ESTROGEN BLOCKS PARATHYROID HORMONE-STIMULATED OSTEOCLAST-LIKE CELL FORMATION IN MODULATING DIFFERENTIATION OF MOUSE MARROW STROMAL CELLS IN VITRO

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Background and Purpose: During skeletal development and bone remodeling, bone marrow stromal cells give rise to osteoblasts and provide a critical microenvironment to support osteoclast formation. Estrogen is important for the maintenance of bone balance in adult animals by either increasing bone mass or inhibiting osteoclastic bone resorption. This study sought to determine the role that estrogen plays in coordinating osteogenic differentiation of bone marrow stromal cells and the ability of these cells to support osteoclast formation.

Methods: A conditionally immortalized mouse bone marrow stromal cell line, MS1, was used to examine the effects of estrogen on stromal cell differentiation and on stromal cell-supported osteoclast formation.

Results: On treatment of MS1 cells with 17β-estradiol (E2) (10−12–10−8 M), alkaline phosphatase activity and bone nodule formation were increased in a dose-dependent manner, while the proliferation of MS1 cells was dose-dependently inhibited. 17β-E2 (10−12–10−8 M) also caused a concentration-dependent inhibition of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cell (MNC) formation in parathyroid hormone (PTH)-stimulated MS1 and spleen cell cocultures. Furthermore, estrogen pretreatment of MS1 cells also decreased the number of TRAP-positive MNCs in cocultures. Culturing in PTH-treated conditioned media did not rescue the loss of activity supporting osteoclast-like cell formation in MS1 cells.

Conclusion: These results indicate that 17β-E2-stimulated osteoblastic differentiation of mouse marrow stromal cells results in bone matrix mineralization and a decrease in activity of supporting PTH-induced osteoclast-like cell formation.

Estrogen supplement has been shown to prevent bone loss in postmenopausal women [1]. This loss of bone has been attributed to uncoupling between the function of osteoblasts and osteoclasts [2, 3]. Studies both in vivo and in vitro have shown that estrogen decreases bone resorption and inhibits bone-resorbing osteoclast activities [3, 4]. Although studies in animals have suggested that estrogen may increase bone formation rather than just inhibit bone resorption [5, 6], the evidence for stimulatory effects of estrogen on bone formation is less compelling than evidence for the inhibitory action on bone resorption.

The demonstration of estrogen receptors in human osteoblasts [7] suggests that estrogen may have a direct effect on the regulation of osteoblast function. Estrogen has also been shown to decrease the synthesis of interleukin-1 [8] and interleukin-6 [9] as well as to increase the production of insulin-like growth factor [10] and transforming growth factor-β [11]. More recently, estrogen has been shown to increase synthesis of osteoprotegerin [12, 13]. These cytokines and growth factors are believed to play important roles in regulating osteoclastic bone resorption. In spite of these findings, the mechanism of

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Estrogen in Marrow Stromal Cell Differentiation and Osteoclast Formation

Estrogen action in bone is far from clear, and its actions on different types of osteoblasts are still not clearly established. In cells of osteoblastic lineage from a variety of sources, estrogen has been reported to stimulate, to inhibit, or not to affect osteoblast proliferation and/or differentiation [14].

Although it remains controversial whether estrogen stimulates bone formation, the failure to detect marked modulation by estrogen of differentiated osteoblast function in vitro may imply that osteoprogenitor cells and not mature osteoblasts are the principal estrogen target cell in bone. In support of this, estrogen receptors and estrogen-responsive genes have been identified in bone marrow stromal cells and stromal cell lines [15, 16], which have long been recognized as the source of osteoprogenitor cells in humans and in animal models [17, 18]. In vitro differentiation studies with glucocorticoids induced osteoblastic phenotype differentiation of isolated stromal cells [19]. Depending on differentiation status, stromal cells express mature markers of mature osteoblasts. In order, these markers are type I collagen, alkaline phosphatase (AP), osteocalcin, and mineralized bone matrix [20]. Qu et al demonstrated that estrogen stimulates sequential differentiation of osteoblasts and increased deposition and mineralization of matrix in a mixed culture of mouse bone marrow cells [21]. However, little is known about the effects of estrogen on bone marrow stromal cells.

In addition to being the source of osteogenic cells, it is well established that bone marrow stromal cells provide a critical microenvironment to support proliferation and differentiation of hematopoietic cells, including osteoclasts. Since bone marrow stromal cells or osteoblasts are the major target cells of estrogen in bone, estrogen has long been thought to inhibit osteoclast bone resorption by regulating the production of cytokines and local factors by bone marrow stromal cells or osteoblasts, which then increase or decrease osteoclastic formation and activity via paracrine mechanisms [22]. Nevertheless, the effects of estrogen on coordinating the differentiation of marrow stromal cells and the activity of stromal cells in support of osteoclast formation remain to be established. The purpose of this in vitro study was to determine whether estrogen affects bone marrow stromal cell differentiation, and whether the effect of estrogen on osteoclast formation, if any, is mediated through bone marrow stromal cell differentiation.

Materials and Methods

Chemicals

Rat parathyroid hormone (rPTH)-(1-34) was obtained from Bachem California (Torrance, CA, USA), and recombinant mouse interferon-γ (IFN-γ) from Genzyme (Boston, MA, USA). Eagle’s Minimum Essential Medium (EMEM), αMEM, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies (Grand Island, NY, USA). ICI 182,780, a pure antiestrogen, was provided by Tocris Cookson (Bristol, UK). 17β-Estradiol (17β-E₂), 17α-E₂, dexamethasone, naphthol AS-BI phosphoric acid, and red violet LB salt were products of Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of 17β-E₂ and 17α-E₂ were prepared in ethanol, and of ICI 182,780 were prepared in dimethyl sulfoxide (DMSO). The stock solutions were stored at −20°C until used. All other chemicals used were of analytic grade. All culture dishes and plates were purchased from Becton Dickson Labware (Franklin Lakes, NJ, USA).

Cell culture

Clonal conditionally immortalized MS1 mouse bone marrow stromal cells [23] were maintained in EMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 U/mL mouse IFN-γ at 33°C in a humidified atmosphere of 5% CO₂ in air. The cells were immortalized and grew indefinitely at permissive conditions (33°C, in the presence of IFN-γ) but were still able to differentiate at semipermissive conditions (37°C, in the absence of IFN-γ) [19]. All cells were cultured in phenol red-free αMEM. FBS was heat-inactivated and charcoal-treated to remove any steroids.

Estrogen receptor immunohistochemistry

MS1 cells were seeded at 2 × 10⁴/cm² into multichambered slides (Nunc, Naperville, IL, USA) in phenol red-free αMEM supplemented with 10% heat-inactivated and charcoal-stripped FBS (hcFBS) for 12 hours under permissive conditions. The cells were refed with fresh medium and maintained at 37°C for an additional 3 days, after which the cells were processed for immunohistochemical studies. Estrogen receptor immunohistochemistry was assessed using rabbit and goat polyclonal antibodies against the mouse estrogen receptors α and β, respectively (sc-542 and sc-6821, Santa Cruz Biotechnology, Santa Cruz, CA, USA), according to the manufacturer’s instructions with minor modifications. Briefly, the cells were washed with cold calcium- and magnesium-free phosphate buffered saline (PBS), fixed with methanol at −20°C, air-dried, and rinsed with PBS. The fixed cells were then blocked with 1.5% (vol/vol) normal goat or donkey serum in PBS (blocking serum-PBS) at room temperature for 30 minutes, rinsed with PBS containing 1% (wt/vol) bovine serum albumin (PBS-BSA), and incubated for 30 minutes with 1 mg/mL specific antibody in PBS-BSA.
Uterine tissue from a 9-week-old female C57BL/6 mouse was used as a positive control. MS1 cells or uterine tissue sections were also incubated without primary antibody or with nonimmune rabbit or goat serum as a negative control. After washing three times with PBS, the cells were incubated with a biotin-conjugated goat anti-rabbit or donkey anti-goat immunoglobulin G (IgG) for 30 minutes in 1.5% blocking serum-PBS, rinsed with PBS, and subsequently incubated for 30 minutes with avidin-biotin enzyme reagent (all from Santa Cruz). The cells were further washed with PBS followed by 0.5% Triton X-100 in PBS before incubation with 0.01% (wt/vol) 3,3-diaminobenzoidine and a few drops of 30% hydrogen peroxide in PBS for 2 to 5 minutes. Finally, the cells were washed extensively with distilled water, air-dried, and photographed using an Olympus BH 2 (Tokyo, Japan) microscope.

**Cell proliferation assay**

Cell proliferation was assessed by counting cell numbers after estrogen treatment. MS1 cells were plated into 35-mm culture dishes at 4 x 10^4 cells/dish in phenol red-free αMEM containing 2% hcFBS and incubated at 33°C for 12 hours to let cells settle. The cells were transferred to medium maintained at 37°C and treated with 17β-E2 (10^-6 M), 17α-E2 (10^-8 M), combined with ICI 182,780 (10^-10 M), or ethanol vehicle from the beginning of the assay. The medium combined with treatment was changed every 2 days. After washing twice with PBS, cells were trypsinized and counted on a hemocytometer at the indicated time points. Viable cells, based on trypan blue exclusion, were counted in a hemocytometer.

**Alkaline phosphatase activity**

MS1 cells were plated at 4 x 10^4 cells/well in 24-well culture plates with phenol red-free αMEM containing 10% hcFBS and cultured at 33°C until confluent. To determine the time course effect of estrogen on AP activity, the cells were subsequently transferred to medium maintained at 37°C and treated with 17β-E2 (10^-12-10^-6 M), 17α-E2 (10^-8 M) combined with ICI 182,780 (10^-10 M), 17α-E2 (10^-8 M), or ethanol vehicle in phenol red-free αMEM containing 10% hcFBS. The cells were fed with fresh medium combined with treatment every 2 days and harvested at the indicated time. At the end of treatment, AP activity was measured as previously described [24]. After washing with PBS, cells were dissolved in 0.25% Triton X-100 (0.5 mL/well) for 1 hour with gentle shaking. The samples were kept frozen until needed for AP activity assays. The release of p-nitrophenol from 10 mM p-nitrophenyl phosphate in a buffer containing 1.0 mM MgCl2·6H2O in 0.75 M 2-amino-2-methyl-1-propanol, pH 10.3, was measured at 30°C. The reaction was stopped after 15 minutes with 0.5 N NaOH and the absorbance determined at 405 nm using a microtiter plate reader. The activity was expressed as nM/minute/mg protein.

**Bone nodule formation**

Cells were seeded at 5 x 10^3 cells/well in six-well plates and grown to confluence at 33°C. Thereafter, the medium was changed to mineralization medium (αMEM containing 10% hcFBS, 1% penicillin-streptomycin, and 10 mM β-glycerophosphate). To assess the effects of estrogen on the mineralization of matrix, cultures were treated with 17β-E2 (10^-12-10^-6 M), 17α-E2 (10^-8 M), 17β-E2 (10^-6 M) combined with ICI 182,780 (10^-10-10^-6 M), or vehicle (ethanol or DMSO) alone. Cultures were then transferred to medium maintained at 37°C and cultured with half-changes of medium twice a week. At the end of the treatment, cultures were stained using a modified von Kossa method to assess the formation of mineralized nodules. Briefly, the cells were washed three times with PBS, fixed with neutral formalin solution for 5 minutes, and rinsed with deionized water. After the addition of 5% silver nitrate solution, the plates were exposed to ultraviolet light for 1 hour. The plates were then rinsed with deionized water, and the residual silver nitrate was neutralized by 5% sodium thiosulfate.

**Osteoclast-like cell formation in vitro**

The coculture procedures required to generate osteoclasts in vitro were described in detail previously [19]. C57BL/6 mice were obtained from the Laboratory Animal Center of the Medical College of National Taiwan University (Taipei, Taiwan, Republic of China) and the Animal Center of the Medical College of National Taiwan University (Taipei, Taiwan, Republic of China). Animal handling and treatment were performed according to protocols provided by the Center. Briefly, the MS1 cells (4 x 10^4 cells/well) were plated in 24-well plates and cultured at 33°C overnight before being overlaid with spleen cells (10^6 cells/well) from 8- to 11-week-old C57BL/6 mice. Bone marrow cells were cultured in 0.5 mL phenol red-free αMEM supplemented with 10% hcFBS and 10^-7 M dexamethasone with 10^-7 M rPTH-1-34) at 37°C for 3 weeks.

To examine the effects of estrogen treatment on PTH-stimulated osteoclast formation, 17β-E2 (10^-12-10^-10 M), 17α-E2 (10^-8 M), 17β-E2 (10^-6 M) combined with ICI 182,780 (10^-10 M), ICI 182,780 (10^-6 M), or vehicle (ethanol or DMSO) alone was added to the cultures from the first day of coculture. The total concentration of organic solvents in the medium was less than 0.1% and the same volumes of solvents were added to the control medium.

To determine whether the differentiation status of MS1 cells induced by estrogen affects PTH-stimulated osteoclast formation, MS1 cells were cultured in phenol red-free αMEM with 10% hcFBS at 33°C until
confluent. The cells were then treated with 17β-E2 (10⁻⁸ M) alone or combined with ICI 182,780 (10⁻⁶ M) for 1, 2, 3, 5, and 7 days at 37°C and then prepared for coculture. The cocultures were stimulated with rPTH-(1-34) (10⁻⁷ M) as described above. All cultures were refed by half-changes of fresh medium and treatment every 3 days for 3 weeks, when they were stained for tartrate-resistant acid phosphatase (TRAP).

Preparation of MS1-conditioned media
In order to collect the conditioned medium of PTH-treated MS1 cells, MS1 cells (10⁵ cells/10-cm dish) were plated and allowed to grow in phenol red-free αMEM and 10% hFBS. At confluence, the media were removed and fresh hFBS-free and 10⁻⁷ M dexamethasone-containing media with or without 10⁻⁷ M rPTH-(1-34) were added and the cells were transferred to medium maintained at 37°C. The conditioned media were harvested after 48 hours of culture and centrifuged at 250 g for 5 minutes; the supernatant was stored at -80°C until use.

The conditioned media were added to cocultures of mouse spleen cells and MS1 cells pretreated for various periods with 10⁻⁶ M 17β-E2, every 3 days in the absence of 10⁻⁷ M rPTH-(1-34), and their effects on the formation of TRAP-positive multinucleated cells (MNCs) were examined.

TRAP-positive multinucleated cell quantitation
At the termination of the experiments, the cultures were fixed with ethanol-acetone (50:50, vol/vol) then stained for TRAP. TRAP-positive MNCs containing three or more nuclei were scored as osteoclasts as previously described [19]. All experiments were done in six replicates and repeated four times for each condition.

Statistical analysis
Data are expressed as the mean ± standard error of the mean. The data shown in the Figures and the significance within each set of data are representative of at least six separate cell preparations. The significance of a difference between comparable groups was determined by Student’s t-test.

Results

Demonstration of estrogen receptors in MS1 cells
Estrogen receptors α and β were readily detected by immunohistochemistry in control uterus and MS1 cells. Within the epithelial compartment of the uterus, glandular epithelial cells showed the strongest signal with virtually all cell nuclei staining strongly positive. Strong staining for estrogen receptor was also observed in stromal cells and myometrial smooth muscle cells, with more than 70% of cell nuclei staining positive (Fig. 1A). Nuclear staining for estrogen receptors α and β was also observed in nearly all MS1 cells, whereas cytoplasm staining was present in very few cells (Fig. 1C).

Effects of 17β-E2 on growth and alkaline phosphatase
Because estrogen receptors were demonstrated to be present on MS1 cells, experiments were performed to investigate whether 17β-E2 could modulate the proliferation and differentiation of MS1 cells. Treatment with 17β-E2 alone or combined with ICI 182,780 did not change the morphology of MS1 cells (data not shown). Table 1 shows that 17β-E2 dose-dependently inhibited MS1 cell proliferation. When MS1 cells were treated with 10⁻⁸ M 17β-E2 for 1, 2, 3, 5, and 7 days, AP activity was found to be significantly enhanced by 1.1-, 1.2-, 1.8-, 2.0-, and 5.7-fold, respectively (Table 2).

The effect of 17β-E2 is specific since the isomeric 17α-E2 had no effect on either proliferation or AP activity in MS1 cells. Co-treatment with the pure anti-estrogen ICI 182,780 (10⁻⁶ M) reversed the stimulatory effect of 17β-E2 on AP activity and the inhibitory effect on cell proliferation, showing that the effects of 17β-E2 on proliferation and AP activity were specifically mediated through estrogen receptors.

Effects of 17β-E2 on matrix mineralization
In the presence of β-glycerophosphate and 17β-E2, but not control, MS1 cells mineralized the extracellular matrix. Mineralization became apparent in cultures receiving 2 weeks of treatment (Fig. 2A). Maximal mineralization was observed after 5 weeks of treatment (data not shown). In both control and cells co-treated with 17β-E2 (10⁻⁸ M) and ICI 182,780 (10⁻¹⁰–10⁻⁶ M), nodule formation did not occur during 5 weeks of treatment (Fig. 2B). The mineralization induced by 17β-E2 was not the result of necrosis or cell death, because no dead cells were detected in the cultures, especially under and in the vicinity of mineralization areas, as determined by trypan blue staining (data not shown).

Effects of 17β-E2 on PTH-stimulated osteoclast-like cell formation
To determine the potential role of estrogen in the regulation of osteoclast formation, we initially assessed its ability to suppress the formation of TRAP-positive MNCs stimulated by PTH in cocultures of MS1 cells and normal mouse spleen cells. These MNCs demon-
strated various characteristics of osteoclasts, including responsiveness to calcitonin and osteoclastic bone resorption [23]. As shown in Fig. 3, 17\(\beta\)-E\(_2\) caused a concentration-dependent inhibition of PTH-stimulated TRAP-positive MNC formation in cocultures. The minimally effective concentration of 17\(\beta\)-E\(_2\) was 10\(^{-10}\) M, and a 55% reduction in PTH-stimulated TRAP-positive MNC formation was seen with 10\(^{-8}\) M 17\(\beta\)-E\(_2\). The inactive

**Table 1.** Effect of 17\(\beta\)-estradiol (E\(_2\)), 17\(\alpha\)-E\(_2\), and ICI 182,780 (ICI) on proliferation of MS1 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.8 ± 0.5</td>
<td>28.3 ± 3.3</td>
<td>72.4 ± 5.5</td>
<td>182.3 ± 7.8</td>
</tr>
<tr>
<td>Control + ICI</td>
<td>5.2 ± 0.9</td>
<td>27.6 ± 2.1</td>
<td>75.3 ± 6.9</td>
<td>195.8 ± 9.8</td>
</tr>
<tr>
<td>17(\beta)-E(_2), 10(^{-8}) M</td>
<td>5.6 ± 0.5</td>
<td>18.4 ± 1.6</td>
<td>42.5 ± 3.6</td>
<td>81.4 ± 6.2</td>
</tr>
<tr>
<td>17(\beta)-E(_2), 10(^{-10}) M</td>
<td>5.2 ± 0.4</td>
<td>22.5 ± 1.6</td>
<td>56.6 ± 3.3</td>
<td>102.3 ± 7.6</td>
</tr>
<tr>
<td>17(\beta)-E(_2), 10(^{-12}) M</td>
<td>5.2 ± 0.7</td>
<td>21.6 ± 1.4</td>
<td>62.3 ± 4.5</td>
<td>172.3 ± 10.3</td>
</tr>
<tr>
<td>17(\alpha)-E(_2), 17(\beta)-E(_2) + ICI, 10(^{-8}) M</td>
<td>5.4 ± 0.9</td>
<td>30.7 ± 3.2</td>
<td>78.8 ± 6.2</td>
<td>179.4 ± 13.6</td>
</tr>
<tr>
<td>17(\beta)-E(_2) + ICI, 10(^{-10}) M</td>
<td>5.5 ± 0.4</td>
<td>26.2 ± 2.6</td>
<td>78.6 ± 6.7</td>
<td>190.3 ± 13.3</td>
</tr>
<tr>
<td>17(\beta)-E(_2) + ICI, 10(^{-8}) M</td>
<td>5.0 ± 0.6</td>
<td>24.9 ± 2.2</td>
<td>64.8 ± 5.4</td>
<td>146.5 ± 14.1</td>
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<tr>
<td>17(\beta)-E(_2) + ICI, 10(^{-10}) M</td>
<td>5.3 ± 0.9</td>
<td>20.8 ± 1.5</td>
<td>48.8 ± 4.9</td>
<td>82.5 ± 10.3</td>
</tr>
</tbody>
</table>

Values shown are mean ± standard error of the mean for six replicate dishes. *Significantly different from controls (p < 0.05); †significantly different from 10\(^{-8}\) M 17\(\beta\)-E\(_2\) (p < 0.05).
Table 2. Effect of 17β-estradiol (E₂), 17α-E₂, and ICI 182,780 (ICI) on alkaline phosphatase (AP) activities of MS1 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AP activity (nmol/10⁴ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Control</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Control + ICI</td>
<td>4.2 ± 0.2</td>
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<tr>
<td>17β-E₂</td>
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<tr>
<td>10⁻⁸ M</td>
<td>4.8 ± 0.2</td>
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<td>10⁻¹⁰ M</td>
<td>4.2 ± 0.5</td>
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<td>10⁻¹² M</td>
<td>4.2 ± 0.6</td>
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<tr>
<td>17α-E₂</td>
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<tr>
<td>10⁻⁸ M ICI</td>
<td>5.0 ± 0.3</td>
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<tr>
<td>10⁻⁶ M ICI</td>
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<tr>
<td>10⁻⁸ M ICI</td>
<td>4.4 ± 0.3</td>
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<tr>
<td>10⁻⁹ M ICI</td>
<td>4.8 ± 0.9</td>
</tr>
<tr>
<td>10⁻¹⁰ M ICI</td>
<td>5.1 ± 0.5</td>
</tr>
</tbody>
</table>

Values shown are mean ± standard error of the mean for six replicate dishes. *Significantly different from controls (p < 0.05); †significantly different from 10⁻⁸ M 17β-E₂ (p < 0.05).

stereoisomer, 17α-E₂ (10⁻⁸ M), failed to inhibit PTH-stimulated TRAP-positive MNC formation (Fig. 3). Further evidence of specificity derived from observations that ICI 182,780 (10⁻⁶ M) caused a reversal of the inhibitory effect of 17β-E₂ upon PTH-induced TRAP-positive MNC formation (Fig. 3).

Fig. 2. Effect of 17β-estradiol (E₂) on mineralization of the matrix secreted by MS1 cells. MS1 cells were maintained at 37°C for A) 2 weeks or B) 5 weeks in phenol red-free αMinimum Essential Medium containing 10% heat-inactivated and charcoal-stripped FBS supplemented with β-glycerophosphate (10 mM) A) alone or plus either 17α-E₂ at concentrations of b) 10⁻⁸ M, c) 10⁻⁹ M, d) 10⁻¹⁰ M, e) 10⁻¹¹ M, f) 10⁻¹² M, or B) 10⁻⁸ M 17β-E₂ and ICI 182,780 at concentrations of b) 10⁻⁶ M, c) 10⁻⁷ M, d) 10⁻⁸ M, e) 10⁻⁹ M, f) 10⁻¹⁰ M before von Kossa staining.
Effects of 17β-E2 pretreatment of MS1 cells on PTH-stimulated osteoclast-like cell formation

Table 3 shows the results of estrogen pretreatment, which suppressed the ability of MS1 cells to support PTH-stimulated osteoclast-like cell formation. A significant decrease was found in the number of TRAP-positive MNCs in cocultures of MS1 cells that had been pretreated with 10⁻⁸ M 17β-E2 for 3 days. Maximal inhibition occurred with 17β-E2 pretreatment of MS1 cells for at least 5 days. Parallel studies of MS1 cells pretreated with 10⁻⁸ M 17β-E2 and 10⁻⁶ M ICI 182,780 abolished the blocking effects (Table 3).

**Discussion**

In the present study, stimulation of AP activity during inhibition of cell proliferation with estrogen was demonstrated in a clonal mouse bone marrow stromal cell culture. 17β-E2 treatment decreased the number of 10⁻⁷ M rPTH-(1-34)-stimulated osteoclast-like TRAP-positive MNCs in MS1 and normal mouse spleen cell cocultures. The decrease in PTH-stimulated TRAP-positive MNCs was dependent on the differentiation status of MS1 cells. To our knowledge, this is the first demonstration of the inhibitory effects of 17β-E2 on osteoclastic cell formation through modulating the differentiation of accessory cells in mouse marrow stromal and spleen cell cocultures.

Earlier studies have demonstrated that 17β-E2 maintains bone mass by inhibition of bone resorption, which leads to a reduction in bone turnover [3, 25]. More recently, evidence from experimental studies suggests that 17β-E2 may also directly promote bone formation in vivo [5, 26, 27]. However, the results of 17β-E2 on osteoblasts from different sources have been confusing due to discrepant findings. 17β-E2 has been reported to enhance the proliferation and differentiation of cultured osteoblast-like cells enzymatically digested from calvaria of newborn rats [28, 29]. In the rat...
Fig. 4. Effect of conditioned media from MS1 cell cultures treated with rat parathyroid hormone (rPTH) (1-34) for 48 hours on tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cell (MNC) formation in MS1 spleen cell cocultures. The MS1 cells were treated with 10^-8 M 17β-E2, for the indicated time before coculture. Conditioned media (CM) of MS1 cell cultures without (–) or with (+) 10^-7 M rPTH (1-34) for 48 hours were harvested and the conditioned media were added to cocultures of 17β-E2-pretreated MS1 cells and mouse spleen cells. After being cultured for 3 weeks, TRAP-positive MNC formation was determined. Data are expressed as mean ± standard error of the mean of six determinations. *p < 0.01, compared with respective control; †p < 0.01, compared with 17β-E2-un treated MS1 cells in conditioned media from PTH-treated MS1 cells.

Estrogen in Marrow Stromal Cell Differentiation and Osteoclast Formation

osteogenic sarcoma cell line UMR-106, 17β-E2 was found to inhibit cell proliferation but to increase AP activity [30]. In a human osteogenic sarcoma cell line, HTB-96, 17β-E2 was found to inhibit proliferation but not to affect AP activity [31]. In human osteoblast-like cells, no direct effect of 17β-E2 on proliferation or differentiation was identified [32]. The reasons for these divergent and conflicting results have been widely discussed [3, 33]. Variable numbers of 17β-E2 receptors expressed in osteoblasts obtained from different sources or in osteosarcoma cells representing different levels of differentiation may have contributed to these conflicting results.

Expression of AP, widely used as a marker for osteoblastic differentiation and bone formation, in osteoblasts appears to be differentiation-stage dependent. Osteoprogenitor cells that are initially AP-negative mature into less proliferative cells that are AP-positive [34]. In differentiating isolated rat osteoblasts, AP expression occurs transiently, coincident with the down-regulation of proliferation [35]. In the present study, 17β-E2 significantly and dose-dependently stimulated AP activity and bone nodule formation in MS1 cells while inhibiting cell proliferation. The increase in AP activity and bone nodule formation occurred concurrently, thus confirming that 17β-E2 enhances the osteoblastic differentiation and potential for calcification of the extracellular matrix of MS1 cells.

These findings suggest that 17β-E2 treatment of MS1 cells first inhibits proliferation and promotes differentiation, and then enhances calcification of extracellular matrix and bone nodule formation. In the present study, this series of events was demonstrated by following the expression of AP. Late formation of calcified nodules confirmed the differentiation and maturation of MS1 cells. Thus, this culture system fulfills the requirements for an in vitro model of both osteoblastic proliferation and differentiation and would be useful in studies aimed at clarifying the regulation of osteoblastic differentiation of bone marrow stromal cells by estrogen.

Several findings from this study suggest that the regulation of osteogenic differentiation seen in the 17β-E2-treated MS1 cultures was due to the addition of the hormone. First, medium used did not contain phenol red, which is known to mimic estrogen effects, and FBS was charcoal stripped to remove residual steroids in the serum. Second, the experimental results of 17β-E2 effects were always compared with corresponding controls grown in the absence of 17β-E2. Third, the maximum responses to 17β-E2 were still obtained when the hormone was added in a concentration of 10^-10 M, which is within the physiologic serum range measured in female rats (0.1 nmol/L) [36]. Fourth, MS1 cells were demonstrated to express estrogen receptors and the 17β-E2 effect could be blocked by a pure antiestrogen ICI 182,780. These findings suggest that the effects of 17β-E2 on both proliferation and differentiation are mediated by the activation of estrogen receptors, since ICI 182,780 has been shown to block all estrogen receptor-mediated effects examined [37, 38].

A previous study found that coculture of MS1 cells with mouse spleen cells supported PTH-dependent formation of MNCs that exhibited major functional criteria for osteoclasts: ie, they were TRAP-positive, expressed calcitonin receptors, and formed resorption lacunae on dentine slices [23]. In the present study, pretreatment of MS1 cells with 17β-E2 significantly decreased the number of TRAP-positive MNCs when the stromal cells were cocultured with normal mouse spleen cells and treated with 10^-7 M rPTH (1-34).

Since bone marrow stromal cells are essential to growth and differentiation of osteoclasts, they are known to be, together with hematopoietic cells, the sources of
various secreted or cell membrane-bound cytokines and growth factors [39, 40]. In the present study, the inhibitory effect of 17\(\beta\)-estradiol (\(17\beta\)-E\(_2\)) pretreatment on osteoclast formation may have been mediated by changes in production of these cytokines or growth factors by MS1 cells. Furthermore, the loss of stimulating activity to osteoclast formation may occur via a cell membrane-bound form rather than via a soluble form because conditioned media collected from PTH-treated MS1 cells did not restore the decrease in TRAP-positive multinucleated cell (MNC) formation in cocultures. An alternative mechanism by which 17\(\beta\)-E\(_2\) pretreatment of MS1 cells may inhibit PTH-stimulated osteoclast formation would be to render the MS1 cells less responsive to PTH through the downregulation of PTH receptors. Future study of the relationship between estrogen-induced differentiation status and cytokine production as well as the responsiveness to PTH in MS1 cells is needed to determine the mechanism responsible for the loss of stimulating activity to osteoclast formation.

The results of our study provide a line of evidence which suggests that the inhibiting action of estrogen on PTH-stimulated osteoclast formation is at least partly mediated through action on bone marrow stromal cells. Delineation of how 17\(\beta\)-E\(_2\) regulates the marrow microenvironment for bone-forming cell differentiation and bone-resorbing cell formation at the molecular level may lead to an understanding of its effects in vivo.

In conclusion, our results support the previous findings that estrogen can enhance osteoblastic differentiation and bone formation in mouse bone marrow stromal cells [5, 28, 29]. We further demonstrated that 17\(\beta\)-estradiol inhibits osteoclast formation in PTH-treated MS1 and spleen cell cocultures. The inhibitory effect on osteoclast formation was shown to be dependent on the differentiation status of MS1 cells. Furthermore, the findings of this study indicate that the MS1 cell is a useful model for analysis of the effects of estrogen and estrogen analogs on marrow stromal cells.

ACKNOWLEDGMENT: The authors would like to thank Ms. Pei-Rong Chung for preparation of the manuscript. This work was supported in part by a research grant from the National Science Council of the Republic of China (NSC-89-2316-B-002-021, NSC-89-2320-B-002-239).

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