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Effects of 17β-Estradiol on the Expression of Matrix Metalloproteinase-1, -2 and Tissue Inhibitor of Metalloproteinase-1 in Human Osteoblast-like Cell Cultures

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Estrogen can effectively prevent estrogen deficiency-induced bone loss in animals and humans. However, its mechanism remains unknown. Osteoblast-derived Matrix metalloproteinse-1 (MMP-1), MMP-2, and tissue inhibitor of metalloproteinase-1 (TIMP-1) recently were implicated as playing important roles in initiating bone resorption. Therefore, we tested the effects of 17β-estradiol (E_2) on MMP-1, MMP-2, and TIMP-1 production in cultures of human osteoblastic MG-63 cells and normal human osteoblasts (hOB). MMP-1, MMP-2 and TIMP-1 concentrations in the culture medium were determined by ELISA, and activity of MMP-2 was assessed by ELISA. After 12–48 h of treatment, E_2 at 10^{-8}M decreased MMP-1 level in cultures of MG-63 cells or hOB. Treatment with increasing dose of E_2 in MG-63 cells or hOB caused a dose-dependent decrease in MMP-1 synthesis. E_2 had no influence on MMP-2 and TIMP-1 production in MG-63 cells or hOB cultures, as well as activation of latent MMP-2. In conclusion, E_2 represses MMP-1 synthesis, and this effect may contribute to its action on the inhibition of bone resorption, followed by prevention of bone loss. Increasing MMP-1 production followed by estrogen deficiency may contribute to the mechanisms involved in postmenopausal osteoporosis.

Key Words: Estrogen; matrix metalloproteinase; osteoblast.

Introduction

Postmenopausal osteoporosis is characterized by a reduction in circulating levels of estrogen, resulting in bone loss *(1)*. Estrogen replacement therapy is believed to effectively prevent bone loss by reducing osteoclast-mediated bone resorption *(1)*, but the mechanism is unclear.

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Over the past decade, evidence has accumulated for an active participation for osteoblast-derived matrix metalloproteinases (MMPs) in initiation of bone resorption by degrading the unmineralized osteoid layer of the bone surface to allow osteoclasts to attach to the mineralized matrix *(2–4)*. MMPs are a family of zinc-dependent endopeptidases marked by their ability to degrade essentially all extracellular matrix components *(5)*. Studies suggested that osteoblast-derived MMP-1 and MMP-2 played an important role in degradation of osteoid *(2–6)*. MMP-1 (interstitial collagenase) can cleave the native triple helix of type I collagen into three quarter and one quarter length fragments (gelatin) *(5)*. The denatured gelatin fragments can be degraded by the highly efficient MMP-2 (gelatinase A) *(5)*. Recently, it was reported that not only denatured, but also native type I collagen could be degraded by MMP-2 *(7)*. MMP-1 and MMP-2 are secreted from cells in a proform requiring activation, and their activity is inhibited by tissue inhibitor of metalloproteinase-1 (TIMP-1) which is constitutively synthesized by osteoblasts in culture *(2,5)*. Estrogen has been shown to modulate several physiology aspects of osteoblasts and osteoblast-like cells *(1,6)*, but the effect of estrogen on MMP-1, MMP-2 and TIMP-1 in human osteoblast-like cells is unknown. We postulated that the action of E_2 on bone resorption involves regulation of MMP-1, MMP-2 and TIMP-1 expression.

The present study was undertaken to determine whether 17β-estradiol (E_2) regulates the expression of MMP-1, MMP-2 and TIMP-1 in human osteoblastic MG-63 cells, and normal human osteoblasts (hOB) cultures, to examine the mechanisms by which estrogen acts on bone.

Materials and Methods

Materials

Water soluble 17β-estradiol (E_2), phenol red-free α-MEM, type IV collagenase, ascorbic acid, aprotinin and alkaline phosphatase (ALP) kit were purchased from Sigma Chemical Co. (St. Louis, MO). Trizol reagent and fetal bovine serum (FBS) were purchased from GIBICO-BRL Co. (Grand Island, NY). Osteocalcin (OC) RIA kit was purchased from DiaSorin Co. (Stillwater, MN). MMP-2 ELISA kit, TIMP-1

ELISA kit, mouse antihuman MMP-1 monoclonal antibody, mouse antihuman MMP-2 monoclonal antibody, mouse antihuman MMP-2 monoclonal antibody, mouse antihuman monoclonal TIMP-1 antibody, peroxidase-labeled rabbit antimouse IgG, and enhanced chemiluminescence (ECL) Kit were purchased from Amersham Pharmacia Biotech Co. (Arkington Heights, IL).

Cell Culture and Treatment with E_2

Human bone cell cultures were prepared according to the method of Robey and Termine *(8)*, with modifications. Briefly, surgical specimens of were rinsed extensively with serum-free α-MEM. The trabecular bones were harvested with a size 00 curette, minced, cut into small pieces, and then washed several times to remove bone marrow. The bone chips thus obtained were incubated with type IV collagenase for 2 h at 37°C. The digested chips were washed extensively to remove released cells and debris. Chips were cultured in 25cm^3 flask in phenol red-free α-MEM containing 15%FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL ascorbic acid at 37°C. After 15 days, cells migrated from within the bone particles, and reached confluence after 25 days. The cells were then released from the bone chips with 0.05% trypsin-EDTA (Sigma), counted and subcultured at 2.0×10^5 cells/cm^2 in 25cm^2 flask in phenol red-free α-MEM containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL ascorbic acid, and reached confluence after 7 days.

The human osteosarcoma cells lines (MG-63) were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in phenol red-free α-MEM containing 10%FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL ascorbic acid in 25 cm^2 flask. The medium was changed every 2 days.

In experiments of cell treatment with E_2, MG-63 cells and hOB were plated in 25 cm^2 flask in phenol red-free α-MEM containing 10%FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL ascorbic acid. After reaching confluence, the cells were then cultured in phenol red-free α-MEM containing 1% FBS for 4 days. Cells were subsequently incubated for 48 h in phenol red-free α-MEM containing 0.25% BSA (Sigma). The cells were subsequently exposed to α-MEM or 10^{-8}M E_2 for 6–48 h. Cells were also treated with vehicle or E_2 for 48 h in phenol red-free α-MEM containing 0.25% BSA. Conditioned media of MG-63 cells and hOB cultures were collected and stored frozen at −70°C until assayed by ELISA, Western immunoblot analysis, and gelatin zymograms. The cell layers were harvested for total protein determination.

ALP and OC Assay

HOB and MG-63 cells cultures were grown to confluence in 25cm^2 flask as described above. The cells were then washed three times with PBS, then the cells layer was scraped into solution containing 20 m*M* Tris-HCl, pH 8.0, and 150 m*M* NaCl, 1%Triton X-100, 0.02%NaN^3, 1 μg/mL aprotinin. The lysates were homogenized by sonication for 20 s. The ALP activity was measured using an ALP kit. OC in culture media was measured using a RIA kit. To normalize protein expression to total cellular protein, a fraction of the lysate solution was used in a Bradford protein assay.

ELISA

Total MMP-1 (latent and active enzyme), latent MMP-2 (proMMP-2), and TIMP-1 levels in various conditioned media were measured using an ELISA kit. Normalization to cell protein was performed using harvested cells in a Bradford assay.

Activation of proMMP-2 Assay

To evaluate the effects of E_2 on the activation of proMMP-2, the levels of active MMP-2 in various conditioned media were measured using an MMP-2 activity assay system (Amersham Pharmacia). According to the manufacturer, the assay uses the pro form of a detection enzyme that can be activated by captured active MMP-2, converting proform into an active detection enzyme through a single proteolytic event. The natural activation sequence in the pro detection enzyme has been replaced using protein engineering, with an artificial sequence recognized by specific matrix metalloproteinases. MMP activated detection enzyme can then be measured using a specific chromogenic peptide substrate. In brief, standards and samples are incubated in microtiter wells precoated with anti-MMP-2 antibody. Any MMP-2 present will be bound to the wells, with other components of the samples being removed by washing and aspiration. The endogenous levels of free active MMP-2 can be detected. This assay is specific, quantitative, and can be applied to determine the endogenous levels of active MMP-2 in cell culture medium. Active MMP-2 captures were performed at 4°C overnight. After washing thoroughly, samples were incubated with detection reagent at 37°C for 1.5 h, and then read at 405nm in a microtiter plate spectrophotometer. The sensitivity of the assay is 0.75 ng. Cell protein determination for normalization was done by the Bradford method. Active MMP-2 levels were also normalized to cell total protein.

Statistical Analysis

Data are presented as the mean ± SEM. Values were analyzed using one-way ANOVA.

Results

Characterization of Human Osteoblasts

Cells obtained from collagenase digestion of human trabecular bone were characterized as osteoblast-like cells by several criteria including high intrinsic ALP activity and secretion of OC. ALP levels in normal human osteoblasts were nearly 20× greater than those in MG-63 cells (74.3 ± 4.7 versus 3.58 ± 0.28 nmol/min·mg protein). RIA of cul-

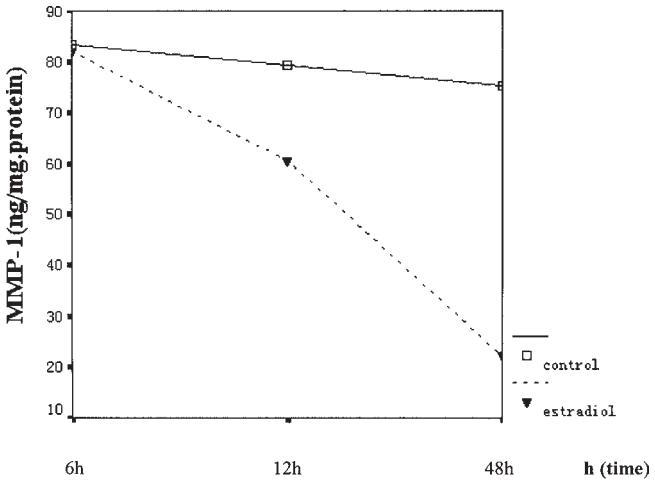

Fig. 1. Time course of MMP-1 secretion regulated by 17β-estradiol (E_2) in cultures of MG-63 cells. Cells were cultured as described in Material and Methods, and were exposed to various 10^{-8}M E_2 in indicated time. Conditioned media were subjected to ELISA analysis. Values are mean ± SEM obtained from four cultures and normalized to cell total protein.

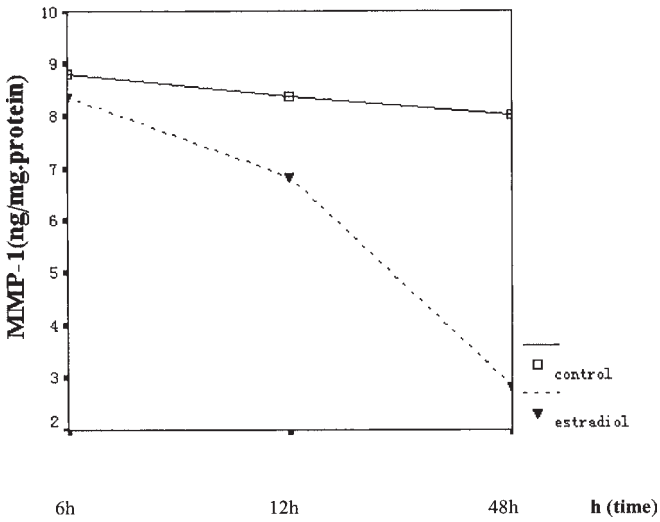

Fig. 2. Time course of MMP-1 secretion regulated by 17β-estradiol (E_2) in cultures of normal human osteoblasts (hOB). Cells were cultured as described in Material and Methods, and were exposed to various 10^{-8}M E_2 in indicated time. Conditioned media were subjected to ELISA analysis. Values are mean ± SEM obtained from four cultures and normalized to cell total protein.

Table 1
ELISA of MMP-1, proMMP-2 and TIMP-1 Production in Culture of MG-63 Cells and Normal Human Osteoblasts (hOB)

	MMP-1 (ng/mg·protein)		ProMMP-2 (ng/mg·protein)		TIMP-1 (ng/mg·protein)	
	MG-63 cells	hOB	MG-63 cells	hOB	MG-63 cells	hOB
Control	86.0 ± 6.1	8.0 ± 0.7	6949 ± 577	5938 ± 740	991 ± 21	639 ± 18
10^{-10}M E_2	49.8 ± 7.7*	4.3 ± 0.3***	5578 ± 313	5568 ± 125	913 ± 40	641 ± 9
10^{-8}M E_2	30.4 ± 4.2**	2.1 ± 0.3***	5616 ± 509	5487 ± 41	1037 ± 34	670 ± 14
10^{-6}M E_2	21.4 ± 3.7***	1.2 ± 0.2***	7135 ± 675	5863 ± 102	986 ± 18	643 ± 17

Cells were cultured as described in Material and Methods, and were exposed to various concentration of E_2 for 48 hours. Conditioned media were subjected to ELISA analysis. Values are mean ± SEM obtained from four cultures and normalized to cell total protein. *, $p < 0.05$, vs control; **, $p < 0.001$, vs control; ***, $p < 0.0001$, vs control.

ture supernatants from unstimulated human bone cells cultures revealed a detectable OC levels (3.84 ± 0.39 ng/mg protein).

Effect of E_2 on MMP-1, MMP-2 and TIMP-1 Protein

To determine the effect of E_2 on MMP-1, MMP-2 and TIMP-1 protein synthesis in cultures of MG-63 cells and normal hOB, ELISA were performed. A small decrease was observed after 12 h of exposure to E_2 in cultures of MG-63 cells and hOB, whereas treatment for 48 h caused a maximal inhibition of MMP-1 production (Fig. 1 and Fig. 2). The amounts of MMP-1 synthesized by MG-63 cells decreased with increasing doses of E_2 (Table 1). The levels of MMP-1 at 10^{-8}M E_2 concentration in hOB cultures were nearly 5× lower than control. E_2 had no effect on the levels of proMMP-2 and TIMP-1 protein in either MG-63 cells or hOB cultures.

Western blot analysis (Fig. 1) demonstrated a decrease in the levels of an approximately 57-kDa protein secreted by MG-63 cells with increasing doses of E_2. The intensity of the band at 10^{-8}M E_2 concentration was lower than control. At 10^{-6}M E_2 concentration, the intensity of the band decreased dramatically. Notably, the approximately 57-kDa bands were undetectable at 10^{-8}M E_2 concentration in hOB cultures, but appeared in hOB cultures without E_2 treatment. The mol wt of this band is consistent with that of the previously reported latent MMP-1 *(2)*. The results revealed that E_2 had no obvious effects on 72-kDa latent form of MMP-2 changes in MG-63 cells or hOB cultures. E_2 also had no effects on 28-kDa TIMP-1 in cultures of MG-63 cells or hOB.

Effect of E_2 on Activition of proMMP-2

The ability of E_2 to activate proMMP-2 in MG-63 cells or hOB cultures was determined by ELISA. The results

Table 2
ELISA of Activated MMP-2 Production in Culture of MG-63 Cells and Normal Human Osteoblasts (hOB)

	Activated MMP-2 (ng/mg·protein)	
	MG-63 cells	hOB
Control	7.1 ± 0.3	7.3 ± 0.3
10^{-10}M E_2	7.3 ± 0.5	6.8 ± 0.1
10^{-9}M E_2	6.6 ± 0.2	7.2 ± 0.2
10^{-8}M E_2	7.1 ± 0.6	7.4 ± 0.5

Cells were cultured as described in Material and Methods, and were exposed to various concentration of E_2 for 48 h. Conditioned media were subjected to ELISA analysis. Values are mean ± SEM obtained from four independent cultures and normalized to cell total protein. All $p > 0.05$, compared with control.

(Table 2) revealed that when treated with various concentration of E_2, MG-63 cells and hOB still produce unchanged activated MMP-2 ($p > 0.05$) These results strongly demonstrated that E_2 had no effect on the activation of proMMP-2.

Discussion

The present investigation was undertaken to determine whether E_2 regulates MMP-1, -2 or TIMP-1 expression in cultures of human osteoblastic MG-63 cells and normal hOB. We found that after 12–48 h of treatment, E_2 at 10^{-8}M decreased MMP-1 level in cultures of MG-63 cells and hOB, treatment with increasing dose of E_2 caused a dose-dependent decrease in expression of MMP-1 and protein in cultures of MG-63 cells and hOB. E_2 had no effect production of MMP-2 and TIMP-1 protein in MG-63 and hOB cultures. proMMP-2 activation assay revealed that MG-63 cells and normal hOB under the condition of E_2 treatment only secreted a 72kDa gelatin-degrading enzyme presenting proMMP-2, which indicated that E_2 has no effect on the activition of proMMP-2.

MMP-1 plays a important role in bone resorption. It was hypothesized that the activition of the osteoclast requires the direct contact of the osteoclast with a mineralized bone matrix *(6, 9, 10–13)*. Indeed, the mineralized bone surfaces are covered by a continous layer of cells of the osteoblastic lineage, either active ostoblasts at places of bone formation, inactive, resting osteoblasts, or lining cells that remain on the resting surfaces of the bone matrix when its formation and mineralization have ceased. The osteoclasts have to infiltrate themselves through this first cellular barrier in their progression toward the bone matrix, and may be prevented from reaching the bone surface by that barrier. Moreover, this osteoblastic cell layer, the mineralized bone matrix may be lined by a layer of osteoid or of a related type 1 collagenous matrix that is not mineralized and could prevent the efficient activation and action of the osteoclast, as these seem to require contact with the mineral. Osteoblasts under the influence of bone-resorption-stimulating hormones secrete MMP-1 which subserves the degradation of surface unmineralized type 1 collagen and thereby promotes osteoclast attaching to minerilized bone matrix and activation. Holliday et al. *(14)* recently reported that Collagenase cleavage of collagen, which resulted in a loss of helical collagen structure at 37°C, could expose $\alpha_V\beta_3$ binding sites, an event that may be involved in osteoclast activation. These results indicated that MMP-1 can function as a "coupling factor", allowing osteoclasts to initiate bone resorption by generating collagen fragments or degrading unmineralized collagous matrix, then promoting osteoclast attachment to mineralized collagenous matricies that activate osteoblasts. MMP-1 is the crucial collagenase known to degrade type 1 collagen at neutral pH in human osteoblast-like cells cultures *(15)*. The inhibiting action of E_2 on MMP-1 synthesis has pivotal significance in inquiries of the mechanism involved in postmenopausal osteoporosis, and estrogen replacement therapy in osteoporosis.

Our results of repression by E_2 on MMP-1 in human osteoblasic cells cultures suggested that in postmenopausal women, estrogen deficiency depresses the inhibiting action of E_2 on MMP-1 in osteoblasts, in turn leading to activation of osteoclasts by facilitated attachment to mineralized matrix, followed by acceleration of bone loss. Estrogen replacement could reduce osteoclast-mediated bone resorption by repressed release of MMP-1, which is considered a "coupling factor" that initiating bone resorption.

In conclusion, the present study has provided evidence that treatment with E_2 in human osteoblast-like cells caused a decrease in MMP-1 production. E_2 had no influence on the MMP-2 and TIMP-1 production in human osteoblast-like cells cultures, or activation of proMMP-2. It is probable that estrogen plays an important role in reducing bone resorption by inhibition of MMP-1 synthesis in osteoblasts. Increased MMP-1 production by osteoblasts, followed by estrogen deficiency, probably contribute to the mechanisms involved in postmenopausal osteoporosis.

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