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d-pinitol inhibits RANKL-induced osteoclastogenesis

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ABSTRACT

Numerous studies have indicated that inflammatory cytokines play a major role in osteoclastogenesis, leading to the bone resorption that is frequently associated with osteoporosis. D-pinitol, a 3-methoxy analogue of D-chiroinositol, was identified as an active principle in soy foods and legumes. Here we found that D-pinitol markedly inhibited the receptor activator of nuclear factor kappa B ligand (RANKL)-induced osteoclastic differentiation from bone marrow stromal cells and RAW264.7 macrophage cells. In addition, D-pinitol also reduced RANKL-induced p38 and JNK phosphorylation. Furthermore, RANKL-mediated increase of IKK, IκBα, and p65 phosphorylation and NF-κB-luciferase activity was inhibited by D-pinitol. However, D-pinitol did not affect the proliferation and differentiation of osteoblasts. In addition, D-pinitol also prevented the bone loss induced by ovariectomy *in vivo*. Our data suggest that D-pinitol inhibits osteoclastogenesis from bone marrow stromal cells and macrophage cells via attenuated RANKL-induced p38, JNK, and NF-κB activation, which in turn protect bone loss from ovariectomy.

1. Introduction

Osteoporosis ensues from an imbalance bone resorption and bone formation with a net bone loss that may be induced by several conditions, such as hormonal imbalance, diseases, or medications (e.g. corticosteroids or anti-epileptic agents) [1]. Current drugs used to treat osteoporosis include bisphosphonates, calcitonin, and estrogen. These drugs are all bone resorption inhibitors, which maintain bone mass by inhibiting the function of osteoclasts [2]. Since the osteoclasts are responsible for bone resorption, therefore they are one of the main targets for treatment of osteoporosis.

Osteoclasts are multinucleated cells formed by the fusion of mononuclear progenitors of the monocyte/macrophage family [3]. *In vitro* maturation of macrophages into osteoclasts requires the presence of stromal cells or their osteoblast progeny [4]. Extensive research in the last few years has indicated that these accessory cells express macrophage colony stimulating factor (M-CSF) and receptor for activation of NF-κB (RANK) ligand (RANKL) that are essential for osteoclastogenesis [5]. RANKL, a member of the TNF superfamily, interacts with the cell surface receptor RANK and in turn recruits TNFR associated factors (TRAF) 1, 2, 3, 5, and 6 [6]. The receptor deletion analysis has shown that sequential recruitment of TRAF6 and NF-κB-inducing kinase by RANK leads to NF-κB activation, and recruitment of TRAF2 leads to JNK, p38, and ERK activation [6,7]. Thus agents that can suppress RANKL signaling can suppress osteoclastogenesis-induced bone loss.

D-pinitol, a 3-methoxy analogue of D-chiroinositol, was identified as an active principle in soy foods and legumes [8]. The mature and dried soybean seeds contain up to 1% D-pinitol. D-pinitol functions as an osmolyte by improving the tolerance to drought stress or heat stress and is involved in reducing the negative effects of osmotic stress and increasing the tolerance of plant tissues to water deficiencies. In addition, D-pinitol has been suggested to possess multifunctional properties, including feeding stimulant, anti-inflammatory, cardioprotective, anti-hyperlipidemic, and creatine retention promotion properties [9–11]. However, the molecular mechanism of D-pinitol on the osteoclast formation has not been reported. Here, we report that D-pinitol inhibits RANKL-induced osteoclast formation from bone marrow stromal cells and RAW264.7 macrophages. D-pinitol also inhibits the RANKL-induced p38, JNK, and NF-κB activation in macrophages. On the other hand, D-pinitol did not affect the proliferation and differentiation of osteoblasts. Therefore, our data provide evidences that D-pinitol may be an anti-resorption agent for treatment of osteoporosis.

2. Materials and methods

2.1. Materials

D-pinitol was purchased from Wako Chemicals (Osaka, Japan). Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for p-IKK, IKK, p-IκBα, IκBα, p-p38, p38, p-JNK, JNK, p-p65, and p65 were purchased from Santa

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Cruz Biotechnology (Santa Cruz, CA, USA). JNK and p38 kinase assay kit were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). Recombinant human M-CSF and RANKL were purchased from R&D Systems (Minneapolis, MN, USA). pSV-β-galactosidase vector, luciferase assay kit was purchased from Promega (Madison, MA, USA). The NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA, USA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Bone marrow cells were prepared by removing from femurs of 6–8 week-old Sprague–Dawley rats and flushing the bone marrow cavity with α-MEM which was supplemented with 20 mM HEPES and 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The non-adherent cells (hematopoietic cells) were collected after 24 h and used as osteoclast precursors. Cells were seeded at a density of 1×10^6 cells/well in 24-well plates in the presence of human recombinant soluble RANKL (50 ng/ml) for 7 days. The culture medium was replaced every 3 days.

Murine RAW264.7 cells (a mouse macrophage cell line obtained from American Type Culture Collection) were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. For differentiation of osteoclasts, RAW264.7 cells (2×10^4, in 24-well plate) were cultured in the presence of RANKL (50 ng/ml) for 5 days. The culture medium was replaced every 3 days.

The human osteoblast-like cell line MG-63 and MC3T3E-1 were purchased from American Type Culture Collection. Cells were cultured in α-MEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

2.3. Osteoclast differentiation assay

Osteoclast formation was measured by quantifying cells positively stained by tartrate-resistant acid phosphatase [TRAP (Acid Phosphatase Kit 387-A; Sigma-Aldrich, St. Louis, MO, USA)]. Briefly, the cells were fixed for 30 s and then stained with Naphthol AS-BI phosphate and a tartrate solution for 1 h at 37 °C, followed by counterstaining with a hematoxylin solution. Osteoclasts were determined to be TRAP-positive staining multinuclear (>3 nuclei) cells using light microscopy. The total number of TRAP-positive cells and the number of nuclei per TRAP-positive cell in each well were counted. The morphological features of osteoclasts were also photographed [12].

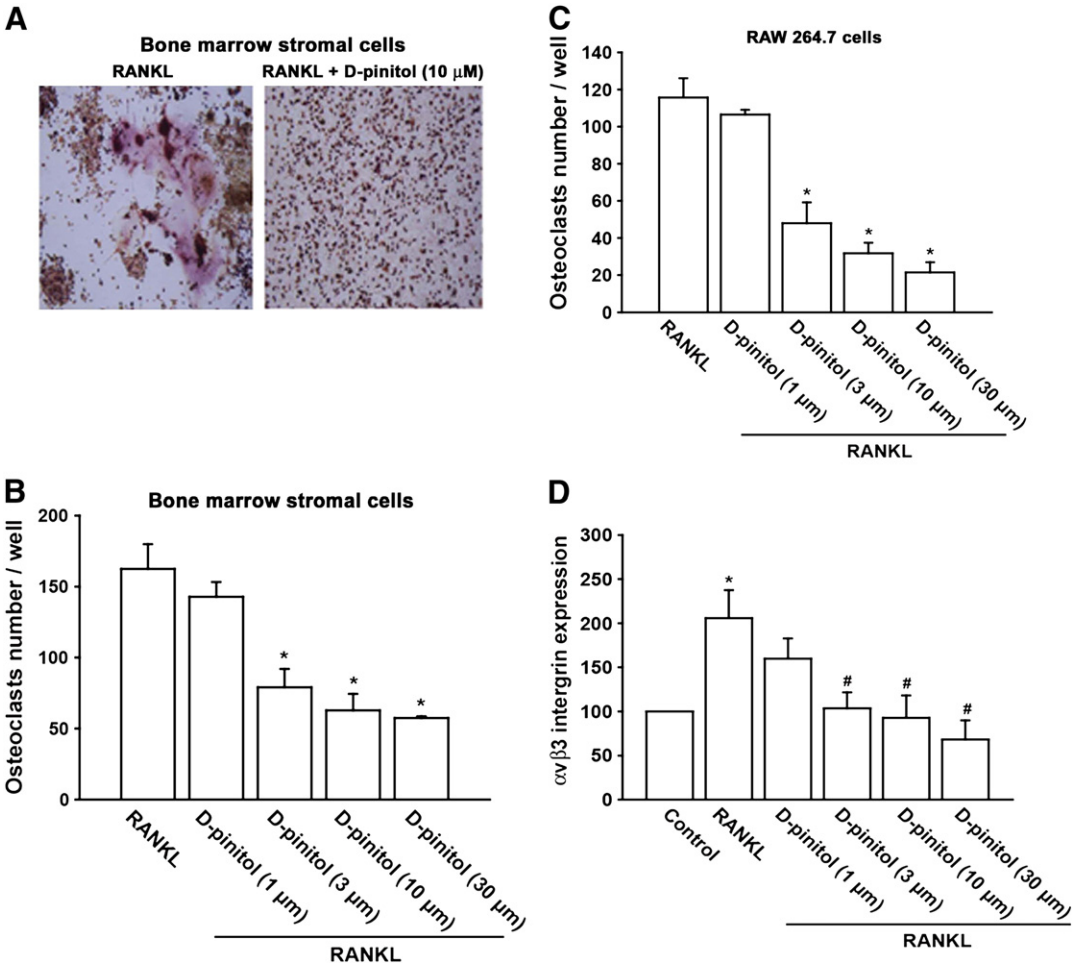

Fig. 1. Inhibition of osteoclast differentiation by D-pinitol.Osteoclast precursors isolated from long bones of adult male rats were plated on a 24-well plate at 1×10^6 cells/well and cultured in the presence of RANKL (50 ng/ml) for 7 days. Following TRAP staining, the cells with more than 3 nuclei were counted (A). Compared with RANKL, D-pinitol treatment markedly inhibited the differentiation of osteoclast. The quantitative data are shown in B. RAW264.7 cells were seeded at 2×10^4 and incubated for 5 day with RANKL (50 ng/ml) without or with D-pinitol. Treatment with D-pinitol inhibited osteoclastogenesis in a concentration-dependent manner *: $p<0.05$ as compared with RANKL-treated group. (C). RAW264.7 cells were treated with RANKL (50 ng/ml) without or with D-pinitol for 5 days. After incubation, cells were treated with anti-αvβ3 integrin antibody and analyzed by flow cytometry *: $p<0.05$ as compared with RANKL-treated group. (D). Results are expressed as the mean ± S.E.M. of four independent experiments. *: $p<0.05$ as compared with control group. #: $p<0.05$ as compared with RANKL-treated control group.