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LC, a novel estrone–rhein hybrid compound, promotes proliferation and differentiation and protects against cell death in human osteoblastic MG-63 cells

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ABSTRACT

Estrogen analogs are promising drugs for postmenopausal osteoporosis, but because of their possible side effects, estrogens which exert their estrogenic effects selectively on bone are desired. Based on our previous studies that rhein had high affinity for the bone mineral, we synthesized estrone–rhein hybrid compounds and confirmed that one of these hybrid compounds, LC, exhibited a selective profile in the bone and prevented bone loss but had no effect on endometrium growth in ovariectomized rats. However, the mechanisms underlying its actions on human bone cells have remained largely unknown. Here we show that LC increases proliferation and differentiation and opposes cisplatin-induced apoptosis in human osteoblastic MG-63 cells containing two estrogen receptor (ER) isoforms. LC promotes proliferation by altering cell cycle distribution whereas LC-mediated survival may be associated with up-regulation of X-linked inhibitor of apoptosis (XIAP) expression. Treatment with the ER antagonist ICI 182,780 abolishes the above actions of LC on osteoblast-derived cells. Using small interfering double-stranded RNAs technology, we further demonstrate that the effects of LC on proliferation and survival are mediated by both ERα and ERβ but those on differentiation primarily by ERα. Moreover, we demonstrate that LC may promote activation of the classic estrogen response element (ERE) pathway through increasing steroid receptor coactivator (SRC)-3 expression. Meanwhile, we find that regulation of osteoblastic proliferation and survival by LC involves Ras/MEK/ERK and PI3K/Akt signaling. Therefore, using rhein for conjugating compounds is a promising method of effectively targeting estrogens to the bone.

1. Introduction

Estrogen analogs are promising drugs for postmenopausal osteoporosis, but because of their possible side effects such as increased risk of cancer, estrogens which exert their estrogenic effects selectively on bone are desired. To target the drugs to the bone effectively, tetracycline (Orme and Labroo, 1994; Neale et al., 2009), bisphosphonate (Bauss et al., 1996; Tsushima et al., 2000), small heterocycles (Willson et al., 1996), and acidic oligopeptide (Yokogawa et al., 2001; Yokogawa, 2006), which have high affinity for the bone mineral, are used as carriers. Rhein, an anthraquinone compound, can be found in the rhizome of rhubarb, a traditional Chinese medicine herb. Recently, we found that rhein also has high affinity for the bone mineral (Cui et al., 2008; Zhang et al., 2008). In addition, studies have shown that rhein itself can inhibit osteoclast formation and bone resorption activity (Wang et al., 2006; Boileau et al., 2008), suggesting that rhein and estrogen may play potential synergistic roles in the anti-osteoporosis. Furthermore, several recent studies have addressed the antitumor role of rhein in several types of cancers (Lin and Zhen, 2009; Ip et al., 2007; Lin et al., 2009; Shi et al., 2008), indicating that rhein may have potential inhibitory effects on endometrial cancer and breast cancer caused by using estrogen replacement therapy. Therefore, we synthesized ten estrone–rhein hybrid compounds and confirmed that some target compounds including LC exhibited a selective profile in the bone (Li et al., 2004) and were able to promote long bone growth in fetal mouse *in vitro* (Li et al., 2007). Meanwhile, the further study showed that LC prevented bone loss but had no effect on endometrium growth in ovariectomized rats (Zhang et al., 2010). However, the mechanisms underlying its actions on human bone cells have remained largely unknown.

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Estrogen has an important role in the development and growth of bones and later in the maintenance of bone mass. It is believed that the major action of estrogen on the skeleton *in vivo* is through the inhibition of bone resorption (Riggs et al., 2002). Although some of the anti-resorptive effects of estrogen are via direct actions on bone-resorbing osteoclasts, estrogen has also been shown to have indirect effects by regulating bone-forming osteoblasts and bone marrow stromal cells (Zallone, 2006). As one of these effects, 17β-estradiol has been suggested to protect osteoblasts from apoptosis (Zallone, 2006; Kousteni et al., 2001; Chen et al., 2006).

The role of osteoblastic/osteocytic apoptosis in the regulation of bone metabolism has been demonstrated by several investigators (Urayama et al., 2000; Nakashima et al., 1998; Weinstein et al., 1998). Especially apoptosis of osteoblasts is currently getting more attention since it has been considered to be an important determinant of bone formation and, therefore, of skeletal integrity (Manolagas, 2000; Weinstein and Manolagas, 2000). Disorders that promote osteoblastic apoptosis are associated with increased bone fragility (Weinstein et al., 1998), and treatments that inhibit it are associated with anti-fracture efficacy (Jilka et al., 1998).

The aim of the present study was to examine whether LC affects proliferation, alkaline phosphatase (ALP) activity, collagen content and cisplatin-induced apoptosis of human osteoblastic MG-63 cells, which have been well-characterized as an *in vitro* model for osteoblast function (Luo and Liao, 2003; Feng et al., 2008) and to have both ERα and ERβ (Luo and Liao, 2003). Moreover, we also explored the mechanisms by which LC involved in the regulation of the proliferation, differentiation and survival of osteoblast-derived cells.

2. Materials and methods

2.1. Cell transfection and generation of cells stably transfected with pGenesil-ERα small hairpin RNA (shRNA), pGenesil-ERβ shRNA and pGenesil-scrambled shRNA vectors

Human osteoblastic MG-63 cell line expressing endogenous ERα and ERβ was cultured as described previously (Wang et al., 2011). MG-63 cells were transfected with pGenesil-ERα shRNA, pGenesil-ERβ shRNA or pGenesil-scrambled shRNA construct by Lipofectamine™ 2000 (Invitrogen, San Diego, CA). MG-63/ERα shRNA, MG-63/ERβ shRNA and MG-63/scrambled shRNA stable cell lines were cloned as described previously (Wang et al., 2011).

2.2. MTT assay

Cell proliferation was measured with MTT assay. Briefly, 6×10^3 cells per well were plated in 96-well plates overnight, and then switched to medium containing 1% charcoal-stripped FBS (sFBS) (Hyclone Laboratories, Inc.) and cultured for 24 h. The cells were treated daily with different concentrations (10, 100 or 1000 nM) of LC, estrone or rhein in DMSO (Sigma, St. Louis, MO). The final DMSO concentration was 0.1%. Control cells were treated with DMSO vehicle as described above. After 4 days of treatment, the cells were incubated with 100 μl of MTT solution (0.5 mg/ml, Sigma) for 4 h at 37 °C. After centrifugation, 100 μl of 0.04 M HCl–isopropanol was added. The absorbance was measured at 490 nm using ELISA microplate reader.

2.3. Flow cytometric assay

Cell cycle analysis was performed as previously described (Li et al., 2008). MG-63 cells were cultured in 6-well plates at 3×10^5 cells per well overnight, and then switched to medium containing 1% sFBS and cultured for 24 h. The cells were treated daily with LC (1000 nM) or DMSO for 24, 48, 72, or 96 h. The cells were collected, and resuspended in a propidium iodide solution containing 0.1 M propidium iodide, 0.1% Triton X-100 (Sigma), and 20% RNase A, in PBS. After incubation on ice, in the dark, for 2 h, the samples were analyzed by flow cytometry. 10,000 events were recorded and the proportion of cells in various phases of the cell cycle were analyzed using the ModFitLT DNA analysis program (Becton Dickinson, San Jose, CA).

2.4. Analysis of ALP activity and type 1 collagen secretion

ALP activity was measured using a commercial kit (Nanjing Jianchen Technologies Inc., China) according to the manufacturer's instructions. Briefly, cells were seeded in 12-well plates at a density of 4×10^4 cells per well. The following day, cells were placed in 1% sFBS-containing medium culture for 24 h, and then treated with either LC, estrone, or rhein at the concentrations indicated in the results for 2, 4, or 6 days. After the culture medium was removed, the cell layers were washed with PBS, and then sonicated for 10 sec on ice three times in 50 mM Tris–HCl, pH 7.5, with 0.1% Triton X-100. The cell lysates were collected and clarified by centrifugation as the enzyme sample. Reaction was started by adding the enzyme sample to 1 ml of 5 mM disodium phenylphosphate in 0.05 M bicarbonate buffer, pH 10.15, containing 2.25 mM 4-aminoantipyrine. After 15 min of incubation at 37 °C, reaction was stopped by adding 1.5 ml of 5.8 mM potassium ferricyanide containing 0.21 M boric acid. After mixing immediately, absorbance was measured at 520 nm. One unit of ALP activity was defined as the activity that produced 1 mg of phenol, and the activity was expressed as units/100 ml sample. The level of type 1 collagen secretion in the cell culture supernatants was analyzed using enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Senxiong Technologies Inc., China) according to the manufacturer's instructions. Relative ALP activity and type 1 collagen content were estimated by dividing the value of ALP activity (U/100 ml) or type 1 collagen content (ng/ml) by of absorbance A490 of MTT.

2.5. Quantification of apoptotic cells

Apoptotic cells were quantified by visualization of changes in nuclear morphology by DAPI staining. Cells were grown in 6-well plates (2×10^5 cells/well) with coverslips overnight. Culture media were replaced with media containing 1% sFBS for 24 h, and then pretreated with either LC (100 nM), estrone (100 nM) or DMSO. After culture for 24 h, cisplatin (10 μM) was added to cultures for 24 h to induce apoptosis. After treatment, the cells were fixed with 3% paraformaldehyde and the coverslips were mounted on the slides with Vectashield mounting medium with DAPI (Roche Applied Science, Germany). Microscopic imaging was carried out on a Zeiss Wide-Field microscope (Zeiss, Jena, Germany). Fifteen areas of 1300 × 1000 pixels in one sample were randomly selected from the image for quantification of apoptotic cells. Data of three independent experiments are presented as the percentage of dead cells for each treatment.

2.6. Semiquantitative RT-PCR

Total RNA was isolated from cells with TRIzol (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Primer sequences were designed by Vector NTI 8 software and synthesized by TaKaRa Biotechnology Co., Ltd. (Dalian, China). The primer sequences were as follow: X-linked inhibitor of apoptosis (XIAP), 5′-ATGATACCATCTTCCAAAA TCC-3′ (forward) and 5′-TTTCTGTAA TGAAGTCTGACTT-3′ (reverse), for steroid receptor coactivator (SRC)-3, 5′-GGACAGGCATTAGAGCCCAAACA-3′ (forward) and 5′-CTGCTG GCCCTGCAGCCTCTGCT-3′ (reverse), for β-actin, 5′-TGGAA