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Enhancing effects of myricetin on the osteogenic differentiation of human periodontal ligament stem cells via BMP-2/Smad and ERK/JNK/p38 mitogen-activated protein kinase signaling pathway

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phenylmethane sulfonyl fluoride (PubChem CID: 4784)
sodium hydroxide (PubChem CID: 14798)
polyvinylidene fluoride (PubChem CID: 6369)
sodium dodecyl sulfate (PubChem CID: 3423265)
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ABSTRACT

Myricetin is a flavonoid that found in berries, onions, and red grapes. It has been reported to have various pharmacological effects such as anti-inflammation, anti-oxidant and anti-cancer activities. However, the underlying mechanisms of myricetin on osteogenic differentiation remain unknown in human periodontal ligament stem cells (hPDLSCs). In this study, we investigated the ability of myricetin to increase osteogenic differentiation and its underlying molecular mechanisms. Myricetin significantly increased cell proliferation, alkaline phosphatase (ALP) activity, and alizarin red-mineralization activity in hPDLSCs in a dose-dependent manner. Furthermore, myricetin dose-dependently increased osteogenic-related mRNA and protein levels. Interestingly, it enhanced osteogenesis by up-regulating bone morphogenetic protein-2 (BMP-2), which induced the expression of BMP receptor type IB, Smad-1/5/9. It also enhanced the phosphorylation of extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinases (MAPKs) and Smads. We confirmed that the treatment of myricetin increased phosphorylated GSK-3β and β-catenin which is related to osteogenesis. In our studies, myricetin-induced increment of ALP activity was decreased by ERK (PD98059), JNK (SP600125), p38 (SB203580), and Smad 1/5/9 (LDN193189) inhibitors. ERK and p38 inhibitors showed the greatest inhibition among the four kinds of inhibitors.

These results demonstrate that myricetin promoted osteogenic differentiation by the up-regulation of ALP activity and expression of osteogenic-related factors through BMP-2/Smad and ERK/JNK/p38 MAPK pathways.

1. Introduction

Periodontal disease is common and major health issue in an aged population. Almost 48% of American adults have periodontal disease (Dikbas et al., 2013; Ricardo et al., 2015), and similar proportions were reported in other nations (Albandar and Rams, 2002). Periodontal disease results from oral infection and destruction of connective tissues (Pihlstrom et al., 2005). It usually causes toothache, swollen gums and bad breath (Ng and Leung, 2006). These symptoms reduce quality of life and could lead to tooth loss (Hynes et al., 2012). In the treatment of periodontal disease, there are antibiotics agents (Walker et al., 2004) and surgical interventions such as barrier membranes, soft tissue grafts, and guided tissue regeneration (Bosshardt and Sculean, 2009). Although, it can help stop the progression of disease, both treatments still have side effects, complications and financial burden (Abu-Ta'a et al., 2008; Hynes et al., 2012; Miller, 1988; Slots, 2004). Therefore, the aim of this study is to contribute to the development of natural therapeutic agents for prophylactic treatment and faster recovery of patients who have undergone periodontal disorders.

Periodontal ligament (PDL) is the connective tissue located in between the cementum and alveolar bone, that helps anchoring the tooth root to the alveolar bone socket tightly. It can rapidly turnover matrix and the ability to adapt to alterations in mechanical loading (Berkovitz, 1990). According to a previous study, PDL cells showed many similarities to osteoblast, including the ability to form mineralized nodules (Seo et al., 2004). PDL stem cells have been considered as promising resources for the regeneration of periodontium and can improve the potential of cell-based therapy in dentistry (Hynes et al., 2012). Human periodontal ligament stem cells (hPDLSCs) are more accessible than human bone marrow stem cells (Han et al., 2014). The hPDLSCs also

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have outstanding abilities for differentiation into periodontal ligament, cementum and bone (Liu et al., 2009; Park et al., 2011; Seo et al., 2004).

Osteogenic differentiation is mediation by various pathways, such as bone morphogenetic protein (BMP) pathway, mitogen-activated protein kinase (MAPK) signaling pathway and Wnt/β-catenin pathway. BMPs are members of the transforming growth factor-beta superfamily. Notably, BMP-2, 4, and 7 enhance the differentiation of mesenchymal stem cells into osteoblasts. In the Smad-dependent pathway, BMP-2 binds to heteromeric receptor complexes, promoting the phosphorylation of specific Smads, including Smad −1/5/9 (Lattanzi and Bernardini, 2011). Several MAPK targets, such as extracellular signal-regulated kinases (ERK)1/2 and c-Jun N-terminal protein kinase (JNK), are involved in the Smad-dependent pathway. Also, phosphorylated glycogen synthase kinase (GSK)-3β and β-catenin are key mediator of the Wnt/β-catenin pathway which is an important mechanism for stimulating osteoblast differentiation (Karner and Long, 2017).

In this study, we aimed to find the effective compound on osteogenic differentiation in hPDLSCs. We compared the effectiveness among 50 flavonoid compounds through in vitro screening, then, we found that myricetin showed outstanding effect on osteogenic differentiation. In previous studies, myricetin reported that has anti-oxidant, anti-diabetic (Ong and Khoo, 1997) and anti-inflammation (Ko, 2012). However, we focused on exploring the effect of myricetin on regenerative regulation of hPDLSCs and its underlying mechanisms. In addition, there is no study investigating effect of myricetin on osteogenic differentiation in hPDLSCs.

2. Materials and methods

2.1. Reagents

Fetal bovine serum (FBS), penicillin-streptomycin, trypsin-ethylene diamine tetraacetic acid (EDTA), and α-minimal essential medium (α-MEM) were supplied by Gibco-BRL (Carlsbad, CA, USA). Phosphatase and protease inhibitor cocktails were purchased from Roche (Mannheim, Germany). Antibodies to RUNX2, p-Smad-1/5/9, ERK, p-ERK, p38, p-p38, JNK, p-JNK, p-GSK-3β, GSK-3β and β-catenin were purchased from Cell Signaling Technology (Danvers, MA, USA). Smad-1/5/9, collagen type I, OSX, and BMP-2 were purchased form Abcam (Cambridge, UK). OPN, BMPR-IA, BMPR-IB, BMPR-II and β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor) were purchased from Cell Signaling Technology (Danvers, MA, USA). LDN193189 (Smad 1/5/9 inhibitor) was purchased from BioScience (Irvine, CA, USA). Nuclear extract kit was purchased from Abcam (Cambridge, UK). Unless otherwise specified, chemicals and laboratory supplies were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Falcon Labware (Franklin Lakes, NJ, USA), respectively.

2.2. Cell culture and myricetin treatment

hPDLSCs were purchased from Cell Engineering For Origin (CEFO Co., Ltd, Seoul, Korea). They were cultured in α-minimal essential medium (α-MEM) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. All experiments were carried out with passage 4–7 cells. For osteogenic differentiation, cells were seeded onto 6-well plates and cultured for 48 h in α-MEM to reach 80% confluence. The α-MEM medium was replaced with osteogenic media containing 5% FBS, 50 μg/ml ascorbic acid, 1 μM dexamethasone, and 3 mM β-glycerophosphate with myricetin (3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl-4-chromenone) at different concentrations (0.01 μM to 1 μM) every other day.

Myricetin was dissolved in dimethyl sulfoxide (DMSO); the final culture concentration of DMSO did not exceed 0.1% in any of the experiments. DMSO at a concentration of 0.1% was used as the control.

2.3. Cell proliferation analysis

For the cell proliferation analysis, hPDLSCs were seeded onto 96-well plates at a density of 5×10^3 cells/well. After 48 h incubation, the cells were treated with myricetin (0, 0.01, 0.1, 1, 5, 10, 20 μM) for various times (0, 12, 24, 48 h), and the proliferation was evaluated using a Cell Counting Kit-8 (Dojindo Molecular Technologies, ml) according to the manufacturer's protocol.

2.4. Alkaline phosphatase (ALP) activity assay

The cells were washed twice with cold phosphate-buffered saline and lysed on days 7 and 14 with lysis buffer containing 50 mM Tris-HCl (pH 7.0, 1% (v/v) Triton X-100, 1 mM of phenylmethane sulfonyl fluoride (PMSF), and a protease inhibitor cocktail tablet. The protein content was then quantified using the Bradford method (Bradford, 1976). The cell lysates were assayed by adding 200 μl of p-nitrophenyl phosphate (pNPP) as a substrate (Sigma) for 30 min at 37 °C. The reaction was stopped by the addition of 3 N sodium hydroxide (NaOH), and the absorbance was read spectrophotometrically at 405 nm. The enzyme activity was expressed as mM/100 μg of protein.

2.5. Alizarin red S staining of mineral deposition

For alizarin red S staining, the osteogenic media were removed and the cells were fixed for 30 min in 4% paraformaldehyde at room temperature, washed twice with deionized water, stained with 2% alizarin red solution (Sigma) for 10 min, and observed under a light microscope. For matrix calcification estimates, the stain was solubilized in 10 mM sodium phosphate buffer (pH 7.0) with 1 ml 10% (w/v) cetylpyridinium chloride. The absorbance of the solubilized stain was subsequently measured using a spectrophotometer.

2.6. RNA isolation and quantitative real-time polymerase chain reaction (PCR)

After the 4 and 7 day of myricetin treatment, total RNA was harvested from the hPDLSCs using easy-BLUE reagent (iNtRON, Seongnam, Republic of Korea), according to the manufacturer's instructions. The cDNAs were synthesized from 5 μg of RNA using a cDNA synthesis kit (TaKaRa, Tokyo, Japan). After cDNA synthesis, quantitative real-time PCR was performed with 2 μl of SYBR Premix Ex Taq (TaKaRa) using an ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction mixtures were incubated for an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 60 s, and 72 °C for 30 s. The expression level of the target gene was normalized to β-actin, which was the reference gene. The primer sequences are shown in Table 1.

2.7. Western blot analysis

To investigate the signaling pathways affected by myricetin, hPDLSCs were incubated with various concentrations of myricetin (0.01, 0.1, 1 μM) for 7 days, washed twice with cold PBS, and lysed in lysis buffer as described in Section 2.4. Equal amounts of proteins were separated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (8%) and transferred to polyvinylidene fluoride (PVDF) membranes. The transferred membranes were incubated for 1 h in blocking solution [5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T)] at room temperature and immunoblotted with primary antibodies at a dilution of 1:1000 at 4 °C overnight. The membranes were washed with TBST for 1 h, incubated with horseradish peroxidase-conjugated secondary antibodies used at a dilution of 1:5000 for 2 h, and then washed with TBST for 1 h. The