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8-Prenylkaempferol accelerates osteoblast maturation through bone morphogenetic protein-2/p38 pathway to activate Runx2 transcription

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ABSTRACT

Aims: In this study, we investigated the effect of 8-prenylkaempferol (8-PK), a prenyl-flavonoid isolated from *Sophora flavescens*, on osteoblast differentiation and maturation.
Main methods: MC3T3-E1 cells were exposed to 8-PK and the cytotoxicity was assayed. Osteoblast differentiation and maturation were evaluated by analyzing alkaline phosphatase (ALP) activity and cell mineralization, respectively. RT-PCR and Western blot were executed to determine the effects of 8-PK on osteoblast differentiation-related gene expression and signaling pathway.
Key findings: 8-PK significantly promoted ALP activity, up-regulated mRNA expressions of osteocalcin, osteopontin, and type I collagen, and induced bone nodules formation. Induction of differentiation by 8-PK was associated with increased bone morphogenetic protein (BMP)-2 expression, and sequentially up-regulated the phosphorylations of Smad1/5/8 and p38, and increased the nuclear translocation of runt-related transcription factor 2 (Runx2). Addition of BMP-2 antagonist noggin blocked 8-PK and recombinant mouse BMP-2-induced ALP activity, reconfirming that BMP-2 production is required in 8-PK-mediated osteoblast differentiation. Noggin also abrogated 8-PK evoked phosphorylations of Smad1/5/8 and p38, suggesting that BMP-2 signaling is required for p38 activation in 8-PK-treated cells. Application of p38 inhibitor SB203580 repressed not only 8-PK-mediated activation of ALP, but also the nuclear translocation of Runx2 and bone nodules formation.
Significance: The present results suggested that BMP-2/p38/Runx2 pathways were involved in 8-PK-induced differentiation/maturation of MC3T3-E1 osteoblasts and firstly demonstrated that 8-PK might be a promising agent for inducing osteogenesis.

Introduction

Many plant-derived compounds, the so-called phytoestrogens, have the potential to counteract the deleterious effects of estrogen deficiency on bone. These include the flavonoids family comprising isoflavone and flavonol derivatives, such as genistein, quercetin and kaempferol, which have been reported to induce osteoblast differentiation (Okumura et al., 2006; Prouillet et al., 2004). Ku-Shen (*Sophora flavescens*) is an oriental herbal drug used in Asian countries as functional food ingredient for centuries because of its potential health beneficial properties such as improving mental health and free radical scavenging and anti-inflammatory activities (Kim et al., 2002; Hwang et al., 2005; Jung et al., 2005). Chemical constituents such as alkaloids and flavonoids have been described from *S. flavescens* (Chen et al., 2004). A prenyl-flavonoid named 8-prenylkaempferol (8-PK) was recently isolated from this herb and displayed anti-inflammatory property (Chiou et al., 2010). Interestingly, Hillerns and Wink (2005) reported that 8-PK possesses estrogenic activity in rat uterus by binding to estrogen receptor (ER) although the relative binding affinity was 10^6 fold weaker than 17β-estradiol. Considering this report, we predicted that 8-PK might probably display protective effect against bone loss. Our preliminary study proved that 8-PK potently stimulated the differentiation of MC3T3-El pre-osteoblasts as measured by an increase in alkaline phosphatase (ALP) activity. Therefore, we attempted to further examine the molecular mechanisms of action.

Osteogenic differentiation of mesenchymal pluripotent cells is regulated by various soluble proteinous factors. Especially, bone morphogenetic protein (BMP) was originally identified as a molecule that promoted the differentiation of mesenchymal cells into an osteoblastic lineage, as suggested from its abilities not only to induce the expression of bone specific proteins ALP, type 1 collagen (Coll 1), osteopontin (OPN), and osteocalcin (OCN), but also to stimulate mineralization (Canalis et al., 2003). In the BMP signaling pathways,

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the Smad proteins play a major role in osteoblastic differentiation. The canonical Smad pathway in which receptor-specific Smad1, 5, and 8 are activated, form complexes with the common partner, Smad4, and translocated into the nucleus to regulate the transcription of target genes (Sakou et al., 1999). The other signaling pathway that mediated by BMP including runt-related transcription factor 2/core binding factor a1 (Runx2/Cbfa1) (Lian et al., 2006). Runx2 is the main transcription factor required for the activation of osteoblast differentiation and is crucial for the regulation of genes responsible for the production of bone specific proteins (Prince et al., 2001). In some types of cells, BMP-2 can activate MAP kinase kinases kinase (MAPKKK) and consequently phosphorylates and activates p38 and ERK (Lai and Cheng, 2002). Many studies have reported that p38 activation is necessary for differentiation in osteoblasts (Guicheux et al., 2003; Jadlowiec et al., 2004).

The present results showed that 8-PK markedly increased ALP activity and calcium nodules formation, although without notable effect on cell proliferation. We also clarified that 8-PK regulated osteoblast differentiation probably through induction of BMP-2 expression and subsequently triggered Smad1/5/8 and p38 phosphorylations, then forwarded mineralization process by modulating Coll 1, OPN and OCN expression through activation of Runx2 transcription.

Materials and methods

Cell cultures and drug treatments

The murine calvaria-derived osteoblastic cell line MC3T3-E1 was maintained in an alpha modification of Eagle's minimum essential medium (α-MEM, Gibco BRL, Grand Island, NY 14072, USA) supplemented with 10% fetal calf serum (FCS, Gibco), 100 U/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco) (defined as minimal medium) in a humidified 5% CO_2 balanced-air incubator at 37 °C. Cells were subcultured using 0.05% trypsin with 0.01% EDTA. To induce differentiation, MC3T3-E1 cells (5×10^3/well) were seeded into 96-well plates (Corning Costar, Tower 2, 4th Floor 900 Chelmsford Street, Lowell, NY, USA) and cultured in minimal medium containing 50 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO) (defined as differentiation medium). To induce bone nodule formation, cells (1×10^5/well) were seeded into 24-well plates and cultured in differentiation medium further supplemented with 10 mM β-glycerophosphate (Sigma-Aldrich) (Lo et al., 2010).

8-Prenylkaempferol (8-PK) was dissolved in dimethyl sulfoxide (DMSO) at 0.1 M as a stock solution and diluted with medium (Chiou et al., 2010). A final DMSO concentration in the culture was less than 0.05% and did not show observably artificial or cytotoxic effect. Recombinant mouse BMP-2 protein (rmBMP-2) and kaempferol were employed as reference controls. All treatments were initiated after 3 days of incubation (day 0). Drug was added to cells for further 3 to 14 days culture to assess its effect on cell differentiation and/or mineralization, respectively. For bone nodules formation, mediums were replenished with fresh treatments every 3 days, i.e. cells were re-stimulated with tested drug to ensure that tested agent was continuously present in the medium through the observation period. In another experiment, noggin (R&D Systems, Minneapolis, Minnesota, USA) or SB203580 (R&D) was added at 2 h before 8-PK or rmBMP-2 stimulation to block BMP- and p38-related signal pathways, respectively.

Quantitative assay of ALP activity

ALP activity in the cells was measured by incubation in 0.1 M $NaHCO_3$–Na_2CO_3 buffer (pH 10) containing 0.1% Triton X-100, 2 mM $MgSO_4$ and 6 mM p-nitrophenyl-phosphate (PNPP) for 30 min at 37 °C. The reaction was stopped by adding 1 M NaOH, and the absorbance was measured at 405 nm by an automatic biochemistry instrument (Hitachi, Japan) (Lo et al., 2010). Protein concentration was determined by incubation in bicinchoninic acid (BCA) protein assay reagent and measured the absorbance at 550 nm. ALP activity was calculated as PNPP concentration/protein concentration/time (μM PNPP/mg protein/h). ALP activity measured in differentiation medium alone was defined as control. In some set of experiment, results were expressed as relative ratio to control. To preclude the possibility that the attenuation in ALP activity was due to cytotoxicity, cell viability was simultaneous measured by a test based on the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases of metabolically active cells. The cells cultivated in differentiation medium without tested agents served as the control.

Mineralized bone-like tissue observation

Cells were incubated in the differentiation medium for 14 days in the absence or presence of 8-PK, respectively. On days 14, the cultures in the wells were rinsed using an ice-cooled PBS and fixed with 95% ethyl alcohol. They were stained for 1 h with 0.1% Alizarin red S (Sigma-Aldrich) to detect the bone nodules (calcium precipitates). After wash with PBS, the samples were observed under light microscope and the representative pictures were photographed. Finally, 0.1 N NaOH was added to dissolve the calcium precipitates then the absorbance was measured at a wavelength of 548 nm. Alizarin red S stained in cells without 8-PK treatment was defined as control. Results were expressed as relative ratio to control.

Cell proliferation

Effect of 8-PK on cell proliferation was measured by 2, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbox-anilide (XTT) assay (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, cells cultured in minimal medium were seeded into 96-well culture plates (5×10^3 cells/well). After 24 h incubation, the cells were treated with or without 8-PK for 72 h. Fifty microliters of XTT test solution, which was prepared by mixing 5 ml of XTT-labeling reagent with 100 ml of electron coupling reagent, was then added to each well. After 4 h of incubation, absorbance was measured on an ELISA reader (Bio-Tek Instrument, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

RT-PCR analysis

Expressions of OCN, OPN, type 1 collagen and BMP-2 mRNA were examined using RT-PCR, with the amplification of glyceraldehydes 3-phosphate dehydrogenase (GAPDH) as control. MC3T3-E1 cells were seeded in 35 mm dish and cultured for 3 days in the absence or presence of 8-PK, respectively. After incubation, total RNA of cells was extracted using Trizol reagent. cDNA was synthesized using 5 μg total RNA, random primer 1 μl, dNTPs 2 μl, and 200 U M-MLV Reverse Transcriptase (Promega, USA) at 37 °C for 1 h. The specific primers of OCN were: Forward: 5′-GCAGCTTGGTGCACACCTAG-3′; Reverse: 5′-GGAGCTGCTGTGACATCCAT-3′. The specific primers of Coll 1 were: Forward: 5′-TCTCCACTCTTCTAGTTCCT-3′; Reverse: 5′-TTGGGTCATTTCCACATGC-3′. The specific primers of OPN were: Forward: 5′-ATGAGATTGGCAGTGATTTG-3′; Reverse: 5′-GTAGGGACGATTGGAGTGAA-3′. The specific primers of BMP-2 were: Forward: 5′-CCAAGACACAGTTCCCTACA-3′; Reverse: 5′-CACGGCTTCTAGTTGATGGA-3′. The specific primers of GAPDH were: Forward: 5′-GCCATCAACGACCCCTTCATTGAC-3′; Reverse: 5′-ACGGAAGGCCATGCCAGTGAGCTT-3′. Amplification was carried out for 30 cycles, each of which was at 94 °C for 15 s, 59 °C for 15 s, and 72 °C for 30 s in a 25 μl reaction mixture containing 3 μl cDNA, 25 pmol of each primer, 0.25 mmol dNTPs, and 2 U of Taq DNA polymerase (Promega, USA). The products of PCR were analyzed with 1% agarose gel