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Dioscin reduces ovariectomy-induced bone loss by enhancing osteoblastogenesis and inhibiting osteoclastogenesis

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ABSTRACT

Our previous studies showed that dioscin can promote osteoblasts proliferation and differentiation *in vitro*, but its anti-osteoporosis effect *in vivo* and the underlying mechanisms remain unclear. In the present work, the results showed that dioscin significantly increased the viability of MC3T3-E1 cells, ALP level and alizarin red S staining area, markedly decreased the numbers of RANKL-induced TRAP-positive multinucleated cells and bone resorption pits formation, enhanced the levels of some osteogenic markers including COL1A2, ALP and OC, which suggested that dioscin clearly promoted osteoblasts proliferation and suppressed osteoclasts formation. *In vivo* experiments demonstrated that dioscin obviously reduced OVX-induced body weight increase, and improved the biochemical indexes including ALP, StrACP, OC, DPD/Cr, HOP/Cr, BMD, biomechanics and microarchitecture. Moreover, H&E, TB, TRAP staining, and fluorescent double labeling tests indicated that dioscin enhanced osteoblastogenesis and inhibited osteoclastogenesis. Further researches demonstrated that dioscin promoted osteoblastogenesis through up-regulating OPG/RANKL ratio, and inhibited osteoclastogenesis through down-regulating the levels of RANKL induced TRAF6 and the downstream signal molecules including MAPKs, Akt, NF-κB, AP-1, cathepsin K and NFATc1. In addition, dioscin also inhibited TLR4/MyD88 pathway to decrease the levels of TRAF6 and the related proteins. These findings provide new insights to elucidate the effects of dioscin against OVX-induced bone loss, which should be developed as a potential candidate for treating postmenopausal osteoporosis in the future.

1. Introduction

Osteoporosis, a less musculoskeletal disease, is characterized by compromised bone strength inclining to an increased risk of fracture [1]. It is started by estrogen deficiency and particularly common in postmenopausal women [2]. Over 200 million people suffer from osteoporosis worldwide, which leads to 1.6 million people with hip fractures and 7.4 million people with other fractures annually [3,4]. Approximately half of the women and a quarter of the men over 50 years old have the lifetime risk of osteoporosis-related fracture. In American, the direct fracture-caused costs are expected to raise from $17 billion in 2005 to over $22 billion by 2020 [5,6]. Thus, osteoporosis is one serious health and society problem.

Mechanistically, postmenopausal osteoporosis (PMO) is a disorder of unbalanced bone remodeling with the increased bone resorption relative to bone formation, leading to the decreased bone mineral density (BMD) and the disruption of bone microarchitecture [7]. Bone resorption is completed by the polarization and the subsequent attachment to bone surface of osteoclasts. Meanwhile, bone formation occurs following osteoblasts phase that is to be developed into osteocytes [2]. Therefore, correcting the imbalance between bone resorption and formation is an effective therapy for PMO.

It is well known that the factors including the receptor activator for nuclear factor-κB (RANK) and its ligand RANKL, and osteoprotegerin (OPG, the decoy receptor for RANKL) associated with osteoporosis have been discovered [8]. Among them, RANKL is mainly produced by osteoblasts and stromal cells, and OPG is

Abbreviations: Akt, protein kinase B; ALP, alkaline phosphatase; AP-1, transcription activator; BMD, bone mineral density; DPD, deoxypyridinoline; COL1A2, collagen, type I, alpha 2; Cr, creatinine; H&E, hematoxylin-eosin; HOP, hyroxyproline; MyD88, myeloid differentiation primary response gene (88); MAPKs, mitogen activated protein kinases; NFATc1, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; OC, osteocalcin; OPG, osteoprotegerin; OVX, ovariectomy; RANKL, receptor activator for nuclear factor-κB ligand; StrACP, tartrate resistant acid phosphatase; TB, toluidine blue; TLR4, toll-like receptor 4; TRAF6, tumor necrosis factor receptor-associated factor; TRAP, tartrate-resistant acid phosphatase.

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generated by osteoblasts. Binding RANKL to RANK provides a pivotal signal to drive osteoclasts generation from haematopoietic progenitor cells as well as to activate mature osteoclasts [9]. Meanwhile, OPG can negatively regulate the binding and then inhibit osteoclasts induced-high bone turnover [10]. Furthermore, Toll-like receptor 4 (TLR4), one germline-encoded pattern recognition receptor, can recognize multiple microbial pathogens including damage-associated molecular pattern molecules (DAMPs) and lipopolysaccharide (LPS), and promote the secretion of various inflammatory mediators to induce pre-osteoclast fusion and stimulate the survival of mature osteoclasts [11]. Therefore, the OPG/RANK/RANKL axis and TLR4 signal may be the potential anti-osteoporosis targets.

Presently, the methods for the treatment of PMO mainly include five kinds of agents including estrogens, bisphosphonates, calcitonin, selective estrogen receptor modulators (SERMs) and monoclonal antibody [12]. However, these agents may cause many adverse effects including invasive breast cancer, osteonecrosis, diarrhea, venous thromboembolism and serious infections of skin and urinary tract. Besides these, single drug target and high price of these anti-PMO agents also restrict their developing and applying in clinic [4,13]. Thus, it is urgent to exploit efficient agents for treating PMO.

Traditional Chinese medicines (TCMs) with high efficiency and low toxicity have attracted great interest in recent years. Some active natural components including lycopene [14], resveratrol [15], and ginsenoside-Rb2 [16] have excellent activities against PMO. Therefore, it is reasonable to develop effective herbal products for the treatment of PMO.

Dioscin (Supplemental Fig. 1a) is a natural steroidal saponin isolated from some medicinal plants [17,18]. Pharmacological investigations have shown that dioscin exhibits hepatoprotective [19–22], anti-tumor [23,24] and anti-ischemia reperfusion injury activities [25,26]. In addition, dioscin has obvious effects against obesity [27] and carbon tetrachloride-induced liver fibrosis [28,29], which also exerts inhibitory effects on several cytochrome P450 enzymes [30]. Our previous works have shown that dioscin can promote the proliferation and differentiation of pre-osteoblast like MC3T3-E1 cells and human osteoblast-like MG-63 cells *in vitro* [31]. However, its effects on PMO and the underlying mechanisms are unclear in our best knowledge.

Therefore, the aim of the present paper was to further explore the effects of dioscin against PMO and then to investigate the underlying molecular mechanisms.

2. Materials and methods

2.1. Chemicals and materials

Dioscin (purity >98%) was prepared from *Dioscorea nipponica* Makino in our laboratory [32,33]. In cell experiments, dioscin was added into the medium by dissolving with DMSO with final concentration of less than 0.1%, and it was suspended in 0.5% sodium carboxyl methyl cellulose (CMC-Na) in animals experiments. Fetal bovine serum (FBS), penicillin and streptomycin were obtained from Hyclone Laboratories, Inc. (Massachusetts, USA). Dulbecco's minimum essential medium (DMEM) and alpha-minimum essential medium (α-MEM) were purchased from Gibco (California, USA). Recombinant mouse macrophage colony-stimulating factor (M-CSF) and recombinant mouse RANKL were purchased from R&D Systems (Minneapolis, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was provided by Roche Diagnostics (Basel, Switzerland). Alkaline phosphatase (ALP), tartrate resistant acid phosphatase (StrACP), hyroxyproline (HOP), calcium (Ca), phosphate (P) and creatinine (Cr) assay kits and hematoxylin-eosin (H&E), toluidine blue (TB) and TRAP staining kits were obtained from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Osteocalcin (OC) and deoxypyridinoline (DPD) ELISA kits were provided by Bogoo (Shanghai, China). alizarin red and calcein were purchased from Solarbio (Shanghai, China). 4′,6-diamidino-2-phenylindole (DAPI) and LPS were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tissue Protein Extraction Kit was purchased from KEYGEN Biotech. Co., Ltd. (Naijing, China). Bicinchoninic acid (BCA) Protein Assay Kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China).

2.2. Cells culture

The MC3T3-E1 cells were purchased from the Institute of Biochemistry Cell Biology (Shanghai, China) and cultured in DMEM medium supplemented with 10% FBS, 100 U/mL streptomycin and 100 U/mL penicillin at 37 °C in a saturated humidified incubator (Thermo Fisher Scientific, Massachusetts, USA). Meanwhile, whole bone marrow cells were extracted from the femur and tibiae of 6-week-old C57BL/6J mice. These cells were grown in α-MEM with 10% FBS, 100 U/mL streptomycin, 100 U/mL penicillin and 50 ng/mL M-CSF for 3 days to generate bone marrow-derived macrophages (BMMs). To generate osteoclasts, 50 ng/mL M-CSF and 100 ng/mL RANKL were added to α-MEM for 4 days of culture in a constant temperature and humidity incubator [34,35].

2.3. Proliferation and ALP detection in MC3T3-E1 cells

After adhering and growing for 24 h, the MC3T3-E1 cells were treated with dioscin (0, 0.25, 0.5 and 1.0 μg/mL) for 24, 48 and 72 h. Then, the viability of MC3T3-E1 cells was detected by MTT assay. The MC3T3-E1 cells (1×10^4 cells/mL) were seeded in 24-well culture plates, and then treated with dioscin (0, 0.25, 0.5 and 1.0 μg/mL) for 72 h. After the cells were gently washed with iced PBS, permeated with 0.2% TritonX-100, and centrifuged, the supernatant was collected and used for detecting ALP activity and total protein concentration.

2.4. Alizarin red S staining in MC3T3-E1 cells

The MC3T3-E1 cells (1×10^4 cells/mL) were seeded into culture dishes and incubated with different concentrations of dioscin (0, 0.25, 0.5 and 1.0 μg/mL). At the 21 days differentiation, the cells were washed with PBS and fixed with ice-cold 70% ethanol (v/v) for 10 min, and then rinsed thoroughly with distilled water, stained with 40 mM alizarin red S solution in deionized water (pH = 4.2) for 10 min and rinsed with PBS. Eventually, the stained culture dishes were photographed using a digital camera.

2.5. Proliferation assay in BMMs

The BMMs (1×10^5 cells/mL) were treated with various concentrations of dioscin (0, 0.25, 0.5, 1.0 and 2.0 μg/mL) plus M-CSF (50 ng/mL) and RANKL (0 and 100 ng/mL) for 24 h. Next, the absorbance of the samples was quantified according to the MTT method.

2.6. TRAP staining in RANKL-induced osteoclasts

The BMMs (1×10^5 cells/mL) were treated with M-CSF (50 ng/mL) and RANKL (100 ng/mL) for 4 days, and then various concentrations of dioscin (0, 0.25, 0.5, 1.0 μg/mL) were added at the day 1, day 2 and day 3. At the fourth day, the cells were fixed and stained for TRAP activity, and the images were photographed by using an inverted microscope (Nikon, Japan).