UV after ethidium bromide staining.

**Western Blotting** Following treatments, cells were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer (50 mM Tris–Cl, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM NaF, 1 mM Na3NO4, and 1% sodium doxycholate) containing protease inhibitors. Protein concentration was measured by DC Protein Assay kit (Bio-Rad, Hercules, CA, U.S.A.). The lysates were suspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (100 mM Tris–Cl, 4% SDS, 20% glycerol, 0.2% bromophenol blue) containing 200 mM β-mercaptoethanol, boiled for 5 min, and resolved by 10% SDS-PAGE, then transferred to polyvinylidene difluoride membranes (Amersham Biosciences). The membranes were blocked in 5% skim milk, blotted with antibodies to c-Fos, NFATc1, actin, and then incubated with either donkey anti-rabbit or sheep anti-mouse horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). Bound antibody was detected by enhanced chemiluminescent reagents (ECL plus, GE Healthcare).

**Cytotoxicity Assay** BMMs (1×104 cells/well), RAW 264.7 (1×104 cells/well), and HT-29 (7×104 cells/well) were seeded in triplicate in 96-well plates in α-MEM (BMMs and RAW 264.7) or Dulbecco’s modified Eagle’s medium (HT-29) containing 10% fetal bovine serum (Gibco BRL) and antibiotics (Sigma) and then incubated for 3 d in the presence or absence of risedronate in various concentrations. After 3 d incubation, 50 μl of 2,3-bis(2-methoxy-4-nitro-S-sulfophenyl)-2H-tetrazolium-S-carboxyanilide (XTT) reagents were added to each well and incubated for 4 h. Absorbance was measured at 450 nm using an ELISA plate reader.

**LPS-Induced Bone Erosion** Six-week-old ICR mice were orally administered risedronate (10 μg/g body weight) or PBS 1 d before injection of LPS. Risedronate was orally administered every other day for 8 d. Mice were injected intraperitoneally with LPS (5 μg/g body weight) on days 0 and 4. The femurs were obtained on day 8 after the first injection of LPS and fixed in 4% paraformaldehyde for 1 d. Radiographic images were taken with micro-CT scan (NFR-Polaris-S160: Nano Focus Ray, Iksan, Korea). Bone volume/tissue volume was calculated using VG Studio program.

**Statistical Analysis** All quantitative results are expressed as mean±S.D. Statistical differences between experimental groups were analyzed by Student’s t-test. Values of p<0.05 were considered statistically significant.

**RESULTS**

Risedronate Suppresses Osteoclast Differentiation We first examined the effects of risedronate on osteoclast differentiation in the co-cultures of osteoblasts and BMCs. As shown in Fig. 1A, the numbers of TRAP-positive osteoclasts were decreased in the risedronate 0.1 μM and 0.5 μM groups. The number of osteoclasts with ≥3 nuclei was decreased in the risedronate-treated group (Fig. 1B). Also, numbers of osteoclast with ≥10 nuclei were reduced due to risedronate (Fig. 1C). These results suggest that risedronate significantly suppresses osteoclast differentiation. To examine whether risedronate directly inhibits RANKL-induced osteoclast dif-
Differentiation, BMMs were treated with RANKL and M-CSF and cultured for 5 d. The risedronate-treated group showed significantly decreased levels of TRAP-positive osteoclasts compared with the control group (Fig. 2A). Osteoclasts with ≥3 nuclei also reduced significantly due to risedronate (Figs. 2B, C). With this result, it can be concluded that risedronate directly suppresses RANKL-induced osteoclast differentiation.

Effect of Risedronate on Cytotoxicity
To examine whether suppression of osteoclast differentiation was due to toxicity or drug effect, we performed XTT assay. BMMs, RAW 264.7 cells, and HT 29 cells were seeded and then cultured for 3 d in the presence of risedronate. Cell toxicity was not observed in the cell groups (Fig. 3). These results suggest that decreased osteoclast differentiation was not due to toxicity.

Risedronate Inhibits RANKL-Dependent c-Fos and NFATc1 Expression
To investigate whether risedronate-induced inhibition of osteoclast differentiation is involved in the expression of transcription factor induced by RANKL, we examined the expression of c-Fos, NFATc1, and TRAP in BMMs treated with RANKL. When BMMs were stimulated for 12—24 h with RANKL, risedronate significantly inhibited the expression of c-Fos, NFATc1, and TRAP (Fig. 4), indicating that the suppressing mechanism of osteoclast influenced by risedronate is related to c-Fos and NFATc1 expression. Western blot was performed to confirm whether rise-
Risedronate affects c-Fos and NFATc1 expression induced by RANKL. BMMs were stimulated with RANKL and M-CSF in the presence or absence of risedronate. The presence of risedronate significantly suppressed expression of c-Fos and NFATc1 in BMMs when stimulated with RANKL and M-CSF compared with control (Fig. 5). Thus the differentiating mechanism of osteoclast influenced by risedronate is related to decreased expression of c-Fos and NFATc1.

Risedronate Suppresses RANKL-Induced p38 Phosphorylation
Risedronate may inhibit osteoclast differentiation by inhibiting c-Fos expression in response to RANKL. Thus to identify a signaling pathway involved in inhibition of c-Fos expression by risedronate, we examined whether risedronate affects RANKL-mediated signaling pathways. BMMs were stimulated with RANKL in the presence or absence of risedronate. Risedronate significantly inhibited p38 mitogen-activated protein kinase (MAPK) phosphorylation (Fig. 6A). Next, we examined whether risedronate-mediated inhibition of p38 MAPK phosphorylation is involved in the expression of c-Fos induced by RANKL. As shown in Fig. 6B, p38 MAPK inhibitor SB203580 significantly suppressed RANKL-induced c-Fos expression. These results suggest that risedronate-induced inhibition of osteoclast differentiation is involved in suppression of c-Fos by inhibiting p38 MAPK.

Effect of Risedronate on LPS-Induced Bone Erosion in Vivo
Osteoclasts are responsible for resorption of bone, and inhibition of osteoclast differentiation by risedronate treatment may exert anti-bone resorptive effect in vivo. Thus to examine the effect of risedronate on bone erosion in vivo, mice were injected with LPS intraperitoneally and given risedronate orally. On micro-CT scan, trabecular bone loss in the femur was increased after LPS injection. On the contrary, scans from mice in the oral risedronate-treated group showed significant reduction of bone loss (Fig. 7A). Moreover, loss of bone volume (bone volume/tissue volume) induced by LPS was prevented in risedronate-administered mice (Fig. 7B).

DISCUSSION
Historically, bisphosphonates have shown remarkable effectiveness in treating osteoporosis. Nitrogen-containing bisphosphonates, in particular, are known to be highly effective inhibitors of bone resorption. Risedronate, one of the best-known nitrogen-containing bisphosphonates, exerts its in-
hibitory effect by binding to hydroxyapatite in bone tissue, so that the resorptive activity of adjacent osteoclasts on bone is inhibited. Bisphosphonates commonly inhibit farnesyl diphatosine synthase within the mevalonial acid pathway, thereby stimulating apoptosis of osteoclasts. Risedronate also induces macrophage apoptosis at relatively low concentration when compared with other bisphosphonates such as alendronate and pamidronate in vitro. In the study of Halasy et al., it was reported that bone resorption was inhibited with reduced apoptosis of osteoclasts by bisphosphonates. In terms of the treatment of osteoporosis, inhibition of cellular differentiation is crucial as well as inhibition of osteoclastic activity and induction of osteoclast apoptosis. Some recent studies have focused on effects of bisphosphonates on osteoblasts. In our study, osteoblasts cocultured with BNCs followed by risedronate treatment showed marked suppression of cellular differentiation. Since osteoblasts express RANKL essential for osteoclast differentiation, the effects of risedronate on osteoclast differentiation may be related to decreased expression of RANKL or increased expression of osteoprotegerin (OPG) on osteoblasts. Nishikawa et al. have suspected that the inhibitory action on osteoblasts in the presence of a bisphosphonate is mediated through soluble factor. In another report it was demonstrated that the inhibitory action of a bisphosphonate on bone resorption does not involve regulation of RANKL and OPG expression. Ohyaa et al. have also reported that bisphosphonate suppressed the formation of PGE2 and osteocalcin in the osteoblast. Further studies are needed regarding the inhibitory mechanism of risedronate on osteoclastogenesis via regulation of osteoblasts. In this study, we showed that risedronate significantly inhibits RANKL and M-CSF-mediated osteoclast differentiation (Fig. 2). Until now, there has been no report that bisphosphonate affects expression of NFATc1 and c-fos in the course of osteoclast differentiation. We showed that NFATc1, a transcriptional factor that plays an important role in osteoclast differentiation, was significantly decreased in BMMs treated with risedronate (Figs. 4, 5). This may show that risedronate plays a critical role in osteoclast differentiation with direct inhibitory effect through down-regulation of NFATc1.

The MAPK signaling pathway has been shown to play a critical role in RANKL-induced osteoclast differentiation. In particular, the p38 MAPK pathway is activated downstream in RANK signaling and is critical for c-Fos expression in BMMs. c-Fos, a component of AP-1, mediates RANKL-induced osteoclast differentiation by transcriptionally inducing NFATc1. In this study, we found that risedronate inhibits p38 MAPK phosphorylation in BMMs (Fig. 6). However, risedronate did not inhibit activation of JNK and NF-κB induced by RANKL (data not shown). These results suggest that risedronate inhibits RANKL-induced osteoclast differentiation by suppressing c-fos expression via p38 MAPK pathway.

Along with senile and post-menopausal osteoporosis, treatments for osteoporosis caused by chronic inflammation and infection are also clinically important. Osteolysis due to chronic GNB bone diseases such as osteomyelitis, septic arthritis, and periodontitis is accompanied with enhanced osteoclast activity. To study bone loss or destruction in chronic inflammatory conditions, LPS-induced mouse models are commonly used. Although the reason why LPS induces osteoclast differentiation is not fully understood in vivo, it is probable that this is due to LPS inducing TNF-α, which ultimately renders expression of c-Src to increase, and inducing interleukin (IL)-1 and IL-6 expression. Also, some studies demonstrate that NF-κB activation seems essential for LPS-induced osteoclastogenesis and bone resorption. Previously, it was reported that alendronate augmented the effects of LPS on histidine decarboxylase activity. Until now, the effects of risedronate on bone loss model induced by LPS have not yet been studied. We found that LPS significantly induced bone erosion. However, risedronate significantly protected bone erosion induced by LPS (Fig. 7). Although this requires further investigation, we suspect that risedronate decreases inflammatory cytokines and PGE2 stimulated with LPS. Until now, risedronate has been commonly used in the clinical field and its clinical effect is established; however, the mechanism and effect of risedronate for the prevention and treatment of osteoporosis are not fully understood. With our study, through the classical pathway such as RANKL–TRAF6–c-Fos–NFATc1, risedronate directly inhibited osteoclast differentiation without cell toxicity. Especially in the chronic inflammatory condition, risedronate markedly inhibited bone loss.

In conclusion, risedronate exerts inhibitory effects on osteoclast differentiation by direct and indirect means. We assert that risedronate could be used not only for the treatment of post-menopausal osteoporosis and senile osteoporosis but also in numerous other areas such as in inflammatory arthritic and infectious conditions of bone such as osteomyelitis and chronic periodontitis.

Acknowledgements

This paper was supported by Wonkwang University in 2007.

REFERENCES


