Effects of Metabolites of the Lignans Enterolactone and Enterodiol on Osteoblastic Differentiation of MG-63 Cells

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Plant lignans are converted by the intestinal microflora to the mammalian lignans enterodiol and enterolactone, which are associated with beneficial health effects in humans. The mammalian lignans enterodiol and enterolactone are believed to protect against certain diseases, e.g., breast and prostate cancer as well as coronary heart disease. In this study, the effects of enterodiol and enterolactone on osteoblastic differentiation were examined. It was found that enterolactone and enterodiol showed the same effects. They have biphasic effects on cell viability, alkaline phosphatase (ALP) activity, transcriptional level of osteocalcin, and collagen I, showing induction at low doses and inhibition at high doses. They increased transcriptional levels of ALP, osteopontin, and osteocalcin in a dose-dependent manner. The difference may be related to the estrogenic and antiestrogenic character and multiple signaling transduction of phytoestrogen.

Key words enterolactone; enterodiol; osteoblast; differentiation; biphasic

MATERIALS AND METHODS

Cell Culture MG-63 cells (purchased from the American Type Culture Collection), a human osteoblast-like cell line, were used to assess the cellular responses to enterolignans. Cells were cultured in 25 cm² flasks in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% heat-inactivated fetal bovine serum, L-glutamine 2 mM, penicillin 100 U/ml, and streptomycin 100 μg/ml. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. When digested, the cells were washed with phosphate-buffered saline (PBS, Gibco), detached with trypsin-EDTA solution (0.25% trypsin, Gibco) at 37 °C for 5 min, centrifuged, and resuspended for further cell tests.

Cytotoxicity Assay Following incubation of cells with enterolactone and enterodiol (purchased from Sigma) for specified times in 96-well plates, 20 μl of PBS containing 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) was added to each well. Plates were incubated at 37 °C for 4 h, then the medium was replaced with 150 μl of dimethyl sulphoxide (DMSO, Sigma). After 10-min incubation, absorbance in control and treated wells was measured in a plate reader at 490 nm.

Alkaline Phosphatase Test Alkaline phosphatase (ALP) activity was measured colorimetrically using di-sodium phenyl phosphate as the substrate. The enzyme ALP expressed by the cells hydrolyzed the substrate to phenol and an inorganic phosphate. Under alkaline conditions, the phenol was converted to a red product and its absorbance was subsequently measured at 520 nm using a spectrophotometer. The absorbance was directly converted to ALP activity level based on the bovine serum albumin standard level.

Reverse-Transcription Polymerase Chain Reaction Total RNA was extracted using the Trizol agent. Reverse-transcription (RT) was performed for 1 h at 42 °C with Mo-AMV reverse transcriptase 200 U, random oligohexamer primers 62.5 μmol, dNTP-Mix 10 mmol, and total RNA 2 μg. The polymerase chain reaction (PCR) was carried out in a 50-μl reaction volume using 2 μl of the previous RT reaction product, 200 ng of target primers, 25 mmol of dNTP-
Mix, and 2.5 U of Taq polymerase. PCR was performed under the following conditions: 5-min 94 °C preincubation, followed by 30 cycles of denaturation (30 s, 94 °C), annealing (1 min, 64.7 °C), and extension (1 min, 72 °C), followed by a final extension of 10 min at 72 °C. PCR products were separated on 2% agarose gel containing ethidium bromide 0.2 μg/ml. The bands were visualized by UV transillumination and analyzed using Gel-Pro Analyzer. The primer sequences are indicated in Table 1.

**Statistical Analysis** Data are expressed as means±S.D. Statistical analysis was performed with Student's t-test to express the difference between two groups. *p<0.05 was considered significant.

**RESULTS**

**Effects of Enterolactone and Enterodiol on the Cell Viability of MG-63 Cells** Enterolactone and enterodiol were prepared at concentrations of 0.01, 0.1, 1, and 10 mg/ml. Their effects on the viability of MG-63 cells were observed for 2, 4, and 7 d. It was found that the viability of MG-63 cells significantly increased when treated with 0.01 mg/ml of enterolactone or enterodiol on day 7 (*p<0.05). On days 2 and 4, enterolactone or enterodiol also increased cell viability, although the difference was not significant (Fig. 2). At higher concentrations, enterolactone or enterodiol significantly reduced the viability of MG-63 cells in a dose-dependent manner (data not shown). For example, the cell survival rate was 21% and 35% when MG-63 cells were treated with 0.1 mg/ml of enterolactone and enterodiol, respectively, for 7 d.

**Effects of Enterolactone and Enterodiol on the ALP Activity of MG-63 Cells** The activity of ALP is a typical representative of the osteogenic activity of osteoblasts. As shown in Fig. 3, enterolactone and enterodiol both increased the ALP activity of MG-63 cells at low concentrations. Enterolactone significantly increased ALP activity at the concentration of 0.01 mg/ml on day 7 and enterodiol significantly increased ALP activity at the concentration of 0.1 mg/ml on day 7 (*p<0.05). At higher concentrations, similar to their effects on the viability of MG-63 cells, enterolactone or enterodiol decreased ALP activity significantly (data not shown).

**Effects of Enterolactone and Enterodiol on Expression of Osteoblastic Markers** RT-PCR was used to demonstrate the expression of mRNA for collagen (col I), ALP, osteocalcin (OC), osteopontin (OP), and osteonectin (ON), in both enterolactone-treated and enterodiol-treated MG-63 cells on

**Table 1. The Primer Sequences of Human ERCC-1, GAPDH**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers (5′–3′)</th>
<th>Reverse primers (5′–3′)</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPN</td>
<td>CTTTCCAAAGTCAGCCGTGAATTC</td>
<td>ACAGGAGATTTCCATGAAGCCACA</td>
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<tr>
<td>Col I</td>
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<td>AATTTCTGCTGGGGGCACCC</td>
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<tr>
<td>OC</td>
<td>ATGAGGCCCTACACCTCCT</td>
<td>CTAGACCGGGCCGTAGAAGGC</td>
<td>303</td>
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<tr>
<td>ALP</td>
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<td>ATGCAGGCTGCTACGCCC</td>
<td>287</td>
<td>62.7</td>
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<tr>
<td>ON</td>
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<td>CCAAGACCTTAATGTGA</td>
<td>465</td>
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<tr>
<td>GAPDH</td>
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<td>TCCACCACCTGTGCTGTA</td>
<td>247</td>
<td>58.9</td>
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</table>

Fig. 2. Effects of Enterolactone and Enterodiol on MG-63 Cell Viability

Cells were treated with a series of concentrations of enterolactone or enterodiol for 2, 4, and 7 d. Cell viability was measured using the MTT test separately. *p<0.05 compared with control.

Fig. 3. Effects of Enterolactone and Enterodiol on ALP Activity of MG-63 Cells

Cells were treated with a series of concentrations of enterolactone or enterodiol for 2, 4, and 7 d. Cell ALP activity was measured as described in Materials and Methods. *p<0.05 compared with control.
day 7. The level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was analyzed in the same samples as a housekeeping reference gene. When compared with the level of GAPDH mRNA, the densitometric ratios of col I, ALP, OC, OP, and ON mRNA levels were calculated and indicated in volume graphs. As shown in Fig. 4, the mRNA levels of OCN, ALP, and OP increased with the increase in the concentration of enterolactone or enterodiol. However, the change in the mRNA level of col I and ON was different (Fig. 5). Similar to the effects on cell viability and ALP activity, enterolactone or enterodiol increased mRNA levels at low concentrations and decreased levels at higher concentrations.

DISCUSSION

Osteoporosis is characterized by a reduced bone mass, which results in increased bone fragility and fracture risk. Estrogen deficiency that occurs at menopause plays a major role in the development of osteoporosis in elderly women. Therefore estrogen replacement therapy (ERT) has been widely used for the prevention of postmenopausal osteoporosis. However, ERT also shows undesirable side effects including increased incidence of breast cancer and heart disease. Recently, there has been growing interest in searching for alternatives from dietary natural plant products.

The mammalian phytoestrogens enterodiol and enterolactone are produced in the colon by the action of bacteria on the plant precursors matairesinol and secoisolariciresinol. In the present study, we tried to evaluate the antiosteoporosis potentials of these enterolignans. We employed osteoblast-like MG-63 cells as an in vitro assay system and evaluated the effects of lignans on osteoblast differentiation by measuring several osteoblast differentiation markers such as ALP, col I, and OC. Despite being a tumor cell line, which proliferates more rapidly than human bone-derived cells, MG-63 cells exhibit many osteoblastic traits characteristic of bone-forming cells. MG-63 cells are believed to be relatively immature osteoblasts. However, they exhibit a number of osteoblast-like phenotypic markers.

The effects of enterolactone and enterodiol on the viability and ALP activity of MG-63 cells suggested that enterolactone and enterodiol have biphasic effects on osteoblast-like cells. At low doses, they induce cell proliferation and ALP activity but show inhibition at high doses. Wang and Kurzer reported that enterolignans have biphasic effects on DNA synthesis in MCF-7 (breast cancer) cells, showing induction at 10—50 μM and inhibition at high concentrations.

The biological effects of estrogens are known to be extremely complex. Estrogens, including phytoestrogens, exert estrogenic effects through binding to estrogen receptors (ERs). Ligands for ERα and ERβ can act as pure agonists, as antagonists, or have partial or selective agonist/antagonist activity. Whether an ER ligand acts as an agonist or antagonist in different tissues is determined by many factors. Although the phytoestrogens bind less strongly to the ER than estradiol E2, their interaction with this receptor can produce both estrogenic and antiestrogenic responses in various cell lines. At relatively low doses certain phytoestrogens express estrogenic activity and stimulate cell growth, while at higher doses the same phytoestrogens appear to be antiestrogenic and suppress cell growth. Our results are in agreement with this. The biphasic mode of action may represent a viable anabolic therapy for osteoporosis. Because MG-63 is an osteosarcoma cell line, the inhibitory effects of enterolactone and enterodiol on cell proliferation can be further studied to...
explore their antitumor activities.

Collagen is the major constituent of the extracellular matrix in bone. ON has calcium-binding sites and it regulates cell spreading and cell disengagement. ON has affinity for type I collagen and hydroxyapatite. The transcriptional level of the two genes increased at low doses of enterolactone or enterodiol and decreased at high doses, similar to the change in cell viability and ALP activity. There is a close relationship between ON gene level and cell viability. When cultured bone cells were treated with antisense oligonucleotides that effectively blocked the production of a portion of the total ON, they showed a specific decrease in total cell DNA synthesis not associated with general toxicity.18) ON is always coexpressed with col and thus they showed similar changes.

OC has the ability to bind hydroxyapatite and calcium and is highly expressed in growing skeletal tissue. It plays an important role in the mineralization or bone turnover process or both. OP has cell attachment activity and is implicated in binding hydroxyapatite, and thus the crucial role of OP was postulated to be the bridging of bone cells to hydroxyapatite. The OP gene may be controlled by transcriptional and posttranslational mechanisms in response to vitamin D. The expression of OP may also be influenced by growth factors and proteocones. ALP produces phosphate required for mineralization and, in addition, hydrolyzes substances that inhibit calcification.

The transcriptional level of these three genes (ALP, OC, OP) increased with the increase in the concentration of enterolactone or enterodiol. It suggested that enterolactone and enterodiol can induce osteoblast cell differentiation at the mRNA level in a dose-dependent manner. However, the ALP mRNA level is not consistent with the results of ALP activity. This may be because of the effect of posttranslational modification or the degradation effect of proteasomes.

Phytoestrogens are considered to act via classical ER-mediated signaling. Estrogen also can act via nonnuclear receptors that interact with ERs via second messenger systems,19 or through a nongenomic/tyrosine kinase pathway.15 There is in vitro evidence that E2 modulates the functions of neural and vascular cells via nongenomic actions. The molecular mechanisms of the nongenomic effects are still under investigation. To date, the relative importance of these pathways has not been evaluated in osteoblasts. Therefore both the genomic effects through classical ERs and nongenomic effects must be considered. Enterodiol and enterolactone do not appear to act via a single mechanism of action; rather their effects are pleiotropic. Structurally related phytoestrogens have discrete target sites and mechanisms of action.20 This may explain why enterolactone and enterodiol showed different effects on cell viability, ALP activity, transcriptional levels of ALP, OC, OP, ON and col I.

In conclusion, we tested the effects of enterolactone and enterodiol on osteoblast-like MG-63 cells and found that enterolactone and enterodiol showed the same effects. They have biphasic effects on the differentiation of osteoblast cells. Enterolactone and enterodiol increased cell viability, ALP activity, and the transcriptional level of ON and col I at low doses, but inhibited them at high doses. There is no biphasic mode of action in the effect of enterolactone and enterodiol on the transcriptional levels of ALP, OC, and OP. The difference may be related to the multiple signaling transduction of phytoestrogen.

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REFERENCES