Effects of 17β-Estradiol on the Expression of IL-6, IL-11 and NF-κB in Human MG-63 Osteoblast-like Cell Line

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Summary: In order to characterize the effects of 17β estradiol (17β-E2) on the expression of IL-6, IL-11 and NFκB in the human MG 63 osteoblast like cell line, the expression of IL-6 was detected by RT-PCR, Northern blot and Western blot. The expression of IL-11 was determined by RT-PCR, and NFκB by Western blot. The results showed that 17β-E2 down-regulated the expression of IL-6 mRNA and protein, IL-11 mRNA and NFκB protein in MG-63 cells. It was suggested that the expression of NFκB, IL-6 and IL-11 in MG-63 cells could be suppressed by 17β-E2, and this might lend support to estrogen replacement therapy in postmenopausal women.

Key words: IL-6; IL-11; NFκB; MG-63

Estrogen replacement therapy (ERT) is one of the treatment methods preventing rapid bone loss in postmenopausal women. Knowledge is incomplete on the exact mechanism by which estrogen protects bone. Osteoblasts express two kinds of estrogenic receptors (ERα, ERβ) suggesting that estrogen may act directly on osteoblasts and thus regulate bone metabolism. Recent studies have revealed that proinflammatory cytokines in interleukin (IL)-1, IL-6 and IL-11 all participate in regulating function of osteoclasts, accelerating differentiation and maturation of osteoclasts, and bone resorption. Hypercalcemia induced by up expression of IL-6 has been found in IL-6 transgenic mice; IL-1 and IL-6 secreted by human bone marrow cells were increased after menopause; Ligand binding subunit gp130 and signal transduction subunit gp130 of the IL-6 receptor were increased in emasculated rats. Estrogen was found to inhibit the IL-6 expression in human osteoblasts stimulated by IL-1 and tumor necrosis factor-α (TNF-α) and to also inhibit the expression of IL-6 induced by PTH. Although androgen was reported to inhibit the IL-6 expression in osteoblasts, it is unclear whether or not estrogen has the same effect on osteoblasts. Therefore, we used 17β-estradiol on the human osteosarcoma MG-63 cell line to observe the changes in the expression of IL-6, IL-11 and NFκB.

1 MATERIALS AND METHODS

1.1 Reagents

- Fetal bovine serum (FBS) and Trizol were purchased from GIBCO (USA); Phenol red-free DMEM, type I collagenase, ascorbic acid, L-glutamine, bovine serum albumin (BSA), 17β-estradiol (E2), coomassie brilliant blue G-250 and salmon sperm DNA from Sigma (USA); rabbit anti-IL-6 polyclonal IgG, goat anti-NFκB polyclonal IgG, hybrid liquor, nylon and nitrocellulose membranes, HRP labeled goat polyclonal IgG and ECL reagent kit from Santa Cruz Biotechnology Inc. (USA); Prestained protein molecular weight markers from New England Biolabs Inc. (Beverly, USA); Reverse Transcription System from Promega (USA); d32P-dCTP from Beijing Fu Rei Biotech Co. Ltd (China); Dimethylsulfoxide (DM-SO); GeneRuler™ 100 bp DNA Ladder, 4 × dNTP, 3 (N-Morpholine) propanesulfonic acid (MOPS) from Shanghai Shenggong company (China).

1.2 Cell Culture

The human osteosarcoma cell line MG-63 was purchased from ATCC (China) and maintained in phenol red free DMEM containing 10% FBS, 50 mg/mL ascorbic acid at 37 °C under an atmosphere of 5% CO₂. The medium was changed every 3 days.

1.3 Experimental Treatments

MG-63 cells were plated in 75 cm² culture flasks with phenol red-free DMEM containing 10% FBS until confluence. The medium was changed to serum-free medium containing 0.1% BSA for further culture for 24 h, then treated with various concentrations of 17β-E2 for 24 h. The cells treated with reagents were divided into the following 4 groups respectively: (1) control (no 17β-E2 treatment); (2) 17β-E2 10⁻¹⁰ mol/L; (3) 17β-E2 10⁻⁹ mol/L and (4) 17β-E2 10⁻⁸ mol/L. For time course analysis, cells were treated with 10⁻⁸ mol/L 17β-E2 and collected at 0, 3, 6, 12 and 24 h. Total RNA and protein were extracted from cells,
1.4 Semi-quantitative RT-PCR

MG-63 cells were lysed by Trizol reagent and total RNA was prepared. cDNA was synthesized by Gene Amp 2400 PCR system from 2 μg total RNA in 20 μL reaction mixture including 2 μL 10 X RT buffer, 4 μL 25 mmol/L MgCl₂, 1 μL oligo-dT, 2 μL dNTP, 0.5 μL RNase inhibitor and 1 μL AMV reverse transcriptase. The mixture was incubated at 42 °C for 60 min, 99 °C for 5 min, then iced. 20 μL of PCR reaction mixture contained 1 μL of the reverse transcription product as a template for amplifying IL-11 and β-actin gene. The primers and PCR conditions were as follows: for IL-11 sense: GACATGAACTGTGTTTGCCGC-CTGG, antisense: TTGTCAGCACACCTGG-AT and for β-actin: sense CCTCGCCTTTGCCGATCC, antisense: GGATCTTCATGAGGTAGTCAGTC, annealing at 56 °C and 28 cycles. PCR products were separated by electrophoresis in a 1.2 % agarose gel and stained with ethidium bromide. The target bands were analyzed densitometrically with Typhoon 2000 Imagequant 5.1.

1.5 Northern Blot Analysis

Total RNA extraction was conducted as previously described[10]. Then 20 μg of total RNA were electrophoresed on 1.2 % agarose formaldehyde-denaturing gels for 3 h at 60 V and transferred to a nylon membrane for 1.5 h, followed by vacuum drying for 2 h at 80 °C and storage at −20 °C. PCR products of IL-6 and β-actin were purified by purification kit and probes were prepared by RT-PCR. 20 μL of PCR reaction mixture contained 0.2 μL dCTP, 2 μL a32P-dCTP (20 μCi ), 0.5 μL dATP, 0.5 μL dGTP and 0.5 μL dTTP. The PCR condition was described above. After purification by Sephadex-G50, the specific activity of probes was examined by Wallac 1409 DSA liquid scintillation counter. The nylon membrane was inserted into a roller with 10 mL of hybrid liquid and denatured salmon sperm DNA for prehybridization for 1 h in a rotary hybridization oven at 68 °C, followed by hybridization with denatured probes for 3 h. The membrane was washed with 1 X SSC/0.5 % SDS and 0.5 X SSC/0.5 % SDS for autoradiography at −70 °C. The target bands were analyzed densitometrically with Typhoon 2000 Imagequant 5.1.

1.6 Western Blot Analysis

Total protein was extracted using Trizol reagent and quantified by the Bradford method. Western blot analysis for IL-6 and NF-κB has been described previously[11]. Briefly, aliquots of protein with 4X SDS loading buffer were denatured for 5 min at 100 °C, then separated by 10 %−12 % SDS-polyacrylamide gel and transferred to nitrocellulose filters. The filters were blocked with PBS-T containing 5 % skimmed milk, incubated for 4 h with rabbit anti-IL-6 polyclonal IgG (1: 1000 dilution) or goat anti-NF-κB polyclonal antibody (1: 1000 dilution), followed by the addition of HRP conjugate goat anti-rabbit IgG or donkey anti-goat IgG (1: 5000 dilution). Protein was visualized using the ECL chemiluminescence system. The density of bands was analyzed by Typhoon 2000 Imagequant 5.1.

1.7 Statistics

All experiments were repeated three times. The data were calculated and expressed as ±s. SPSS 11.0 statistical analyzing software was used. Single factor variance was used for group comparison where P < 0.05 was considered a significant difference.

2 RESULTS

2.1 IL-6 mRNA and Protein Expression in MG-63

Northern blot analysis showed that after 12 h of treatment with 10⁻⁸ mol/L 17β-E₂, the band of density for IL-6 was lower than that of the control (fig. 1), indicating that the IL-6 expression was affluent in MG-63 cells (the control), and downregulated by treatment with 10⁻⁴ mol/L or 10⁻³ mol/L 17β-E₂. Western blot analysis revealed that, compared to the control, the band density was similar to the control at 10⁻¹⁰ mol/L, but decreased at 10⁻⁸ mol/L or 10⁻⁶ mol/L (fig. 2), indicating the IL-6 protein expression was downregulated at 17β-E₂ at a concentration greater than 10⁻⁴ mol/L.

2.2 IL-11 mRNA Expression in MG-63 Cells

RT-PCR analysis demonstrated that the IL-11 mRNA expression was downregulated after treatment with 10⁻⁴ mol/L 17β-E₂ for 6 h. Compared with the control, downregulation was detected at 10⁻¹⁰ mol/L, showing a dose- and time-dependent increase (fig. 3).

2.3 NF-κB Protein Expression in MG-63 Cells

Western blot analysis indicated that the NF-κB protein expression was downregulated after treatment with 10⁻⁴ mol/L 17β-E₂ for 6 h. Compared with the control, downregulation was detected at 10⁻¹⁰ mol/L, showing a dose- and time-dependent increase (fig. 4).

3 DISCUSSION

Proinflammatory cytokines IL-6 and IL-11 can stimulate recruiting, proliferation and differentiation of osteoclast precursors, resulting in an increase in osteoclast number and enhancement of bone resorption activity. The aim of this study was to investigate the expression of IL-6 and IL-11 and their reaction to 17β-E₂ in the human osteosarcoma MG-63 cell line.

In our study, Northern and Western blot analyses revealed that IL-6 was expressed in MG-63 cells under steady state. However, the expression of IL-6 protein and IL-11 mRNA was downregulated after treatment with 17β-E₂. At present, no co-