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Herbacetin inhibits RANKL-mediated osteoclastogenesis in vitro and prevents inflammatory bone loss in vivo

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ABSTRACT

Herbacetin is an active flavonol (a type of flavonoid) that has various biologic effects such as antioxidant, antitumor, and anti-inflammatory activities. However, one of its novel effects remains to be investigated, that is, the induction of osteoclastogenesis by the receptor activator of nuclear factor-κB ligand (RANKL). In this study, we examined the effects and mechanisms of action of herbacetin on osteoclastogenesis in RANKL-treated bone marrow–derived macrophages (BMMs) and murine macrophage RAW264.7 cells in vitro and on lipopolysaccharide (LPS)-induced bone destruction in vivo. Herbacetin significantly inhibited RANKL-induced osteoclast formation and differentiation in BMMs and RAW264.7 cells in a dose-dependent manner. Moreover, the suppressive effect of herbacetin resulted in a decrease in osteoclast-related genes, including RANK, tartrate-resistant acid phosphatase, cathepsin K, and matrix metalloproteinase-2 and -9 (MMP-9). Consistent with mRNA results, we confirmed that herbacetin treatment downregulated protein expression of MMP-9 and cathepsin K. Herbacetin also decreased induction of the osteoclastogenic transcription factor c-Fos and nuclear factor of activated T cells c1 (NFATc1) and blocked RANKL-mediated activation of Jun N-terminal kinase (JNK) and nuclear factor-κB. Herbacetin clearly inhibited the bone resorption activity of osteoclasts on plates coated with fluorescein-labeled calcium phosphate. More importantly, the application of herbacetin significantly reduced LPS-induced inflammatory bone loss in mice in vivo. Taken together, our results indicate that herbacetin has potential for use as a therapeutic agent in disorders associated with bone loss.

1. Introduction

Bone remodeling is a physiological process that involves osteoclast-induced bone resorption and osteoblast-mediated bone formation (Karsenty and Wagner, 2002). Osteoclasts are known to arise through the fusion of myeloid hematopoietic precursors and macrophage precursor cells formed in the bone marrow (Boyce, 2013a, 2013b). Osteoclast formation leads to the dissolution of inorganic hydroxyapatite and the cleavage of organic collagen fibers in the bone matrix, which may cause pathologic bone diseases such as rheumatoid arthritis, periodontal disease, and osteoporosis (Han et al., 2007).

Many key cytokines, such as macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor-κB ligand (RANKL), are essential in the regulation of osteoclastogenesis (Takayanagi et al., 2002). M-CSF supports cell proliferation and survival during this process, while RANKL induces signaling that is essential for precursor cells to differentiate into osteoclasts (Theill et al., 2002; Park et al., 2008). Binding of RANKL to cell-surface RANK receptor results in complexes made up of RANKL/RANK/tumor necrosis factor receptor–associated factors (TRAF) that sequentially activate nuclear factor-κB ligand (NF-κB) and mitogen-activated protein kinases (MAPK) (Li et al., 2011; Lee et al., 2013). The MAPK family includes extracellular signal–regulated kinase 1/2 (ERK 1/2), c-Jun N-terminal kinase 1/2 (JNK1/2), and p38 (Cargnello and Roux, 2011). RANK/TRAF6 signaling activates JNK/activator protein-1 (AP-1) (including c-Fos), NF-κB, and calcineurin/nuclear factor of activated T cells c1 (NFATc1) signaling to induce osteoclast formation, and at the same time activates both MKK6/p38/microphthalmia-associated transcription factor (MITF) and Src to mediate osteoclast resorption and both ERK and Src to mediate osteoclast survival (Boyce, 2013a, 2013b). In addition, NF-κB, c-Fos, and NFATc1 are the principal inducible transcription factors during osteoclast development mediated by various extracellular stimuli, such as pro-inflammatory cytokines and RANKL. These factors induce the expression of a number of gene-encoding proteins involved in osteoclast activation, such as cathepsin-K, tartrate-resistant acid phosphatase (TRAP), osteoclast-associated receptor (OSCAR), and matrix metalloproteinase-9 (MMP-9) (Asagiri and Takayanagi, 2007).

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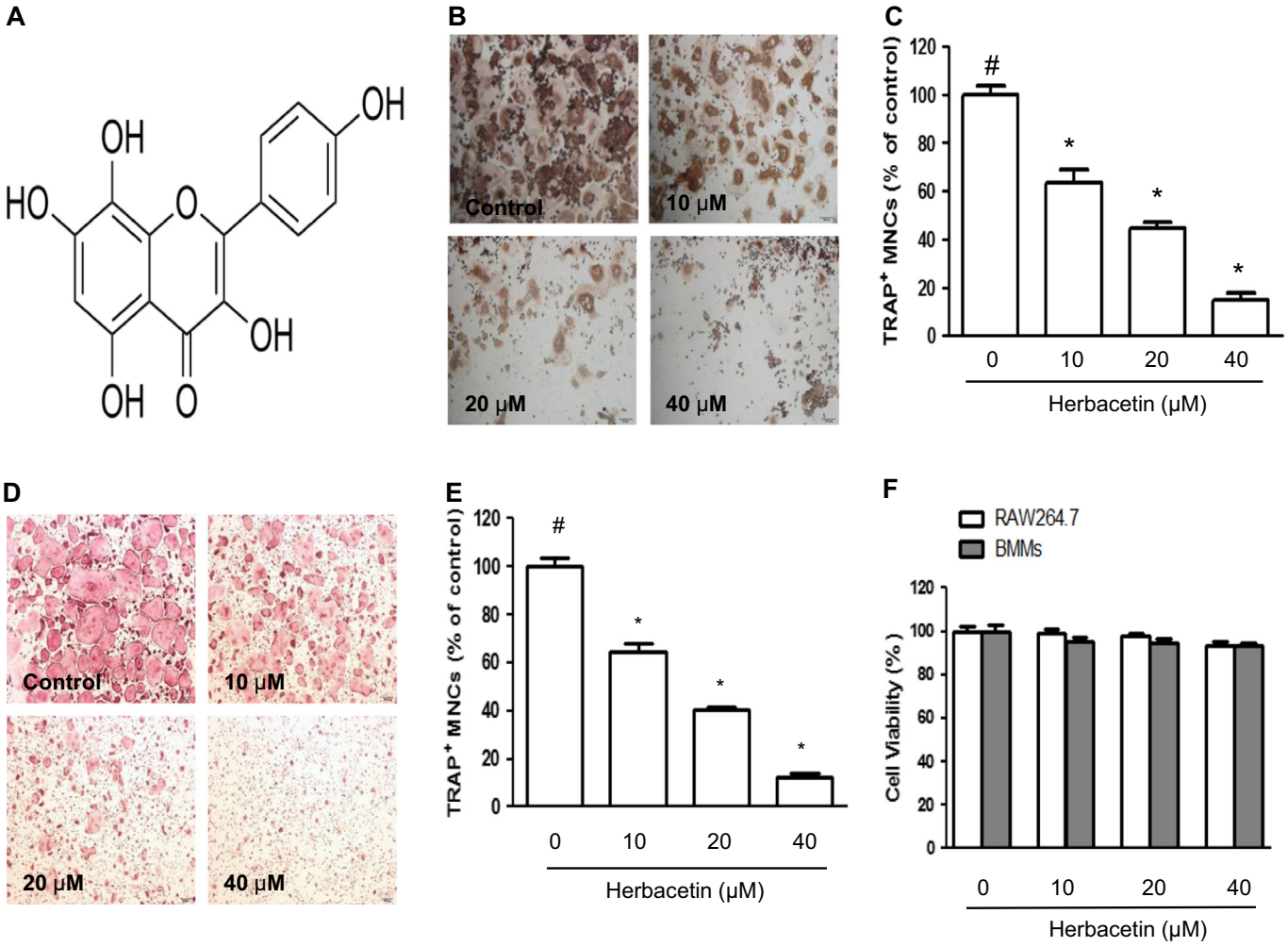

Fig. 1. Effects of herbacetin on RANKL-induced osteoclast differentiation in RAW264.7 cells and bone marrow macrophages (BMMs). (A) Chemical structure of herbacetin. (B) RAW264.7 cells were cultured for 4 days in the presence RANKL (50 ng/ml) with or without herbacetin. Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). (C) TRAP-positive multinucleated cells ($TRAP^+$) were counted in the RAW264.7 cells. (D) BMMs were cultured with various concentrations of herbacetin in the presence M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 4 d. The cultured cells were then fixed, and TRAP staining was performed. (*E*) TRAP+were counted in the BMMs. (F) The effect of herbacetin on the viability of RAW264.7 cells and BMMs at 3 d. RAW264.7 cells and BMMs were treated with various concentrations of herbacetin. Cell viability was measured by MTT assay. Each histogram represents the mean ± S.E.M. (n=3), and the asterisk (*) indicates a significant difference ($P < 0.05$) when compared with the RANKL-treated cells (#).

Recent studies have focused attention on flavonoids because of their role in improving renal function, their protective effect in renal failure, and their ability to promote bone formation according to one patent claim (Fliniaux et al., 2014). In recent years, herbacetin (3, 4′, 5, 7, 8-pentahydroxyflavone), a natural flavonoid of the flavonol family, has been widely used in cancer research (Fig. 1A). Many researchers have reported that the inhibition of osteoclastogenesis by these compounds may be attributed in part to their anti-inflammatory and antioxidant properties (Bu et al., 2008; Choi et al., 2010; Lee et al., 2010). Accordingly, dietary components and natural compounds with anti-inflammatory and antioxidant activity may stimulate bone formation and optimize bone health (Nepal et al., 2013). Hence, we studied the anti-osteoclastogenic effects and signaling pathways of herbacetin in RANKL-induced macrophages. Here, for the first time, we suggest that herbacetin significantly inhibits RANKL-stimulated osteoclast differentiation by modulating transcription factors, osteoclast-specific genes, and signaling molecules in vitro. We also determined that herbacetin had inhibitory effects on lipopolysaccharide (LPS)-induced bone resorption in vivo in a mouse model.

2. Materials and methods

2.1. Materials

Herbacetin was purchased from Tongtian Biotechnology (Shanghai, China) (purity ≥ p) and was prepared as a 100-mM stock in dimethyl sulfoxide (DMSO). The DMSO made up < 0.05% of the volume of the culture medium. Fetal bovine serum (FBS) and cell culture medium were obtained from Gibco (Gaithersburg, MD, USA). The primary antibodies used were anti-phospho-ERK, anti-phospho-p38, anti-phospho-JNK, anti-ERK, anti-JNK, anti-p38, anti-c-Fos (all from Cell Signaling, MA, USA) and anti-NFATc1, anti-MMP-9, anti-cathepsin K, and anti-β-actin (all from Santa Cruz, CA, USA). PCR primers were purchased from Bioneer (Daejeon, Korea). RANKL was purchased from PeproTech (Rocky Hill, NJ, USA) and M-CSF was purchased from R&D Systems (Minneapolis, MN, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified, as described in a previous publication (Choi et al., 2008).