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Research Article

Glycyrrhiza uralensis (licorice) extracts increase cell proliferation and bone marker enzyme alkaline phosphatase activity in osteoblastic MC3T3-E1 cells*

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ABSTRACT

Purpose: The *Glycyrrhiza uralensis* species (Leguminosae) as a medicinal biocompound, and one of its root components, isoliquritigenin (ISL), which is a flavonoid, has been reported to have anti-tumor activity *in vitro* and *in vivo*. However, *its* function in bone formation has not been studied yet. In this study, we tested the effect of *Glycyrrhiza uralensis* (ErLR) and baked *Glycyrrhiza uralensis* (EdLR) extracts on osteoblast proliferation, alkaline phosphatase (ALP) activity, and bone-related gene expression in osteoblastic MC3T3-E1 cells. **Methods:** MC3T3-E1 cells were cultured in various levels of ErLR (0, 5, 10, 15, 20 μg/mL), EdLR (0, 5, 10, 15, 20 μg/mL), or ISL (0, 5, 10, 15, 20 μM) in time sequences (1, 5, and 20 days). Also, isoliquritigenin (ISL) was tested for comparison to those two biocompound extracts. **Results:** MTT assay results showed that all three compounds (ErLR, EdLR, and ISL) increased osteoblastic-cell proliferation in a concentration-dependent manner for one day. In addition, both ErLR and EdLR compounds elevated the osteoblast proliferation for 5 or 20 days. Extracellular ALP activity was also increased as ErLR, EdLR, and ISL concentration increased at 20 days, which implies the positive effect of *Glycyrrhiza* species on osteoblast mineralization. The bone-related marker mRNAs were upregulated in the ErLR-treated osteoblastic MC3T3-E1 cells for 20 days. Bone-specific transcription factor Runx2 gene expression was also elevated in the ErLR- and EdLR-treated osteoblastic MC3T3-E1 cells for 20 days. **Conclusion:** These results demonstrated that *Glycyrrhiza uralensis* extracts may be useful for preventing osteoporosis by increasing cell proliferation, ALP activity, and bone-marker gene expression in osteoblastic cells.

KEY WORDS: *Glycyrrhiza uralensis*, MC3T3-E1 cells, cell proliferation, ALP activity, Runx2

Introduction

Bone is remodeled continuously during adulthood and it is composed of a tightly coupled group of osteoclasts and osteoblasts that sequentially carry out resorption of old bone and formation of new bone.[1] These two closely coupled events are responsible for renewing the skeleton, and any imbalance between bone resorption and bone formation leads to abnormal bone metabolism.[2] Bone loss with increasing age induces osteoporosis, and this loss is sometimes due to increased bone resorption and decreased bone formation.[3] Osteoblasts are the cells responsible for bone formation, and they originate from multipotent mesenchymal progenitors that replicate as undifferentiated cells but can differentiate into different lineages of mesenchymal tissues, including bone, cartilage, fat, muscle, and marrow stroma.[4,5] Osteoblasts express high levels of alkaline phosphatase and osteocalcin, which are the enzyme for inorganic phosphate for mineralization and the component of extracellular matrix, respectively, and the level of these proteins in blood reflects the rate of bone formation.[6] Osteoblasts control bone formation not only by synthesizing bone-matrix proteins and regulating extracellular mineralization, but also by orchestrating the process of bone resorption by modulating receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) expression.[4,7]

The *Glycyrrhiza uralensis* species (Leguminosae) is roots of licorice that have been widely used as supplementary treatments in both traditional herbal medicine and modern medicine.[8,9] It has been reported that *Glycyrrhiza* plants produce not only glycyrrhizin but also many saponins, alkaloids, polysaccharides, polyamines, andflavonoids.[10] Many species-specific flavonoids were also reported in

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the underground parts of *Glycyrrhiza* species.[10-13] In oriental medicine, it has pharmaceutical functions, including detoxification, antiinflammation, antiviral, antiatherogenic, anticarcinogenic, and antioxidant activities.[14-18] It has been reported that liquiritigenin isolated from *Glycyrrhiza uralensis* accelerated the growth and differentiation of osteoblasts.[19] However, the potential function of *Glycyrrhiza uralensis* Fisch in bone formation has not been evaluated. Therefore, we assessed the potential function of *Glycyrrhiza uralensis* extracts on osteoblast function in osteoblastic MC3T3-E1 cells.

In this study, we investigated whether *Glycyrrhiza uralensis* extracts can improve osteogenic function through osteoblast proliferation, bone-related mRNAs expression, ALP activity and bone-specific transcription factor modulation. Therefore, we tested the effect of *Glycyrrhiza uralensis* ethanol extracts (ErLR) and baked *Glycyrrhiza uralensis* Fisch ethanol extracts (EdLR) on osteoblast cell proliferation and alkaline phosphatase (ALP) activity, bone-related mRNAs (ALP and COLI) and bone-specific transcription factor Runx2 expression in MC3T3-E1 cells, a mouse osteoblast-cell line. In addition, we tested isoliquritigenin (ISL), which is a flavonoid from *Glycyrrhiza uralensis*, as the positive control.

Methods

Cell culture

The MC3T3-E1 subclone 4 (high osteoblast differentiation, ATCC, CRL-2593) was purchased from the American Type Culture Collection. MC3T3-E1 cells were grown in a humidified incubator under 95% air and 5% CO_2 at 37°C in α-MEM with 10% FBS, 1 mM sodium pyruvate, and 1% penicillin and streptomycin, as previously described.[20] At 80% confluence, the cells were cultured in osteoblastic differentiation media [growth media plus 10 mmol/L β-glycerolphosphate (Sigma) and 50 μg/mL L-ascorbic acid (Sigma)]. ErLR (0, 5, 10, 15, 20 μg/mL) ethanol extracts or EdLR (0, 5, 10, 15, 20 μg/mL) ethanol extracts or ISL (0, 5, 10, 15, 20 μM) was added to each culture. The medium was changed every three days, and the cells were harvested every 1, 5, and 20 days.

Cell proliferation

Cell proliferation was assessed by MTT assay as recently described.[20,21] Briefly, a proliferation assay was done by seeding MC3T3-E1 cells (1×10^4 cells/well in 96-well plates) and maintaining them in growth media for 24 h at 5% CO_2, 37°C. At 80% confluence, the cells were cultured with ErLR (0, 5, 10, 15, 20 μg/mL) or EdLR (0, 5, 10, 15, 20 μg/mL) or ISL (0, 5, 10, 15, 20 μM) compounds for 1, 5, and 20 days. MTT solution was removed, and filtered DMSO was added to each well, dissolving the formazan crystals. The plates were read on a Micro Elisa reader (TECAN, Austria) at 570 nm. Results were expressed in relation to control values specified for each experiment.

Alkaline phosphatase (ALP) activity assay

The activity of ALP in cellular (synthesized) or medium (secreted) was measured using *p*-nitrophenyl phosphate (pNPP) as the substrate, as previous described.[20,21] The activity of cellular ALP was expressed as nmol PNP/mg of protein/min. The activity of medium ALP was expressed as nmol PNP/mL/min.

Real-time PCR analysis

Transcription levels for ALP, collagen type 1 (COLI), and Runx2 were measured using quantitative real-time PCR (qRT-PCR) as described.[20] Total RNA was extracted from cultures by using Trizol reagent (Invitrogen, USA). Reverse transcription reactions were performed in a 20-μL reaction volume containing Taq DNA polymerase, dNTPs, Tris-HCl (pH 9.0), KCl, MgCl2, Oligo-p (dT) 1X random primer, and total RNA (1 μg) using the reaction condition specified for the reverse transcription kit (Bionner, Korea). The cDNA was stored at -20°C until future use. The resulting cDNAs were PCR-amplified by using a mixture kit (Bionner, Korea). The primer sequences were as follows:

ALP gene forward primer 5'-GCT GAT CAT TCC CAG GTT TT-3' and reverse primer 5'-CTG GGC CTG GTA GTT GTT GT-3';

COLI gene forward primer 5'-ACG TCC TGG TGA AGT TGG TC-3' and reverse primer 5'- CAG GGA AGC CTC TTT CTC CT-3';

Runx2 gene forward primer 5'-CCG GTC TCC CCC GGG TAC C-3' and reverse primer 5'- TCC TAT CTG AGC CAG ATG ACA TCC -3';

GAPDH gene forward primer 5'-TCC ACT CAC GGC AAA TTC AAC G-3' and reverse primer 5'-TAG ACT CCA CGA CAT ACT CAG C-3'.

The PCR conditions were 95°C for 2 min and then 30

cycles at 95°C for 30 s, 54°C for 45 s and 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were separated on 1.8% agarose gel, and band intensity was measured and presented by normalizing with the housekeeping gene GAPDH intensity.

Statistical analysis

Data were analyzed with SPSS 17.0 program (SPSS Inc., Chicago, IL, USA) and differences were considered significant at $p < 0.05$. Statistical analysis of the data was performed by one-way ANOVA to test the effect of different compound levels. Once significance was detected, Turkey's HSD test was used to compare difference between groups.

Results

Glycyrrhiza uralensis compound treatment and osteoblast morphological change

Firstly, to find out the effect of ErLR and EdLR compounds on the morphological change of osteoblastic MC3T3-E1 cells, cells were cultured in various concentrations of ErLR and EdLR, along with the positive control ISL for 5 and 20 days. The cell morphology showed that cell layers (5 days) and the spindle-like morphology as the typical sign of osteoblast cultures (20 days) increased as the concentration of all three compounds (ErLR, EdLR, and ISL) increased (Fig. 1).

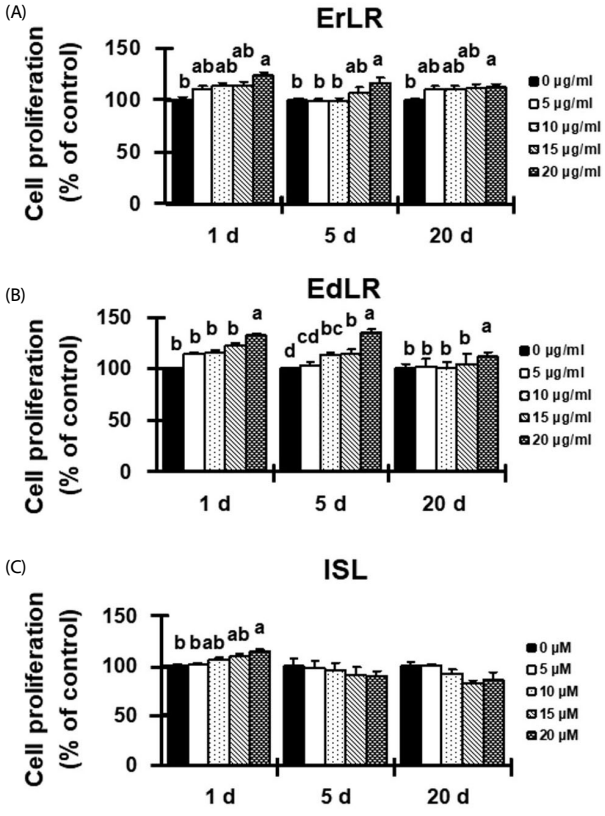

Fig. 2. Effect of ErLR (A), EdLR (B), and ISL (C) compounds on the proliferation of osteoblastic MC3T3-E1 cells. Significant differences between various treatments were found by one-way ANOVA. Labelled characters without a common letter represent significant differences from the other group(s). The values are presented as % of control at 0 h.

Glycyrrhiza uralensis compounds and osteoblast proliferation

Next, we tested whether ErLR, EdLR, and ISL compounds elevated the cell proliferation during the osteoblast proliferation period (1 ~ 5 days), as well as during the osteoblast differentiation and mineralization period (20 days) in MC3T3-El cells. ISL compound was increased in osteoblastic proliferation at day 1 (Fig. 2C). However, there were no significant changes during the osteoblast mineralization period of 5 or 20 days (Fig. 2C). The ErLR and EdLR compounds elevated osteoblastic proliferation at day 1 and the stimulation of proliferation by ErLR and

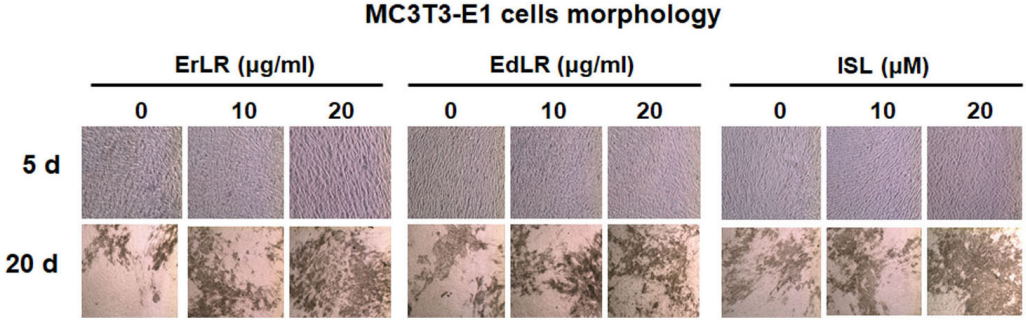

Fig. 1. Morphological characteristics of osteoblastic MC3T3-E1 cells treated with various ErLR, EdLR, and ISL compounds. Photographs were taken with a phase-contrast microscope at 100 × magnification.

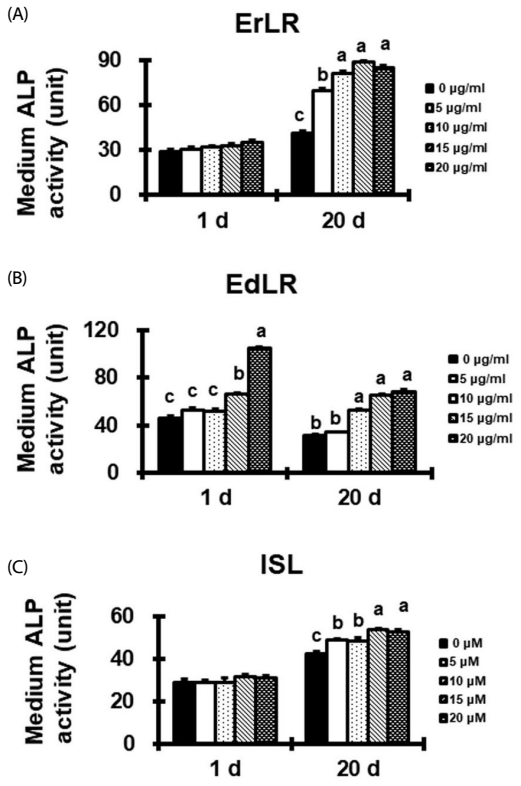

Fig. 3. Effect of ErLR (A), EdLR (B), and ISL (C) compounds on the medium ALP activity of osteoblastic MC3T3-E1 cells. Significant difference between treatments were found by one-way ANOVA. Labelled characters without a common letter represent significant differences from the other group(s) at $p < 0.05$.

EdLR extended up to 20 days (Fig. 2A and B). These results suggested that *Glycyrrhiza uralensis* extracts stimulated the elevation of osteoblast proliferation in MC3T3-El cells.

Glycyrrhiza uralensis compounds and secreted ALP activity

ALP activity as the indication of producing inorganic phosphatate for extracellular matrix mineralization is a typical representative for osteoblast differentiation and bone formation by osteoblasts. To investigate the effect of ErLR, EdLR, and ISL compounds on the differentiation of osteoblastic MC3T3-E1 cells, we measured the activity of ALP during the osteoblast proliferation, differentiation and mineralization period (1 to 20 days).

Cellular (synthesized) ALP activity was not changed in ErLR, EdLR, and ISL compound-treated MC3T3-El cells for 1 or 20 days (data not shown). Medium (secreted) ALP activity was increased by ErLR and EdLR compounds within the concentration range of 5 to 20 μg/mL during the 1 or 20 days, compared to the control (0 μg/mL) (Fig. 3). Our results showed that *Glycyrrhiza uralensis* extracts elevated the secreted ALP activity, leading to osteoblast differentiation and extracellular matrix mineralization in MC3T3-El cells.

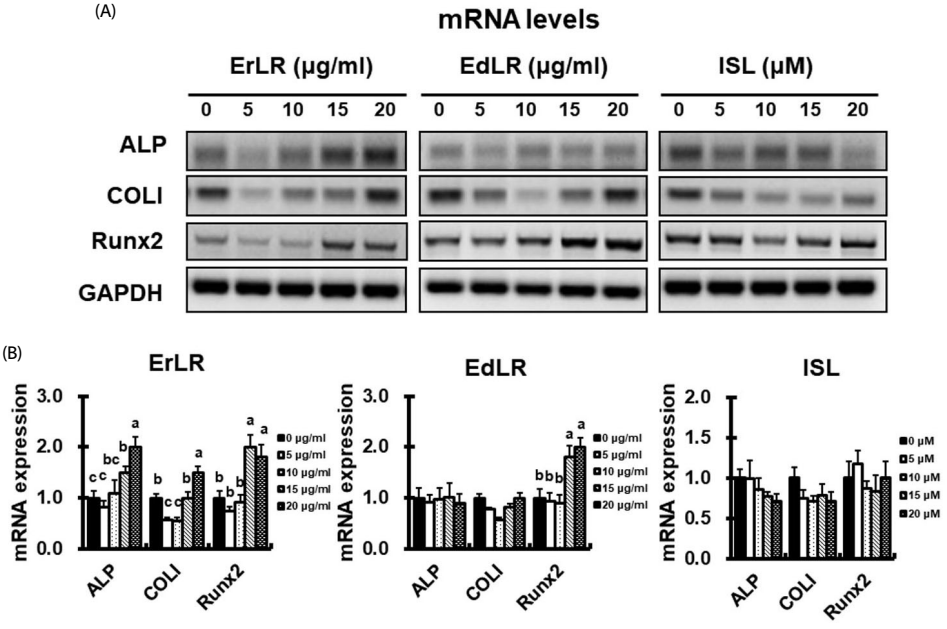

Fig. 4. Gene expression of bone-marker genes (ALP and COLI) and transcription factor Runx2 in osteoblastic MC3T3-E1 cells treated with various ErLR, EdLR, and ISL compounds for 20 days. (A-B) mRNA transcription level of bone markers was measured using quantitative real-time PCR (qRT-PCR). Significant difference between treatments were found by one-way ANOVA. Labelled characters without a common letter represent significant differences from the other group(s).

Glycyrrhiza uralensis compounds and bone-related gene expression

Here, we investigated whether ErLR, EdLR, and ISL compounds modulate the bone-related gene expression (alkaline phosphatase as ALP and collagen type I as COLI) and Runx2 as a transcription factor that is associated with osteoblast differentiation in osteoblastic MC3T3-E1 cells. We observed that gene expression of ALP and COLI was significantly elevated by the ErLR compound treatment within the concentration range of 15 to 20 μg/mL for 20 days as compared to the control (0 μg/mL) (Fig. 4). Interestedly, Runx2 mRNA expression was upregulated by both ErLR and EdLR compounds within the concentration range of 15 to 20 μg/mL for 20 days (Fig. 4). However, the ISL compound did not change bone-related gene and Runx2 expression (Fig. 4). These results showed that *Glycyrrhiza uralensis* extracts upregulated the expression of bone-related genes (ALP and COLI) and Runx2 which is the transcription factor for osteoblast differentiation in MC3T3-E1 cells.

Discussion

Recently, with the advent of global interest in complementary and alternative medicinal foods, various natural biocompounds serve as a viable source to offer benefits for the improvement and maintenance of bone health.[21] There have been many nutraceutical and pharmacological studies, both *in vitro* and *in vivo*, demonstrating that a wide variety of natural products might have beneficial effects by maintaining or promoting bone health.[22] The Choi group reported that liquiritigenin isolated from *Glycyrrhiza uralensis* caused elevation of cell growth, ALP activity, collagen synthesis, and glutathione content in osteoblast.[19] In the present study, we investigated the effects of both *Glycyrrhiza uralensis* (ErLR) and baked *Glycyrrhiza uralensis* (EdLR) compounds on cell proliferation, ALP activity, and bone-related gene expression in osteoblastic MC3T3-E1 cells. Also, we used isoliquritigenin (ISL) which is a flavonoid from *Glycyrrhiza uralensis*, as the positive control.

In this study, we found that ErLR and EdLR extracts markedly increased osteoblast proliferation, the secreted ALP activity, and bone-related genes (ALP and COLI) and Runx2 expression in osteoblastic MC3T3-E1 cells. Our findings support the potential function that *Glycyrrhiza uralensis* Fisch ethanol extracts increased the osteogenic effect in osteoblastic MC3T3-E1 cells, and these stimulated osteogenic functions are mediated by increasing osteoblast proliferation and osteoblast differentiation. Proliferation, extracellular matrix maturation, and mineralization are three sequential processes in the differentiation of osteoblasts.[23] It is known that pre-osteoblastic cells produce proteins for the extracellular matrix, including type I collagen, and then successively produce ALP during differentiation for mature osteoblasts. As the final stage of bone formation, osteoblasts deposit calcium and increase late bone-marker proteins, such as osteocalcin.[24] In fact, ALP is present in osteoblast plasma membranes, as well as within and outside osteoblasts, and plays an important role in bone formation and mineralization. Bellows *et al.* reported that ALP is one of the osteoblast differentiation markers as an essential enzyme for mineralization.[25] Our data showed that ErLR and EdLR compounds increased the osteoblast proliferation and ALP activity in media, which is the enzyme activity in secretion outside osteoblasts during osteoblast differentiation periods (1, 5, and 20 days). In addition, ISL compound also elevated the osteoblast proliferation for 1 day and the ALP activity in secretion for 20 days. Therefore, these results suggest that *Glycyrrhiza uralensis* extracts increased the osteoblast proliferation and the secreted ALP activity, which is a critical factor for osteoblast differentiation as well as extracellular mineralization. However, *in vivo* studies are needed to confirm these effects for future study.

The transcription factor Runx2 is a critical regulator of osteoblast differentiation.[26] Runx2 determines the osteoblast lineage from multipotent mesenchymal cells, induces osteoblastic differentiation at the early stage, but inhibits it at the late stage.[26,27] Further, Runx2 has been shown to induce ALP activity, expression of bone-matrix protein genes, and mineralization in immature mesenchymal cells and osteoblastic cells.[28-30] We found that Runx2 mRNA expression was increased by ErLR and EdLR compounds within the concentration range of 15 to 20 μg/mL for 20 days. Additionally, ALP and COLI were elevated by the ErLR compound within the concentration range of 15 to 20 μg/mL for 20 days. Therefore, these results demonstrate that *Glycyrrhiza uralensis* extracts modulated both the transcription factor Runx2 and bone-related genes, compared to ISL as the positive control in osteoblastic MC3T3-E1 cells.

Summary

Taken all together, our findings imply that *Glycyrrhiza uralensis* extracts (1) increased the proliferation of osteoblasts, (2) stimulated ALP activity, and (3) upregulated gene expression of bone-related genes (ALP and COLI) and the Runx2 transcription factor, which triggers osteoblastic differentiation in MC3T3-E1 cells. These findings imply that *Glycyrrhiza uralensis* extracts may be good candidate for protecting against of osteoblast dysfunction.

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