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Inhibitory effects of obovatol on osteoclast differentiation and bone resorption

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ABSTRACT

Osteoclasts are polykaryons that have the unique capacity to degrade bone. Modulation of osteoclast formation and function is a promising strategy for the treatment of bone-destructive diseases. Here, we report that obovatol, a natural compound isolated from *Magnolia obovata,* inhibits receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL)-induced osteoclast differentiation in vitro and inflammatory bone loss in vivo. We found that obovatol strongly inhibited osteoclast formation from bone marrow-derived macrophages in a dose-dependent manner without cytotoxicity. Obovatol significantly suppressed RANKL-induced activation of NF-κB, c-Jun-N-terminal kinase, and extracellular signal-regulated kinase signaling pathways. Obovatol also inhibited RANKL-induced expression of the genes c-Fos and nuclear factor of activated T cells c1, which are transcription factors important for osteoclastogenesis. In addition to osteoclast differentiation, obovatol blocked cytoskeletal organization and abrogated the bone resorbing activity of mature osteoclast. Obovatol also accelerated osteoclast apoptosis through the induction of caspase-3 activation. Consistent with its in vitro anti-resorptive effect, obovatol prevented bone loss induced by lipopolysaccharide in vivo. Together, our data suggest that obovatol may be a useful therapeutic agent for the treatment of pathological bone disorders characterized by excessive osteoclastic bone resorption.

1. Introduction

Adult skeletal mass and integrity are maintained by balancing osteoclast-mediated bone resorption and osteoblast-induced bone formation during bone remodeling (Anderson et al., 1997; Lacey et al., 1998; Martin and Sims, 2005; Wong et al., 1997; Yasuda et al., 1998). Abnormal increases in osteoclastic bone resorption can lead to excessive bone destruction as observed in osteoporosis, rheumatoid arthritis, and metastatic cancers (Baron and Hesse, 2012; Boyle et al., 2003; Bruzzaniti and Baron, 2006). Osteoclasts are multinucleated cells capable of degrading bone matrix. These polykaryons are generated from hematopoietic progenitor cells in the presence of two key cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) (Teitelbaum, 2007; Teitelbaum and Ross, 2003).

RANKL, which belongs to the tumor necrosis factor (TNF) superfamily, binds to its receptor, RANK, on osteoclast precursor cells, recruits the adaptor molecules such as TNF receptor-associated factor 6 (TRAF6), and triggers osteoclast differentiation. Binding of RANKL to RANK activates downstream signaling pathways including NF-κB and mitogen activated protein kinases (MAPKs), c-Jun-N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 (Novack and Teitelbaum, 2008; Tanaka et al., 2005; Teitelbaum and Ross, 2003). These signaling cascades lead to the induction and activation of the transcription factor complex activator protein-1 (AP-1) (Asagiri and Takayanagi, 2007; Wagner, 2010). As a component of AP-1, c-Fos plays a critical role in osteoclast differentiation (Grigoriadis et al., 1994; Johnson et al., 1992; Wang et al., 1992) and induces the expression of the nuclear factor of activated T cells c1 (NFATc1), which is another important factor for RANKL-induced osteoclastogenesis (Takayanagi et al., 2002). NFATc1, in turn, cooperates with other transcriptional partners and mediates the expression of osteoclast-specific genes such as tartrate-resistant acid

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phosphatase (TRAP) and cathepsin K (Asagiri and Takayanagi, 2007; Nakashima and Takayanagi, 2011).

Obovatol is a compound isolated from *Magnolia obovata*, a plant that has been traditionally used as a folk medicine for the treatment of gastrointestinal disorders, anxiety and allergies in Asian countries (Lee et al., 2011). It has been reported that obovatol possesses various biological activities, such as anti-tumor (Lee et al., 2008a, 2008b), anti-Alzheimer (Choi et al., 2012a, 2012b), anti-platelet (Park et al., 2011; Pyo et al., 2002), and anti-oxidative (Lee et al., 2012) properties. In addition, obovatol has been shown to inhibit lipopolysaccharide (LPS)-induced inflammation in microglial BV-2 (Ock et al., 2010) and Raw 264.7 cells (Choi et al., 2007). However, the effect of obovatol on osteoclast development has not been investigated.

In this study, we report that obovatol inhibits osteoclast differentiation through the suppression of RANKL-stimulated activation of NF-κB and MAPKs, thereby down-regulating the gene expression of c-Fos and NFATc1 in osteoclast lineage cells. We also report the inhibitory effect of obovatol on activity and survival of mature osteoclast and its protective effect against inflammatory bone destruction.

2. Materials and methods

All experiments were performed following the approval of the Ethics Committee for Animal Experiments at Kyungpook National University.

2.1. Reagents

RANKL and M-CSF were purchased from R&D Systems (Minneapolis, MN). Antibodies for phospho-IκBα, phospho-JNK, phospho-p38, phospho-ERK, IκBα, JNK, p38, ERK, cleaved caspase-3 and cleaved caspase-9 were purchased from Cell Signaling Technology (Beverly, MA). The anti-Bim antibody was obtained from BD Biosciences (San Jose, CA). The anti-c-Fos antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-NFATc1 antibody was obtained from BD Pharmingen (San Diego, CA). Obovatol was isolated from the leaves of *M. obovata* in Daejeon, Korea and identified by Dr. Byoung-Mog Kwon (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea). The isolation method was described previously (Lee et al., 2008a). The chemical structure of obovatol was shown in Fig. 1A. Obovatol was dissolved in dimethyl sulfoxide (DMSO) and added to cell cultures to the desired concentration. DMSO was used as a vehicle control.

2.2. Osteoclast culture and tartrate-resistant acid phosphatase (TRAP) staining

Osteoclast cultures were performed as previously described (Kim et al., 2012). Briefly, primary bone marrow-derived macrophages (BMMs) were isolated from C57/BL6 mice and cultured in α-minimal essential medium (MEM) containing 10% FBS and M-CSF for 3 days. To generate osteoclasts, BMMs were cultured in a 96-well plate at a density of 5×10^3 cells/well in α-MEM containing 10% FBS with M-CSF (10 ng/ml) and RANKL (20 ng/ml). After 4 days, cells were fixed in 4% paraformaldehyde for 20 min and then stained with tartrate-resistant acid phosphatase (TRAP) staining solution (0.1 mg/ml of naphthol AS-MX phosphate, 0.3 mg/ml of Fast Red Violet LB stain). TRAP-positive multinucleated cells containing three or more nuclei were counted.

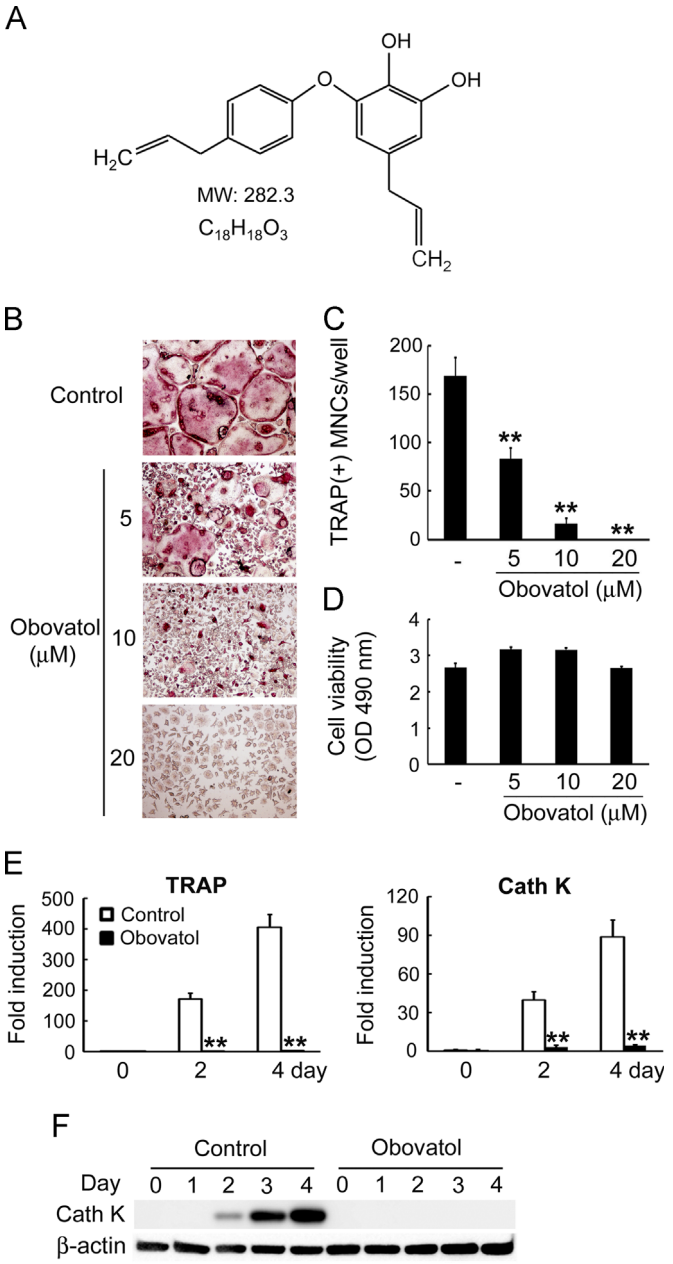

Fig. 1. Obovatol inhibits RANKL-induced osteoclast differentiation. (A) The chemical structure of obovatol. (B)–(D) BMMs were cultured with the indicated concentrations of obovatol in the presence of M-CSF (10 ng/ml) and RANKL (20 ng/ml). (B) After 4 days, the cells were fixed and stained for TRAP. (C) The number of TRAP-positive multinucleated cells (MNCs) was counted. Data are expressed as the means ± S.D. $^{**}P < 0.001$ versus vehicle-treated control. (D) After 3 days, cell viability was evaluated by measuring the OD values at 490 nm. (E)–(F) BMMs were cultured in the presence of M-CSF (10 ng/ml) and RANKL (20 ng/ml) with vehicle or obovatol (20 μM) for the indicated number of days. Expression of the indicated genes was analyzed by real-time qPCR (E) or immunoblotting (F). β-Actin served as the loading control. Data are expressed as the means ± S.D. $^{**}P < 0.001$ versus vehicle-treated control.

2.3. Cell viability assay

Cell viability was measured using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay. BMMs were seeded onto 96-well plates and cultured with various concentrations of obovatol in the presence of M-CSF (10 ng/ml) and RANKL (20 ng/ml). After 3 days, the media was replaced with 100 μl fresh medium containing 20 μl