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Decursin from *Angelica gigas* suppresses RANKL-induced osteoclast formation and bone loss

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ABSTRACT

Osteoclasts are the only cells capable of breaking down bone matrix, and excessive activation of osteoclasts is responsible for bone-destructive diseases. In this study, we investigated the effects of decursin from extract of *Angelica gigas* root on receptor activator of nuclear factor kappa B ligand (RANKL)-induced osteoclast formation using mouse bone marrow-derived macrophages (BMMs). Decursin inhibited RANKL-induced osteoclast formation without cytotoxicity. In particular, decursin maintains the characteristics of macrophages by blocking osteoclast differentiation by RANKL. Furthermore, the RANKL-stimulated bone resorption was diminished by decursin. Mechanistically, decursin blocked the RANKL-triggered ERK mitogen-activated protein kinases (MAPK) phosphorylation, which results in suppression of c-Fos and the nuclear factor of activated T cells (NFATc1) expression. In accordance with the *in vitro* study, decursin reduced lipopolysaccharide (LPS)- or ovariectomy (OVX)-induced bone loss *in vivo*. Therefore, decursin exerted an inhibitory effect on osteoclast formation and bone loss *in vitro* and *in vivo*. Decursin could be useful for the treatment of bone diseases associated with excessive bone resorption.

1. Introduction

Bone homeostasis is a dynamic process delicately regulated by osteoclast-mediated bone resorption and osteoblast-induced bone formation. Disruption of this balance incurs various bone-related diseases including postmenopausal osteoporosis, rheumatoid arthritis, periodontitis, Paget's disease, hypercalcemia, and bone metastasis (Novack and Teitelbaum, 2008; Tanaka et al., 2005). Therefore, it would be useful to target osteoclasts and/or osteoblasts for the development of new drugs against bone-destructive diseases.

Osteoclasts, bone-specialized multinucleated cells, are derived from hemotopoietic progenitors of the monocyte/macrophage lineage through a differentiation process primarily governed by two key cytokines, namely macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) (Asagiri and Takayanagi, 2007). M-CSF is important for the proliferation and survival of osteoclast precursors, and up-regulates RANK expression, which is a prerequisite for osteoclastogenesis (Arai et al., 1999). RANKL, a member of the tumor necrosis factor (TNF) family, is expressed on the surface of osteoblasts, stromal cells, and T cells, and plays crucial roles in osteoclast differentiation and activation (Asagiri and Takayanagi, 2007; Takayanagi, 2007). Osteoblasts, which are originated from mesenchymal stem cells, support osteoclast differentiation by increasing RANKL and decreasing its decoy receptor osteoprotegerin (OPG) in response to osteotropic factors such as prostaglandin E_2 (PGE_2), vitamin D_3 ($VitD_3$), and interleukin-1 (IL-1) (Takeda et al., 1999; Miyaura et al., 2003; Wei et al., 2005; Boyle et al., 2003).

Binding of RANKL to its receptor RANK in osteoclast precursor cells induces the activation of multiple intracellular signaling pathways including mitogen-activated protein kinase (MAPK) pathways (ERK, JNK and p38 MAPK), the phospho-inositide-3-kinase (PI3K)/AKT pathway, and the NF-kB pathway (Darnay et al., 1999). These signaling cascades lead to the induction and activation of osteoclastogenic transcription factors such as c-Fos and nuclear factor of activated T cells (NFATc1) (Grigoriadis et al., 1994; Matsumoto et al., 2000; Takayanagi et al., 2002). Therefore, targeted modulation of these signaling pathways to regulate c-Fos and NFATc1 expression may be helpful for the treatment of bone-

Abbreviations: BMM, bone marrow-derived macrophages; M-CSF, macrophage-colony stimulating factor; RANKL, receptor activator of NF-kB ligand; TRAP, tartrate-resistant acid phosphatase; OVX, ovariectomy; BMD, bone mineral density; DXA, dual-energy X-ray absorptiometry; μCT, microcomputer tomography

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destructive diseases.

Angelica gigas Nakai (AG, Umbelliferae), an oriental herbal medicine, has been used traditionally for the prevention of various diseases (Lee et al., 2003a, 2003b; Han et al., 1998). A coumarin compound decursin is the major active component of AG root, and has been identified to exert beneficial effects against disease models such as cancer, oxidative stress, and inflammation (Kim et al., 2003, 2010a, 2010b; Jiang et al., 2006; Yang et al., 2009). Other reports also revealed that decursin might be useful for the treatment of obesity and diabetes (Hwang et al., 2012). However, there was little knowledge about the effects of decursin on lytic bone diseases.

In this study, we investigated the effect of decursin from AG root on RANKL-induced osteoclastogenesis using mouse primary osteoclast precursors. Decursin significantly suppressed RANKL-induced osteoclast differentiation *in vitro* and lipopolysaccharide (LPS)- or ovariectomy (OVX)-induced bone loss *in vivo*. Therefore, decursin may be considered as a promising drug candidate for therapy or protection of bone-destructive diseases.

2. Materials and methods

2.1. Reagents

Decursin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against ERK, phospho-ERK, phospho-p38, p38, IκB, β-actin, and c-Fos were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against NFATc1 were purchased from Santa Cruz Biotechnology (Santa Cruz, TX, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cells and culture system

Bone marrow cells were obtained from the long bones of 8-10-weeks-old ICR mice (Samtako, Inc., Osan, Korea). Bone marrow cells were cultured in the presence of M-CSF (30 ng/ml, PeproTech, Inc., Rocky Hill, NJ, USA) for 3 days to generate the bone marrow-derived macrophages (BMMs). To examine osteoclast formation, BMMs were treated with decursin in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml, PeproTech, Inc., Rocky Hill, NJ, USA) in 96-well culture plates (Corning, MA, USA). For human osteoclast formation, human peripheral blood monocytic cells (PBMC) were prepared using Ficoll-Paque gradient (Amersham Pharmacia, Burkinghamshire, UK). The cells were seeded in a 96-well plate at a density of 1.5×10^5 cells per well and cultured with M-CSF (100 ng/ml) and RANKL (100 ng/ml) in the absence or presence of decursin. Cells were then fixed and permeabilized with an equal volume mixture of acetone and ethanol for 30 s and then treated with tartrate resistant acid phosphatase (TRAP) staining solution (0.01% naphthol AS-MX phosphate (Sigma-Aldrich) and 0.06% Fast Red Violet LB Salt (Sigma-Aldrich) in 50 mM sodium tartrate dehydrate and 45 mM sodium acetate (pH 5.0)). TRAP-positive multinucleated cells (>5 nuclei/cell) were counted as mature osteoclasts.

2.3. RNA extraction and polymerase chain reaction (PCR) assay

Total RNA was purified with Easy-Blue (iNtRON Biotechnology, Inc., Korea). The cDNA was synthesized from 5 μg of RNA by using the Revert Aid™ first-strand cDNA synthesis Kit (iNtRON Biotechnology, Inc., Korea) and amplified using real-time PCR. The following primers of osteoclastogenic genes were used in this study: RANKL, 5′- CCA AGA TCT CTA ACA TGA CG-3′ (forward), 5′-CAC CAT CAG CTG AAG ATA GT-3′ (reverse); OPG, 5′-ACG GAC AGC TGG CAC ACC AG-3′ (forward), 5′-CTC ACA CAC TCG GTT GTG GG-3′ (reverse); calcitonin receptor (CTR), 5′-TTT CAA GAA CCT TAG CTG CCA GAG-3′ (forward), 5′-CAA GGC ACG GAC AAT GTT GAG AAG-3′ (reverse); cathepsin K (CTK), 5′-CTT CCA ATA CGT GCA GCA GA-3′ (forward), 5′-ACG CAC CAA TAT CTT GCA CC-3′ (reverse); ATP6v0d2, 5′-TCA GAT CTC TTC AAG GCT GTG CTG-3′ (forward), 5′-GTG CCA AAT GAG TTC AGA GTG ATG-3′(reverse); DC-STAMP, 5′-TGG AAG TTC ACT TGA AAC TAC GTG-3′(forward), 5′-CTC GGT TTC CCG TCA GCC TCT CTC-3′ (reverse); αV-Integrin, 5′-CCT CAG AGA GGG AGA TGT TCA CAC-3′ (forward), 5′-AAC TGC CAA GAT GAT CAC CCA CAC-3′ (reverse); β3-Integrin, 5′-GAT GAC ATC GAG CAG GTG AAA GAG-3′ (forward), 5′-CCG GTC ATG AAT GGT GAT GAG TAG-3′ (reverse); F4/80, 5′-GAA TCT TGG CCA AGA AGA GAC-3′ (forward), 5′-GAA TTC TCC TTG TAT ATC ATC AGC-3′ (reverse); CD11b, 5′-AAA GCC ATT GTG GCC AGT-3′ (forward), 5′-GTC ATC TCT GAA GCC GTG A-3′ (reverse); c-fms, 5′-GCG ATG TGT GAG CAA TGG CAG T-3′ (forward), 5′-AGA CCG TTT TGC GTA AGA CCT G-3′ (reverse), CD14, 5′-AGG GTA CAG CTG CAA GGA CT-3′ (forward), 5′-CTT CAG CCC AGT GAA AGA CA-3′ (reverse); GAPDH, 5′-AAC GGA TTT GGT CGT ATT GGG-3′ (forward), 5′-CAG GGG TGC TAA GCA GTT GG-3′ (reverse). Real-time PCR reactions were performed in a total volume of 20 μl using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Thermocycling was performed using a 7500 Real-time PCR System (Applied Biosystems) with the following conditions: initial hold 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C, and extension at 60 °C for 1 min. An index mRNA level was assessed using a threshold cycle (Ct) value and normalized against glyceradehyde 3-phosphate dehydrogenase (GAPDH) expression.

2.4. Immunoblot analysis

Total cell lysates were separated by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat-milk in PBS-T, and then immunostained with anti-phospho ERK (1:1000), anti-phospho p38 (1:1000), anti-ERK (1:1000), anti-p38 (1:1000), anti-IκB (1:1000), anti-NFATc1 (1:200), anti-c-Fos (1:1000), and anti-β-actin (1:4000), followed by secondary horseradish peroxidase-conjugated antibody (1:5000). The membranes were developed using an advanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK).

2.5. Bone resorption assay

BMMs were differentiated on dentine slices with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 2 days, and decursin were treated for 4 days. The surfaces of the dentine slices were wiped to remove the cells, and the slices were then stained with toluidin blue (1 μg/ml, J.T. Baker, Phillipsburg, NJ, USA). The number of pits formed by bone resorption on the dentine slices were counted.

2.6. In vivo experiment

To study the effects of decursin on LPS-induced bone loss *in vivo*, 8-weeks-old male ICR mice received injections *i.p.* of LPS (5 mg/kg) or vehicle (PBS) on day 0 and 4, as well as daily injections *i.p.* of decursin (20 mg/kg) (dissolved in DMSO and corn oil) or vehicle (equal volume of DMSO and corn oil to decurin) beginning on day-1 ($n=8$). The left femurs of the mice were collected on day 8 after the first injection. For the effects of decursin on ovarietomy (OVX)-induced bone loss *in vivo*, 10-weeks-old female C57BL/6 mice underwent either ovariectomy (OVX) or sham (sham) operation. After one week, mice were received daily injections *i.p.* of decursin (20 mg/kg) or vehicle for 12 weeks ($n=7$). The extracted femurs were scanned with dual-energy X-ray