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A flavonoids compound inhibits osteoclast differentiation by attenuating RANKL induced NFATc-1/c-Fos induction

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ABSTRACT

Function studies of pectolinarigenin demonstrated that, as a natural product, it possesses the regulatory effects on transcription factors (TFs) such as: signal transducer and activator of transcription 3 (STAT3). Herein, we aimed to identify the regulatroy effects of pectolinarigenin on the osteoclastogenesis TFs such as: NFATc1 and c-Fos, and further identify the relevant up-stream signals activity. We initially found pectolinarigenin inhibited receptor activator of nuclear factor-kappa B ligand (RANKL) induced osteoclast formation during the bone marrow-derived macrophages (BMMs) cultures, suggesting that this natural product could act on osteoclast precursors by inhibiting the down signaling cascades of RANKL signaling. Moreover, mechanistical investigation showed pectolinarigenin inhibits RANKL-mediated osteoclastogenesis by attenuating the nuclear factor of activated T cells cytoplasmic 1 (NFATc-1) and c-Fos following the Akt and mitogen activated protein kinases (MAPKs) signaling costimulatory. These findings identify that pectolinarigenin may act as an anti-resorption agent by blocking osteoclast activation.

1. Introduction

Pathological skeletal disorders such as osteoporosis and osteolysis are a fatal problem that is characterized by fragile bone and impaired bone quality [1]. Elevated levels of inflammatory cytokines acting in concert with the osteoclastogenic factor, receptor activator of nuclear factor-kappaB (NF-κB) ligand (RANKL) and macrophage-colony stimulating factor (M-CSF), drive excessive osteoclast (OC) differentiation, and lead to local joint erosion and systemic bone loss [2, 3]. During early stage of OCs differentiation (osteoclastogenesis), M-CSF induces expression of RANKL receptor (RANK) as well as supports survival and proliferation [4]. Following, RANKL binding to RANK, trimerization and the recruitment of adaptor molecules including tumor necrosis factor receptor-associated factor 6 (TRAF6) is stimulated to activate many downstream signaling pathway such as: three mitogen activated protein kinases (MAPKs) including p38, extracellular signal regulated kinase (ERK) and c-jun-N terminal kinase (JNK) [5]. Signals further activate c-Fos and nuclear factor of activated T cells cytoplasmic1 (NFATc1), two master transcription factors for OCs differentiation [6].

Pharmaceutical treat bone loss focusing on explore compounds derived from natural products regained attentions given by it has fewer side effects, and as precursory studies expected to be a suitable medicine for long-term use than synthetic drugs [7–10]. In that, several natural products have been screened and found possess the effects on bone metabolism [7, 11–13]. A flavonoids compound, pectolinarigenin (Pec, Fig. 1: a) has been shown to possess numerous biologic activities such as anti-inflammation [14] and anti-tumor [15]. Moreover, study also reported pectolinarigenin possesses effective prevent the hepatic injury in vivo [16]. Most recently, an interesting study has showed pectolinarigenin effectively regulated extracellular signaling pathway signal transducer and activator of transcription 3 (STAT3) [17]. Thus, in our current study, we aimed to investigate the effects of pectolinarigenin on osteoclastogenesis in vitro.

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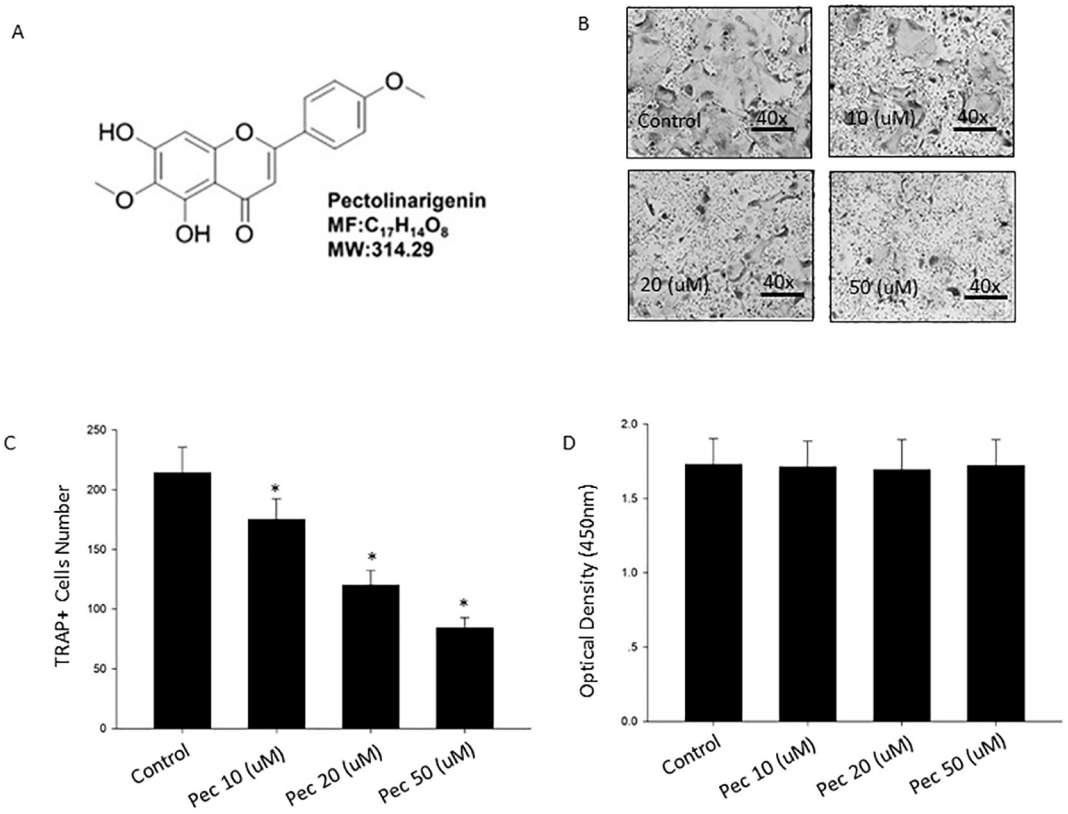

Fig. 1. Chemical structure of Pectolinarigenin and Effect of Pectolinarigenin on RANKL-induced OCs differentiation.
(A) Chemical structure of pectolinarigenin. (B) Effect of pectolinarigenin on RANKL-induced OCs differentiation. TRAP-positive cells were counted as osteoclasts in each experimental group. (C) Number of TRAP-positive OCs (D) Cell viability was determined by XTT assay. Asterisk indicates a statistically significant difference ($p < 0.05$) between control and treated. Similar results were obtained in at least 3 independent experiments.

2. Materials and methods

2.1. Reagents and antibodies

Pectolinarigenin (Molecular formula: $C_{17}H_{14}O_6$, Molecular weight: 314.28, purity > 98%) was purchased from Shanghai Yuan Ye Biotechnology Co. Ltd. (Shanghai, China). M-CSF and human RANKL were obtained from PeproTech EC, Ltd. (London, U.K.). The sodium 3′-[(-1 phenylaminocarbonyl 9-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay kit was obtained from Roche (Indianapolis, IN, USA). Antibodies for c-Fos and NFATc1 were purchased from Santa Cruz Biotechnology, and Western blot antibodies for phosphor-ERK, ERK, phosphor-JNK, JNK, phosphor-p38, p38, U-0126, SB-203580, SP-600125 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); β-actin antibody was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

2.2. Bone marrow-derived macrophages culture

Bone marrow-derived macrophages (BMMs) were used as OC precursors as Kong et al. [18, 19] previously reported. The cells were grown in serum and 1% penicillin-streptomycin solution, and in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C; the media were changed every 3 days. For differentiation, cells were plated at 2×10^3 cells per well of 96-well plates in Dulbecco's modified eagle's medium (DMEM) supplemented with 75 ng/mL recombinant RANKL and 15 ng/mL M-CSF. For drug assays, pectolinarigenin was added at different concentrations to the cell medium. Then cells were incubated in a humidified incubator at 37 °C with 5% CO_2 and fed daily with RANKL and M-CSF medium for 7 days.

2.3. Cell viability assays

Cell viability of BMMs after pectolinarigenin treatments was studied by XTT as previous studies described [20]. Briefly, BMMs were seeded in 96-well plates by the 1×10^4 cells with different concentrations of pectolinarigenin. After incubated for 3 days in the presence of RANKL, XTT solution was added to each well and incubated for 4 h. The plate was read at 450 nm.

2.4. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIzol reagent (QIAGEN, Valencia, CA, USA.) according to the manufacturer's instructions, 1 μg RNA was reverse transcribed using oligo dT primers and dNTPs. Next following the instruction, 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, 100 mM dithiothreitol (DTT), RNase inhibitor, and SuperScript II reverse transcriptase (Invitrogen, Shanghai branch, China). cDNA was amplified by TOPsimple dryMix premix PCR kit (Clontech, CA, USA). Primers listed in Table 1 are employed for amplification in our study. The mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as internal control.

2.5. Western blot analysis

Whole-cell lysates were washed with lysis buffer: 50 mM Tris–HCl, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1% deoxycholate, and protease inhibitors. Lysates were incubated in sodium dodecyl sulfate (SDS) buffer for 5 min, then resolved by 8–10%