Isoliquiritigenin Inhibits Metastatic Breast Cancer Cell-induced Receptor Activator of Nuclear Factor Kappa-B Ligand/Osteoprotegerin Ratio in Human Osteoblastic Cells

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Bone destruction induced by the metastasis of breast cancer cells is a frequent complication that is caused by the interaction between cancer cells and bone cells. Receptor activator of nuclear factor kappa-B ligand (RANKL) and the endogenous soluble RANKL inhibitor, osteoprotegerin (OPG), directly play critical roles in the differentiation, activity, and survival of osteoclasts. In patients with bone metastases, osteoclastic bone resorption promotes the majority of skeletal-related events and propagates bone metastases. Therefore, blocking osteoclast activity and differentiation via RANKL inhibition can be a promising therapeutic approach for cancer-associated bone diseases. We investigated the potential of isoliquiritigenin (ISL), which has anti-proliferative, anti-angiogenic, and anti-invasive effects, as a preventive and therapeutic agent for breast cancer cell-induced bone destruction. ISL at non-toxicity concentrations significantly inhibited the RANKL/OPG ratio by reducing the production of RANKL and restoring OPG production to control levels in hFOB1.19 cells stimulated with conditioned medium (CM) of MDA-MB-231 cells. In addition, ISL reduced the expression of cyclooxygenase-2 in hFOB1.19 cells stimulated by CM of MDA-MB-231 cells. Therefore, ISL may have inhibitory potential on breast cancer-induced bone destruction.

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Key Words: Isoliquiritigenin, Breast cancer, Bone metastasis, RANK ligand, Osteoblastic cells

INTRODUCTION

Bone metastasis from breast cancer leads to severe bone destruction through the interaction between cancer cells and bone cells.¹ Osteolytic bone destruction significantly decreases the survival rate of patients with metastatic breast cancer.² Receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) are key molecules in bone-resorbing osteoclast differentiation and activation. RANKL from osteoblasts binds to receptor activator of nuclear factor kappa-B (RANK) on the surface of preosteoclasts and promotes the maturation of osteoclasts. OPG secreted by osteoblasts obstructs the binding of RANKL and RANK. Whereas a balance between RANKL and OPG is maintained in normal physiology, various cytokines or chemokines secreted by breast cancer cells disrupt the balance between RANKL and OPG.³ Consequently, the number of bone-resorbing osteoclasts and bone destruction are abnormally increased in patients with metastatic breast cancer.⁴ Therefore, RANKL can be a strategic therapeutic target for patients with metastatic breast cancer.⁵

Isoliquiritigenin (ISL) (Fig. 1A) is a flavonoid derived from licorice and shallot.⁶ ISL has diverse pharmacological activities such as anti-inflammatory, anti-diabetic, anti-angiogenic, and anti-osteoclastic properties.⁷⁻¹⁰ In addition, ISL has strong anti-cancer effects in various types of cancers.¹¹⁻¹³ In breast cancer, ISL inhibits the metastasis of human breast cancer cells through...
Figure 1. The effect of isoliquiritigenin (ISL) on the receptor activator of nuclear factor kappa-B ligand (RANKL)/osteoprotegerin (OPG) ratio in hFOB1.19 cells treated with conditioned medium (CM) of MDA-MB-231 cells. (A) Chemical structure of ISL. (B) hFOB1.19 cells were cultured with indicated concentrations of ISL for 24 hours and 48 hours. The cell viability was analyzed by the MTT assay. Data are expressed as the means ± SE. *P < 0.01 vs. untreated cells. (C) hFOB1.19 cells were treated with CM and ISL at noncytotoxic concentrations for 24 hours. RANKL and OPG levels in the culture media were determined using commercially available ELISA kits. Data are expressed as the means ± SE. †P < 0.01 vs. control; ‡P < 0.05; ††P < 0.01 vs. hFOB1.19 cells treated with CM of MDA-MB-231 cells. (D) hFOB1.19 cells were incubated with CM of MDA-MB-231 cells and ISL (1-10 μM) for 48 hours. Then, the cells were harvested and analyzed by fluorescence activated cell sorter (FACS) as described in Materials and Methods. MFI, mean fluorescence intensity; C, control.
preventing anoikis resistance, as well as the migration and invasion of BT-549 and MDA-MB-231 cells. ISL also induces growth inhibition and apoptosis through downregulation of the arachidonic acid metabolic network and the deactivation of PI3K/Akt in MCF-7 and MDA-MB-231 cells.

In the present study to verify the potential of ISL as a promising agent for control of breast cancer bone metastasis, we investigated whether ISL could affect RANKL and OPG expression in osteoblastic cells stimulated with conditioned medium (CM) of metastatic breast cancer cells.

**MATERIALS AND METHODS**

1. **Materials**

   ISL, vitamin C (ascorbic acid), vitamin D₃, vitamin K₃, MTT, dimethylsulfoxide, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Leibovitz's L-15 (L-15) medium, Dulbecco's Modified Eagle's Medium:Nutrient Mixture F-12 (Ham) (1:1) (DMEM/F12), FBS, and antibiotics were purchased from Gibco BRL (Grand Island, NY, USA). Monoclonal antibody (mAb) against human RANKL was obtained from R&D Systems (Minneapolis, MN, USA), and polyclonal anti-human COX-2 antibody was purchased from Cayman Chemical (Ann Arbor, MI, USA). Normal goat immunoglobulin G (IgG) and normal rabbit IgG, as well as an enhanced chemiluminescence (ECL) kit were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

2. **Cell culture**

   MDA-MB-231 human breast cancer cells and hFOB1.19 human fetal osteoblast cells were obtained from American Type Culture Collection (Manassas, VA, USA). MDA-MB-231 cells were cultured in L-15 supplemented with 10% FBS and 1% penicillin/streptomycin. hFOB1.19 cells were maintained DMEM/F12 with 10% FBS and 1% penicillin/streptomycin, and, at 80% to 90% confluence, cultured in differentiation medium for an additional 2 to 3 days prior to treatment with CM of breast cancer cells and/or ISL. Differentiation medium consisted of DMEM:F-12 (1:1), 50 μg/mL vitamin C, 10⁻³ M vitamin D₃, 10⁻⁶ M vitamin K₃, and 10% charcoal-stripped FBS to minimize exposure to hormones and growth factors.

3. **MTT assay**

   hFOB1.19 osteoblasts were seeded into 96-well culture plates at a density of 1 x 10⁴ cells/well. Twenty-four hours later, hFOB1.19 cells were cultured for 24 hours and 48 hours in respective serum-free media with various concentrations of ISL. Cell viability was measured using an MTT assay. The absorbance was determined at 570 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

4. **Preparation of conditioned medium derived from MDA-MB-231 cells**

   MDA-MB-231 cells (1 x 10⁶ cells) were plated in T75 culture flasks with 10 mL DMEM:F-12 (1:1) supplemented with 10% FBS, and incubated at 37°C. When the cells were nearly confluent, the medium was changed to serum-free DMEM:F-12 (1:1). After 24 hours incubation, the medium was collected as CM, aliquoted, and frozen at −20°C. For the following experiments, 70% CM in differentiation medium was used.

5. **ELISA**

   hFOB1.19 cells were cultured in 96-well plates (1 x 10⁵ cells/well) in DMEM/F12 containing 10% FBS for 24 hours and then treated with 70% CM of MDA-MB-231 cells and ISL (0.1-10 μM). After 24 hours incubation, cultured medium of the hFOB1.19 cells was collected. The RANKL and OPG protein levels in the collected medium were quantified using commercially available ELISA kits (EIAab, Guangguguojii, China) according to the manufacturer’s protocols.

6. **Flow cytometric (fluorescence activated cell sorter) analysis**

   hFOB1.19 cells (1 x 10⁵ cells) were treated with 70% CM and ISL at the indicated concentrations for 48 hours. The cells were detached with 2 mM EDTA and subsequently incubated in phosphate-buffered saline (PBS) with 1% BSA on ice for 30 minutes. Aliquots (2 x 10⁶ cells) were incubated with 100 μL RANKL mAb (10 μg/mL) on ice for 1 hour. The cells were washed twice with Mg²⁺- and Ca²⁺-free PBS with 1% BSA and resuspended in Mg²⁺- and Ca²⁺-free PBS containing 1% BSA and a 1:50 dilution of fluorescein isothiocyanate-conjugated IgG. After incubation with secondary antibody for 1 hour at 4°C, the cells were washed again. Ten thousand events were stored as list mode data for further analysis using WinMDI software (Windows Multiple Document Interface Flow Cytometry Application. 1993-1998 Joseph Trotter).

7. **Reverse transcription-PCR**

   hFOB1.19 cells (1 x 10⁵ cells) were treated with 70% CM and ISL for 6 hours. Total RNA from hFOB1.19 cells was extracted using the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). Single stranded cDNA was transcribed from the RNA (2 μg) using
Promega’s reverse transcription system (Madison, WI, USA). PCR was carried out in a reaction mixture containing cDNA (2 μg), MgCl₂ (25 mM), dNTPs (10 mM), primers (1 pmol), and Taq polymerase (1 unit) (Takara, Shiga, Japan) with the following primers: COX-1, forward 5’-CATCCTCGACGGCATCTCAGC-3’; reverse 5’-TTGGGTCAGGGGTGGTTATTG-3’; COX-2, forward 5’-ATGACTTCAAAGCTGGCCGT-3’; reverse 5’-CCTCTTCAAAAAACTTCTCCACACC-3’; GAPDH, forward 5’-GTCAGTGGTGGACCTGACTC-3’; reverse 5’-AGGGGTCTACATGGCAACTG-3’. The amplification consisted of 30 cycles with an annealing temperature of 63°C for COX-1, 57.5°C for COX-2 and 52°C for GAPDH. The PCR products were electrophoresed, and the detected bands were analyzed with the TINA program ver. 2.10e (Raytest. Straubenhardt. Germany).

8. Western blotting

hFOB1.19 cells (1 × 10⁶ cells) were cultured in media containing 70% CM and/or ISL at the indicated concentration for 6 hours. and were lysed with the radioimmunoprecipitation assay buffer. The protein concentration was measured with a bicinchoninic acid (BCA) kit (Pierce. Rockford. IL. USA). Equal amounts of protein (40 μg) were loaded onto a gel for SDS-PAGE. The blots were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) and then incubated with the primary antibodies for COX-2 (1:1,000) and β-actin (1:2,000) in 3% skim milk in TBST for 24 hours at 4°C. After washing, the blots were incubated for 1 hour with secondary antibody coupled to horseradish peroxidase (1:2,000) and visualized with the ECL kit.

9. Statistical analysis

Statistical analysis was performed with SPSS statistical software ver. 21 (IBM. Endicott. NY. USA). Data are expressed as mean ± SE. Data were analyzed by means of the Student’s t-test to express differences between the two groups. Results with values of $P < 0.05$ and $P < 0.01$ were considered statistically significant.

RESULTS

1. Isoliquiritigenin inhibited the receptor activator of nuclear factor kappa-B ligand/osteoprotegerin ratio in osteoblastic cells stimulated with conditioned medium of MDA-MB-231 breast cancer cells

We first examined the cytotoxicity of ISL on hFOB1.19 human osteoblastic cells. When hFOB1.19 cells were exposed to various concentrations of ISL, the viability of hFOB1.19 cells was suppressed at 20 μM by 17% with 24 hours treatment and by 47% with 48 hours treatment (Fig. 1B). Next, we analyzed the secreted levels of soluble RANKL and OPG in hFOB1.19 cells treated with CM of MDA-MB-231 cells and ISL at noncytotoxic concentrations, using ELISA kits. ISL treatment significantly inhibited the secreted levels of RANKL and rescued those of OPG into the culture medium of osteoblastic cells stimulated with CM of MDA-MB-231 cells. Consequently, the elevated RANKL/OPG ratio by CM of MDA-MB-231 cells was inhibited by ISL treatment in a dose-dependent manner (Fig. 1C). In addition, we detected the expression level of membrane-bound RANKL in hFOB1.19 cells using fluorescence activated cell sorter analysis. CM of breast cancer cells increased the membrane-bound RANKL expression, but ISL reduced the CM-induced level of membrane-bound RANKL by 37% at 10 μM (Fig. 1D).

2. Isoliquiritigenin inhibited the expression level of COX-2 in conditioned medium-stimulated osteoblastic cells

COX-2 is one of the well-known molecules that regulate the expression of RANKL.17,18 Thus, we further examined the effect of ISL on the expression of COX-2 in CM-treated hFOB1.19 cells. Reverse transcription (RT)-PCR data showed that CM of MDA-MB-231 cells increased the mRNA expression of COX-2, but ISL at non-cytotoxic concentrations blocked its CM-induced mRNA expression in hFOB1.19 osteoblastic cells. COX-1 mRNA expression was not changed significantly by either ISL or CM of MDA-MB-231 cells in hFOB1.19 cells (Fig. 2A). Western blot analysis also indicated that ISL significantly inhibited the protein levels of COX-2 in hFOB1.19 cells exposed to CM of MDA-MB-231 cells (Fig. 2B).

DISCUSSION

Bone metastasis is frequently observed in patients with advanced breast cancer, and the mortality rate is significantly higher in patients with bone metastasis than in patients without bone metastasis.2 Metastatic breast cancer cells metastasize to bone and secrete various osteolytic factors.1 These factors stimulate osteoblasts that are important regulators of bone metabolism and abnormally increase the expression of RANKL, which is a key promoting factor for osteoclast differentiation and activation.19 Consequently, excessive bone resorption by osteoclasts is rapidly increased in patients with metastatic breast
Figure 2. The effect of isoliquiritigenin (ISL) on mRNA and protein expressions of COX-2 in hFOB1.19 cells stimulated by conditioned medium (CM) of MDA-MB-231 cells. (A) hFOB1.19 cells were incubated with CM of MDA-MB-231 cells and ISL (1-10 μM) for 1 hour. The mRNA expression of COX-1 and COX-2 was analyzed by reverse transcription-PCR. (B) hFOB1.19 cells were incubated with CM of MDA-MB-231 cells and ISL (1-10 μM) for 6 hours. Proteins were extracted and subjected to western blotting using the specific antibody against COX-2. Data are expressed as the means ± SE, aP < 0.01 vs. control; bP < 0.05, cP < 0.01 vs. hFOB1.19 cells treated with CM of MDA-MB-231 cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; C, control.

cancer. Several growth factors released from the bone matrix by osteoclast-mediated bone resorption contribute to the proliferation and survival of cancer cells. These amplified interactions between breast cancer cells and bone cells, referred to as a 'vicious cycle', make the condition difficult to treat. Therefore, controlling osteoblastic RANKL expression in the bone microenvironment with breast cancer metastases can be a promising strategy for the prevention and treatment of cancer-associated bone loss.

ISL has potent anti-inflammatory, anti-angiogenic, and anti-cancer effects. In particular, ISL prevents the metastatic potential of breast cancer cells through inhibition of matrix metalloproteinase activities and the PI3K/Akt signaling pathway. In our previous study, we found that ISL inhibited RANKL-induced osteoclastogenesis. The RANKL and OPG from osteoblasts are important molecules in the differentiation of osteoclasts. Thus, we further found that ISL at non-cytotoxic concentrations inhibited the RANKL/OPG ratio by blocking the expression of soluble and membrane-bound RANKL and the decrease in OPG production in human osteoblastic cells stimulated with CM of metastatic breast cancer cells.

COXs are the enzymes that mediate the conversion of arachidonic acid to prostaglandins (PGs). Whereas COX-1 is expressed constitutively in most tissues and plays an important role in the maintenance of homeostasis, COX-2 is an inducible enzyme contributing to the elevated production of PGs in inflammation and cancer. Recent studies have demonstrated that COX-2 expression regulates the production of PGE2, which is one of the osteolytic factors involved in RANKL expression in osteoblasts. Mice lacking COX-2 showed reduced bone resorption in response to parathyroid hormone. PGE2 produced by cancer cells or osteoblasts binds to the osteoblastic EP4 receptor, which is one of the PGE receptor subtypes, and induces RANKL expression to stimulate bone resorption. Additionally, COX-2 has been known to be involved in breast cancer bone metastasis. In our study, ISL downregulated the expression levels of COX-2 mRNA and protein elevated by CM of MDA-MB-231 cells in hFOB1.19 cells.

Taken together, ISL inhibited the RANKL/OPG ratio and COX-2 expression in human osteoblast hFOB1.19 cells stimulated with...
CM of metastatic breast cancer MDA-MB-231 cells. Thus, ISL can be a beneficial agent to inhibit and treat breast cancer cell-associated bone diseases by blocking the interaction between cancer cells and bone cells via the inhibition of osteoblastic RANKL expression.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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