Bone 46 (2010) 724–731

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Inhibition of osteoclast differentiation and bone resorption by rotenone, through down-regulation of RANKL-induced c-Fos and NFATc1 expression

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ARTICLE INFO

Article history:
Received 18 June 2009
Revised 5 September 2009
Accepted 29 October 2009
Available online 6 November 2009

Edited by: J. Aubin

Keywords:
Rotenone
Osteoclasts
RANK ligand
Osteoporosis
Lipopolysaccharides

ABSTRACT

Osteoclasts are responsible for bone erosion in diseases as diverse as osteoporosis, periodontitis, and rheumatoid arthritis. Natural plant-derived products have received recent attention as potential therapeutic and preventative drugs in human disease. The effect of rotenone in RANKL-induced osteoclast differentiation was examined in this study. Rotenone inhibited RANKL-mediated osteoclast differentiation in bone marrow macrophages (BMMs) in a dose-dependent manner without any evidence of cytotoxicity. The mRNA expression of c-Fos, NFATc1, TRAP, and OSCAR in RANKL-treated BMMs was inhibited by rotenone treatment. Rotenone strongly inhibited p38 and ERK phosphorylation and I-κB degradation in RANKL-stimulated BMMs, and did not inhibit JNK phosphorylation. Further, RANKL-induced c-Fos and NFATc1 protein expression was suppressed by rotenone. Rotenone additionally inhibited the bone resorptive activity of differentiated osteoclasts. A lipopolysaccharide (LPS)-induced bone erosion study was also performed to assess the effects of rotenone *in vivo*. Mice treated with rotenone demonstrated marked attenuation of bone erosion based on Micro CT and histologic analysis of femurs. These results collectively suggested that rotenone demonstrated inhibitory effects on osteoclast differentiation *in vitro* and suppressed inflammatory bone loss *in vivo*. Rotenone may therefore serve as a useful drug in the prevention of bone loss.

Introduction

The human bone is a highly dynamic organ that maintains its homeostasis through a delicate balance between bone formation and resorption, mediated by the bone-forming osteoblasts and the bone-eroding osteoclasts. This balance between these two cells types results in bone remodeling, and this continues unless critical changes in the balance occur. Increased osteoclast activity induces thinning and trabecular bone erosion, resulting in fragile bones. Conversely, increased osteoblast activity increases bone density, which is associated with bone deformity and osteopetrosis [1,2].

Osteoclasts are multinucleated giant cells that originate from the hematopoietic stem cell monocyte/macrophage lineage. Osteoclasts differentiate into multinucleated giant cells that attach to bone tissue and excrete various types of acids and enzymes. Osteoblasts and stromal cells express receptor activators of the nuclear factor-κB (NF-κB) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). M-CSF provides survival signals to osteoclasts and their precursor cells. RANKL is a member of the tumor necrosis factor (TNF) family and binds to the RANK receptor expressed in osteoclast precursor cells [1,3]. Many plant-derived natural products have been used in traditional medicine for the treatment of various diseases. Several compounds derived from natural products have been recently reported to possess inhibitory effects on osteoclast differentiation and function, leading to decreased bone loss *in vivo*. Examples from the current literature include curcumin (a compound derived from *Curcuma aromatica*) *Stewartia koreana* extract (Danshen extract known as tanshinones IIA), an undefined chloroform extract of young deer antlers, and berberine (an element of Coptidis Rhizoma) [4–8]. Rotenone is a compound isolated from the root of *Derris elliptica*. It is a mitochondrial complex I inhibitor that is involved in inhibition of oxidative respiration and ATP synthesis [9]. It has been widely used as

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doi:10.1016/j.bone.2009.10.042

a pesticide. Recent studies have demonstrated that rotenone affects osteoclast differentiation through decrease of mitochondrial function and showed that mitochondrial respiration and subsequent ATP synthesis is essential for osteoclastogenesis [10]. However, the effects of rotenone on RANKL and M-CSF functions vital to osteoclast differentiation are not clarified.

We investigated the effects of rotenone on signaling pathways involved in osteoclast differentiation, activation, and survival in the present study. In addition, the *in vivo* effects of rotenone were observed in mice with induced bone erosion.

Materials and methods

Reagents and antibodies

Rotenone was purchased from Sigma (St. Louis, MO, USA). Human RANKL and M-CSF was obtained from Peprotech (London, UK). The XTT assay kit was obtained from Roche (Indianapolis, IN, USA). Antibodies for c-Fos and nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Western blot antibodies for phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, p38, and I-κB were purchased from Cell Signaling Technology (Danvers, MA, USA). Actin antibody was purchased from Sigma.

Osteoclast differentiation

Bone marrow cells were obtained by flushing the femurs and tibiae of 5-week-old ICR mice with α-minimum essential medium (α-MEM; Gibco BRL, Gaithersburg, MD, USA) and suspended in α-MEM supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA). Non-adherent cells were collected and cultured for 3 days in the presence of M-CSF (30 ng/ml). Floating cells were discarded and adherent cells on dish bottoms were classified as bone marrow-derived macrophages (BMMs). BMMs were seeded at 3.5×10^4 cells/well in α-MEM/10% FBS, and were cultured in the presence of M-CSF (30 ng/ml) and RANKL (50 ng/ml) for 4 days in the presence or absence of rotenone. Osteoclasts were identified by staining for tartrate-resistant acid phosphatase (TRAP) activity, as described below. TRAP-positive multinucleated cells with greater than three nuclei were counted as osteoclasts.

Cytotoxicity assay for rotenone

BMMs were plated in 96-well plates at a density of 1×10^4 cells/well in triplicate. Cells were treated with M-CSF (30 ng/ml) and increasing concentrations of rotenone were added to the mix. Cells were incubated for 3 days. After 3 days, XTT reagent (50 μl) was added to each well. Wells were incubated for 4 h. The optical density at 450 nm was analyzed with an ELISA reader.

Clonogenic assay

RAW 264.7 cells were seeded in 48-well plates at a density of 3,000 cells/well in triplicate and cultured for 4 days in the presence of increasing concentrations of rotenone. After 4 days, the cells were fixed and stained with Hematoxylin (Sigma). Colonies with 50 or greater cells were counted.

Real time RT-PCR analysis for c-Fos, NFATc1, TRAP, osteoclast-associated receptor (OSCAR)

Total RNA was isolated with TRIzol reagent (Invitrogen Inc., USA) per the manufacturer's instructions. RNA (1 μg) was reverse transcribed using oligo dT primers (10 μg) and dNTPs (10 mM). The mixture was incubated at 65 °C for 5 min, and cDNA was produced by incubating at 42 °C for 50 min with first strand buffer (50 mM Tris–HCl, pH 8.3, 75 mMKCl, 3 mM $MgCl_2$), 100 mM DTT, RNase inhibitor, and Superscript II reverse transcriptase (Invitrogen). The cDNA was amplified using the following primer sets: c-Fos, 5′-CTGGTGCAGCCCACTCTGGTC-3′ (forward) and 5′-CTTTCAGCAGATTGGCAATCTC-3′ (reverse); NFATc1, 5′-CTCGAAAGACAGCACTGGAGCAT-3′ (forward) and 5′-CGGCTGCCTTCCGTCTCATAG-3′ (reverse); TRAP, 5′-CTGGAGTGCACGATGCCAGCGACA-3′ (forward) and 5′-TCCGTGCTCGGCGATGGACCAGA-3′ (reverse); OSCAR, 5′-CTGCTGGTAACGGATCAGCTCCCCAGA-3′ (forward) and 5′-CCAAGGAGCCAGAACCTTCGAAACT-3′ (reverse); and GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen) in triplicates according to the manufacturer's instructions. Relative levels of c-Fos, NFATc1, TRAP, and OSCAR were normalized to GAPDH.

Western blot analysis

BMMs or osteoclasts were lysed in a buffer containing 50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1% deoxycholate, and protease inhibitors. The lysates were centrifuged at 14,000×*g* for 20 min and supernatants were collected. Protein concentrations of supernatants were determined. Cellular proteins (30 μg) were resolved by 8–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride membranes (Milipore, Bedford, MA, USA). Non-specific interactions were blocked with 5% skim milk for 2 h and were then probed with the appropriate primary antibodies. Membranes were incubated with the appropriate secondary antibodies attached to horseradish peroxidase, and immunoreactivity was detected with enhanced chemiluminescence reagents. Densitometric values were quantified for each band with the Image Pro-plus program version 4.0.

Resorption pit assay

Mature osteoclasts were prepared by isolating osteoblasts from the calvariae of newborn mice by serial digestion in collagenase (Wako, Japan), as previously described [7]. Bone marrow cells were isolated as described above. Osteoblasts and bone marrow cells were co-cultured on a collagen-coated 90-mm dish in the presence of 1α, 25-dihydroxyvitamin D_3 ($VitD_3$), prostaglandin E_2 (PGE_2) for 6 days. Co-cultured cells were detached from the collagen-coated dishes, were re-plated on dentine slices in 48-well plates, and were treated with rotenone for 24 h. Cells on dentine slices were stained for TRAP and photographs were taken under a light microscope at 40× magnification. To observe resorption pits, cells on dentine slices were completely removed and stained with hematoxylin. Total resorption pit areas were analyzed by the Image Pro-Plus program version 4.0 (Media Cybernetics).

Effect of rotenone on LPS-mediated bone erosion

ICR mice (6 weeks old) were divided into three groups of 5 mice. Mice were injected intraperitoneally rotenone (1 μg/g body weight) or PBS as control 1 day before injection of LPS (5 μg/g body weight). Rotenone or PBS was injected intraperitoneally every other day for 8 days. LPS was injected intraperitoneally on days 1 and 4. All mice were sacrificed 8 days after the initial LPS injection, and the left femurs of all animals were scanned with a high-resolution micro-CT (NFR-Polaris-S160: Nano Focus Ray, Iksan, Korea). Bone histomorphometric analyses were performed with the micro-CT data using the software provided by VGStudio MAX 1.2 software (Volume Graphics Inc., Germany). The bone volume ratio, represented by bone volume/tissue volume (BV/TV) was measured to assess the trabe-