17β-Estradiol Inhibits Expression of Human Interleukin-6 Promoter–Reporter Constructs by A Receptor-dependent Mechanism

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Abstract

We previously reported that 17β-estradiol inhibits cytokine-stimulated bioassayable IL-6 and the steady-state level of IL-6 mRNA. To determine the molecular basis of this effect, the transient expression of chloramphenicol acetyltransferase (CAT) reporter plasmid driven by the human IL-6 promoter was studied here in HeLa or murine bone marrow stromal cells (MBA 13.2). 17β-estradiol (10^{-8} M) completely suppressed stimulated CAT expression in HeLa cells cotransfected with IL-6/CAT constructs and a human estrogen receptor (hER) expression plasmid; but had no effect on reporter expression in HeLa cells not transfected with hER. 17β-estradiol also inhibited stimulated expression in MBA 13.2 cells (which express the estrogen receptor constitutively) without the requirement of cotransfection of the hER plasmid. The hormonal effects were indistinguishable between constructs containing a 1.2-kb fragment of the 5′ flanking region of the IL-6 gene or only the proximal 225-bp fragment. However, yeast-derived recombinant hER did not bind to the 225-bp segment in DNA band shift assays, nor did the 225-bp fragment compete for binding of an estrogen response element oligonucleotide to yeast-derived estrogen receptor. These data suggest that 17β-estradiol inhibits the stimulated expression of the human IL-6 gene through an estrogen receptor mediated indirect effect on the transcriptional activity of the proximal 225-bp sequence of the promoter. (*J. Clin. Invest.* 1994. 93:944–950.) Key words: cytokines • estrogen • stromal cells • osteoporosis • receptors

Introduction

IL-6 plays an important role in the replication and differentiation of hematopoietic cell precursors including the colony-forming units/granulocytes/macrophages (1–3), which are the presumed progenitors of the bone resorbing osteoclasts (4, 5). Moreover, deregulated production of IL-6 might be responsible for increased osteoclastogenesis in pathologic states associated with increased bone resorption, such as multiple myeloma (6) and Paget's disease (7).

Results from previous in vitro (8) as well as in vivo studies (9) of ours have also suggested a link between 17β-estradiol, IL-6, and the bone loss that is associated with loss of ovarian function. Specifically, we have found that IL-6 is produced by murine bone marrow–derived stromal cell lines, normal human bone–derived cells, and nontransformed osteoblast cell lines from mice and rats. In all these cell types, IL-6 production was stimulated in response to the combination of IL-1 and TNF. Addition of 17β-estradiol into these cultures caused inhibition of IL-1–and/or TNF-induced production of bioassayable IL-6 and the levels of the IL-6 mRNA. Based on these in vitro findings, we proceeded to test in vivo the hypothesis that estrogen loss upregulates osteoclastogenesis through an increase in the production of IL-6 in the microenvironment of the marrow. In support of this hypothesis, we found that loss of estrogens (ovariectomy) in mice caused upregulation of the formation of colony-forming units/granulocytes/macrophages colonies in short-term cultures of marrow cells from ovariectomized animals. Similarly, marrow cell cultures from ovariectomized mice exhibited consistently greater numbers of osteoclasts. Furthermore, we found that ovariectomy causes a significant increase in the number of osteoclasts present in sections of trabecular bone. All these changes could be prevented by a neutralizing antibody against IL-6, as well as by estrogen replacement, indicating a mediating role of IL-6 in the upregulated osteoclastogenesis, which ensues upon estrogen deficiency (9).

In the studies presented here, we have investigated the molecular basis of the inhibiting effect of 17β-estradiol on IL-6 production. To do this, we have used HeLa cells, a well-established cell model of induced IL-6 transcription by both phorbol esters and cytokines (10), as well as the murine bone marrow stromal cell line MBA 13.2, which, as we have shown earlier (8), expresses the IL-6 gene. We present evidence that 17β-estradiol, acting via its receptor, inhibits the stimulated expression of human IL-6 promoter–reporter constructs in both cell lines. However, this effect does not appear to be mediated by direct interaction of the human estrogen receptor with the IL-6 promoter.

Methods

Chemicals and reagents. 17β-estradiol, 17α-estradiol, progesterone, testosterone, and diethylstilbestrol (DES),[1] as well as phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human IL-1β was donated by the Biological Resources Branch of the Biological Response Modifiers Program, Division of Cancer Treatment/National Cancer Institute (Bethesda, MD) (specific activity = 1.8×10^7 U/mg). Recombinant murine TNF-α was purchased from Boehringer Mannheim Corp. (Indianapolis, IN) (specific activity = 6×10^7 U/mg). ^{125}I-16-α-iodo-3,17β-estradiol (2,200 Ci/mmol) was purchased from New England Nuclear

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1. *Abbreviations used in this paper:* DES, diethylstilbestrol; E_2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; IL-6 p-225, proximal 225 bp of the human IL-6 promoter; hER, human estrogen receptor; MBA 13.2, murine bone marrow stromal cells; PMA, phorbol 12-myristate 13-acetate.

(Boston, MA). D-[dichloroacetyl-1-^{14}C]chloramphenicol (50–60 mCi/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). Oligonucleotides were synthesized on a DNA synthesizer (model 381A; Applied Biosystems, Foster City, CA). Plasmid pCH110, which encodes β-galactosidase, was purchased from Pharmacia Pine Chemicals (Piscataway, NJ). pUC-19 was purchased from GIBCO/BRL (Gaithersburg, MD). pUCAT-1 was generously provided by Dr. Dan Spandau (Indiana University, Indianapolis, IN). The human estrogen receptor expression plasmid was generously provided by Dr. Pierre Chambon (INSERM, Unit 184, Strasbourg, France) (11, 12). Human estrogen receptor preparations from cytosol of yeast transfected with recombinant human estrogen receptor (hER) expression plasmid were generously provided by Dr. J. W. Pike (Ligand Pharmaceuticals, San Diego, CA).

Construction of reporter plasmids. The IL-6 promoter was amplified using human genomic DNA as the template. The upstream primer extended from bp −1,183 to −1,160 and contained a native restriction site for BamHI at bp −1,179. The downstream oligonucleotide was complementary to bp +9 through +31 of the IL-6 gene and contained an engineered restriction site for KpnI at bp +26. Conditions used for the amplification of this region of the IL-6 promoter were 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C for 30 cycles. The amplified product was electrophoresed on a 1% agarose gel. A discrete band of expected size 1.2 kb was isolated from the gel, digested with BamHI and KpnI, and subsequently cloned into pUCAT-1 to generate pIL-6/CAT 1 (Fig. 1). Confirmation of the insert's identity with the known sequence of the promoter was obtained by restriction mapping and sequencing using the chain termination method. To construct pIL-6/CAT 2, pIL-6/CAT 1 was digested with BamHI and NheI, the ends were filled in with Klenow fragment of DNA polymerase to create blunt ends and then religated.

Cells and culture conditions. HeLa cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in phenol red–free DME supplemented with 5% FCS. The MBA 13.2 bone marrow–derived murine stromal cell line (13) was provided by Dr. Zipori from the Weismann Institute (Rehovot, Israel) and were maintained in phenol red–free McCoy's 5A (Sigma Chemical Co.) medium supplemented with 10% FBS (Hyclone Laboratories, Logan, UT), penicillin (100 U/ml), and streptomycin (100 pg/ml) at 37°C in 5% CO_2 in air.

17β-Estradiol binding studies. The concentration of 17β-estradiol receptor sites in HeLa cells transfected with the hER expression plasmid or wild-type HeLa cells transfected with the vector alone was assayed using ^{125}I-17β-estradiol and a previously described method (14) with slight modifications. Briefly, 40 h after transfection cells were harvested by trypsinization and washed twice in PBS (120 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate pH 7.4), suspended in cold KETD-0.3 buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM leupeptin, and 0.3 M KCl) containing 1 mM sodium molybdate, sonicated on ice with 20-s bursts/10 × 10^6 cells (Sonifer cell disrupter; Heat Systems-Ultrasonics, Inc., Plainview, NY), and centrifuged for 1 h at 105,000 *g* in an ultracentrifuge (Beckman Instruments, Mountain View, CA). Cytosol aliquots (100 μl) were incubated at 4°C for 11 h (in triplicate) in tubes containing 0.05–1.0 nM ^{125}I-16-α-iodo-3,17β-estradiol; the specific activity of the steroid was adjusted to 400 Ci/mmol by the addition of the appropriate amount of unlabeled estradiol. Parallel sets of tubes were incubated in the presence of 100-fold molar excess of nonradioactive DES. At the end of incubation, bound and free steroids were separated by 15-min incubation with dextran-coated charcoal suspension on ice (1.25% Norit A and 0.125% dextran T-70 in 0.1% gelatin buffer containing 0.1 M NaH_2HPO_4, 0.15 M NaCl, and 0.015 M sodium azide), and subsequent centrifugation at 2,500 *g* for 20 min. Supernatants were counted using a gamma counter. Specific binding was determined by subtracting the mean cpm in the presence of DES from the mean cpm of the corresponding tubes containing only labeled estradiol. For the Scatchard analysis, the best fit line was determined by linear regression and a three test influence diagnostic (Cook's distance, leverage coefficient, and DFFITS) was performed to detect outliers that could have a disproportionately strong influence on the calculation of the regression line (15).

Transfections and chloramphenicol acetyltransferase (CAT) assays. HeLa or MBA 13.2 cells were grown on 10-cm tissue culture plates to 50–60% confluency until the day of transfection. 3 h before transfection, the cells were washed with PBS, and the medium was replaced with fresh medium containing no serum. Transfections were performed using the calcium phosphate precipitation method followed by glycerol shock (16). HeLa cells were transfected with 10 μg of the reporter plasmids (either pIL-6/CAT-1 or pIL-6/CAT-2) and either 5 μg of the estrogen receptor expression plasmid or 5 μg of the control plasmid pUC19. MBA 13.2 cells were transfected with the reporter plasmids alone. Both cell types were also transfected with 5 μg pCH110 to provide a measure of transfection efficiency. After transfection, HeLa cells were maintained in serum-free media in the absence or presence of 10^{-8} M of either 17β-estradiol, 17α-estradiol, progesterone, or testosterone for 16 h. MBA 13.2 cells were maintained in media enriched with 0.5% FCS, since complete absence of serum resulted in the detachment from the plastic dishes. At this stage, the cells were stimulated with either PMA (160 nM) or the combination of IL-1 (1.6 nM) and TNF (0.3 nM). 24 h later, cells were harvested and whole-cell extracts were prepared by three freeze/thaw cycles in TEN buffer (0.04 M Tris-HCl [pH 7.4], 1.0 mM EDTA, and 0.15 M NaCl).

Assay of reporter genes. CAT enzyme activity in cell extracts was determined after heat treatment (60°C for 10 min) to inactivate cellular deacetylation activity, as described earlier (17) using 0.1 μCi [^{14}C]-chloramphenicol, 1 μM acetyl CoA, and 50–100 μg of cellular protein extract in a total volume of 100 μl. The reaction was carried for 4 h at 37°C. Subsequently, the acetylated [^{14}C]chloramphenicol was separated from the unacetylated by thin layer chromatography in 95% chloroform/5% methanol. Radioactivity was quantified using a β scanner

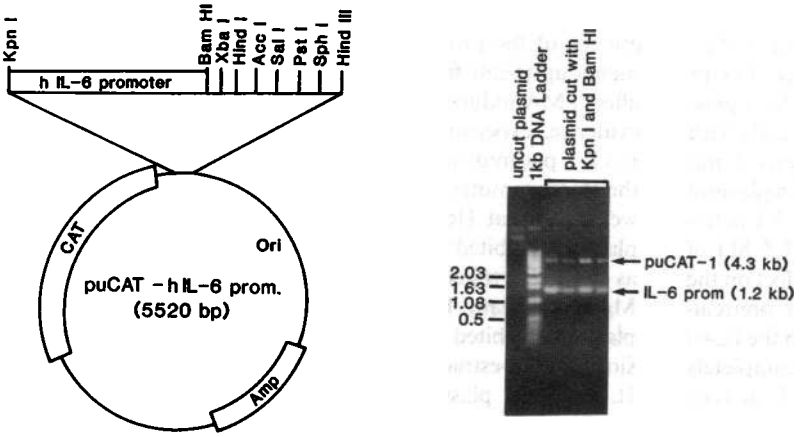

Figure 1. Isolation of the human IL-6 promoter and cloning into pUCAT-1. Primers (20–24 mers) flanking the 1.2-kb sequence of the human IL-6 gene promoter were engineered to contain BamHI and KpnI restriction sites at either end. They were then used to amplify the IL-6 gene promoter from human genomic DNA. The amplified product was subcloned first in pUC-19 and subsequently in pUCAT-1 plasmids. The gel of the right panel illustrates the electrophoretic mobility of the digestion products of the pUCAT/human IL-6 promoter with KpnI and BamHI. The uncut plasmid and a molecular weight DNA ladder are shown in lanes *1* and *2*, respectively. Confirmation of the insert's identity with the known sequence of the promoter was obtained by restriction mapping and sequencing.

(AMBIS Systems, Inc., San Diego, CA). β-Galactosidase activity of the cell extracts was determined colorimetrically from the rate of hydrolysis of *o*-nitrophenylgalactoside. β-Galactosidase activity was not significantly different in control cells (0.1295±0.025 U/mg protein) compared to PMA-treated cells (0.1279±0.037 U/mg protein, $P > 0.5$). Further, β-galactosidase activity was also not affected by treatment with 17β-estradiol alone (0.1187±0.035 U/mg protein), 17β-estradiol + PMA (0.1227±0.042 U/mg protein), IL-1 + TNF (0.1371±0.087 U/mg protein), or IL-1 + TNF + 17β-estradiol (0.1317±0.094 U/mg protein). Values obtained for CAT activity (percent of acetylation) were normalized for β-galactosidase activity. Activities described in the text and figures are measured in units of percent acetylation per units of β-galactosidase activity.

Preparation of nuclear extracts and electrophoretic mobility shift assays. Nuclear extracts were obtained as previously described (18). Briefly, cells were harvested, pelleted at low speed, and disrupted with a Dounce homogenizer in hypotonic buffer. The extract was then obtained by adding high salt buffer (containing Hepes [pH 7.9] $MgCl_2$, 0.8–1.6 M KCl, EDTA, and protease inhibitors). After gentle mixing for 30 min, the nuclear debris was pelleted and the supernatant saved as the extract. The extract was dialyzed to a KCl concentration of 100 mM. An NheI/KpnI fragment (bp −225 to +26) of the IL-6 promoter was gel purified and labeled at the NheI site with T4 polynucleotide kinase and [^{32}P]dATP. The ^{32}P-labeled DNA probe (0.1–1 ng) was then incubated for 15 min at room temperature with 20 μg of nuclear extract along with a final concentration of 300 μg/ml of BSA and 2 μg/ml of poly (dI-dC) in 10% glycerol solution (12 mM Hepes, 4 mM, Tris-HCl, 60 mM KCl, 1 mM EDTA, and 1 nM DTT [pH 7.9]). The resulting DNA-protein complexes were resolved by non-denaturing polyacrylamide gel (4%) electrophoresis. The sequence of the double-stranded estrogen response element (ERE) oligonucleotide used in the electrophoretic mobility shift assays was the vitellogenin consensus ERE {5'GATCTCAGGGCACAGTGACCTGA3'}.

Results

Before determining the effect of 17β-estradiol on the IL-6 promoter activity, the level of the 17β-estradiol receptor expressed by the cells used in these experiments was determined by means of ^{125}I-17β-estradiol saturation binding analysis. Wild-type HeLa cells did not exhibit measurable receptor binding; however, transient transfection of the HeLa cells with the hER expression plasmid caused the appearance of readily measurable 17β-estradiol binding (Fig. 2). Scatchard analysis of this binding yielded a K_d of $\sim 4.5 \times 10^{-10}$ M and a maximal concentration of binding sites of 17.7 fmol/mg protein. In studies reported earlier, we determined that MBA 13.2 cells, unlike HeLa cells, constitutively express estrogen receptors; with a $K_d = 1.2 \times 10^{-10}$ M and a maximal concentration of binding sites of ~ 1.0 fmol/mg protein (19).

The results of a representative experiment examining the effect of 17β-estradiol on the activity of the IL-6/CAT 1 plasmid are illustrated in Fig. 3 *A*. CAT activity was very low in unstimulated HeLa cells transfected with the IL-6/CAT 1 plasmid (lanes *3* and *4*); however, stimulation of the cells with PMA caused a marked increase in CAT activity (lanes *5* and *6*). Cotransfection of the cells with the hER expression plasmid had no discernable effect on the PMA-stimulated CAT activity. Similarly, pretreatment with 17β-estradiol (10^{-8} M) of cells transfected only with the IL-6/CAT 1 had no effect on the activity of the reporter enzyme (lanes *7–9*). However, pretreatment with 17β-estradiol of cells transfected both with the IL-6/CAT 1 plasmid and the hER expression plasmid completely prevented the PMA-induced stimulation of CAT activity (lanes *10* and *11*).

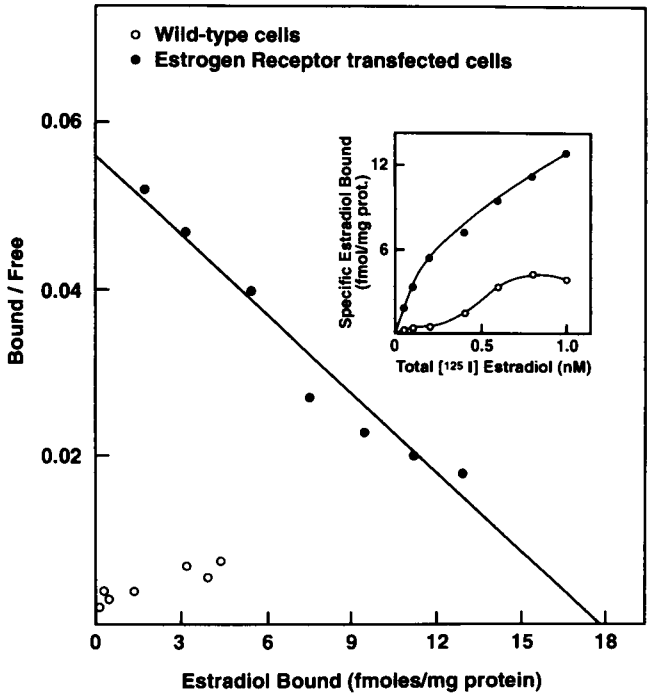

Figure 2. Binding of ^{125}I-estradiol to wild type HeLa cells and HeLa cells transfected with the hER expression plasmid. (*Inset*) 100-μl aliquots of cytosol from each cell preparation were incubated with increasing concentrations of ^{125}I-16-α-iodo 3,17β-estradiol alone or in the presence of 100-fold molar excess of nonradioactive diethylstilbestrol for 11 h at 4°C. Bound estradiol was separated from free estradiol with 1 ml of ice cold dextran-coated charcoal suspension. Specific binding was derived by subtracting nonspecific from total binding and was used to generate the Scatchard plot. Each point represents the mean of triplicate determinations. Analysis of the Scatchard plot demonstrated essentially no specific binding of estradiol to wild type HeLa cells. HeLa cells transfected with the hER expression plasmid expressed estrogen binding sites at a concentration of 17.7 fmol/mg of protein with a K_d of $\sim 4.5 \times 10^{-10}$ M.

Similar results to those shown in Fig. 3 *A* were obtained in five additional experiments, the results of which are summarized in Fig. 3 *B*. The degree of stimulation by PMA in the experiments varied widely. Nevertheless, qualitatively similar observations regarding the inhibitory effect of 17β-estradiol on the expression of the plasmid and the requirement of the ER for this effect, were confirmed in each one.

It had been demonstrated earlier that the PMA response element of the IL-6 gene lies within the proximal 225-bp sequence of the promoter; and that deletion of promoter elements upstream from the −225 bp of the promoter did not affect PMA-induced activation of this gene (10). Based on this evidence, experiments were also performed using the IL-6/CAT 2 plasmid, which contains only the proximal 225 bp of the IL-6 promoter. In agreement with the published evidence, we found that HeLa cells transfected with the IL-6/CAT 2 plasmid exhibited the same level of PMA-stimulated activation as HeLa cells transfected with the full-length promoter (Fig. 4). More important, HeLa cells transfected with the IL-6/CAT 2 plasmid exhibited a similar degree of inhibition of CAT expression by 17β-estradiol as the HeLa cells transfected with the IL-6/CAT 1 plasmid. Moreover, in full agreement with the results of the cells carrying the IL-6/CAT 1 plasmid, the effect

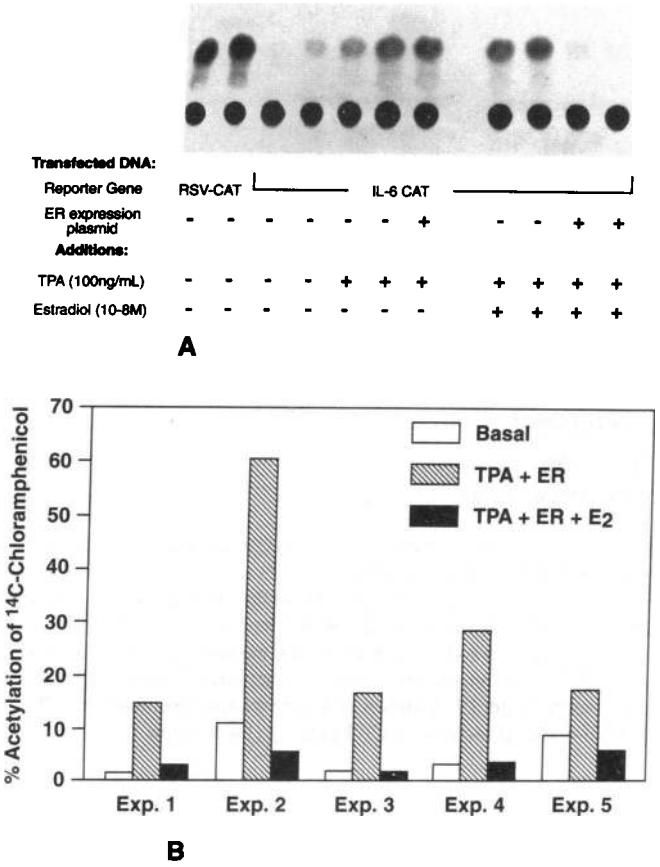

Figure 3. 17β-estradiol inhibits stimulated transcription from the human IL-6 promoter in transfected HeLa cells. HeLa cells were transfected with the IL-6/CAT 1 construct alone or with IL-6/CAT 1 and an hER expression plasmid using the calcium phosphate precipitation method. After transfection, cells were placed in serum and phenol red–free medium in the absence or presence of 10^{-8} M 17β-estradiol (E_2). 16 h later, cells were exposed to 160 nM PMA for 24 h, after which cytosolic extracts were prepared and used for the CAT assay. The results of a representative experiment are shown in (*A*). CAT activity was increased 4.5-fold by the addition of PMA (lanes *5–6*) compared to untreated cells (lanes *3–4*). Cotransfection with hER plasmid in the absence of E_2 or addition of E_2 in the absence of hER did not affect PMA-induced IL-6/CAT 1 activation (lanes *7–9*). Addition of E_2 to HeLa cells cotransfected with the hER blocked the PMA-induced increase in CAT activity (lanes *10–11*). The results of five additional experiments demonstrating the inhibition of PMA-stimulated IL-6/CAT 1 expression by E_2 are summarized in 3(*B*). Although there was significant variation in the degree of PMA-stimulated IL-6/CAT 1 expression in these experiments, E_2 consistently decreased IL-6/CAT 1 expression to baseline levels.

of the hormone on cells transfected with the IL-6/CAT 2 plasmid could only be demonstrated in cells cotransfected with the hER expression plasmid (lane *6*). These results were reproduced in three additional experiments. The mean value of the PMA-stimulated CAT activity in cells transfected both with the IL-6/CAT 2 plasmid and the hER plasmid, and treated with estradiol (1.5±0.5), in these four experiments, was significantly less ($P < 0.05$), as compared to cells transfected only with the IL-6/CAT 2 and not treated with estradiol (9.1±4.3); or cells that were not transfected with hER plasmid and treated with estradiol (5.9±3.4); or cells cotransfected with the hER plasmid but not treated with the hormone (5.3±3.0). 17β-Estradiol also reduced CAT activity in HeLa cells cotransfected with the IL-6/CAT 2 plasmid and the hER plasmid, and stimulated by the combination of the cytokines IL-1 (1.6 nM) and TNF (0.3 nM), instead of PMA (data not shown).

The specificity of the effect of 17β-estradiol on the inhibition of PMA-induced IL-6/CAT 2 expression in HeLa cells cotransfected with hER plasmid was examined by comparing the effect of 17β-estradiol with the effects of 17α-estradiol, progesterone, or testosterone. The results from two replicate experiments are illustrated in Fig. 5. Unlike 17β-estradiol, 17α-estradiol was completely ineffective. However, progesterone and testosterone (10^{-8} M) also inhibited CAT activity but their effect was considerably less pronounced than that of 17β-estradiol. The relative potency of progesterone and testosterone was very similar to the relative potency of these steroids in inhibiting cytokine-stimulated bioassayable IL-6 by murine bone marrow stromal cells (8).

The murine bone marrow–derived stromal cell line MBA 13.2 expresses IL-6 endogenously and constitutively expresses the estrogen receptor. Moreover, cytokine-stimulated production of bioassayable IL-6 by these cells is inhibited by 17β-estradiol (8). Based on this and the results from the experiments with HeLa cells (Figs. 3 and 4), we proceeded to study the transient expression of IL-6/CAT constructs in the MBA 13.2 cells. In agreement with the observations from the HeLa cells, 17β-estradiol completely inhibited PMA-stimulated IL-6/CAT 2 expression (Fig. 6). In four replicate experiments, the mean value of the PMA-stimulated CAT activity in estradiol-treated cells (0.65±0.58) was significantly less ($P < 0.01$) compared to cells not treated with estradiol (8.35±6.58). Similar results were reproduced using the IL-6/CAT 1 plasmid

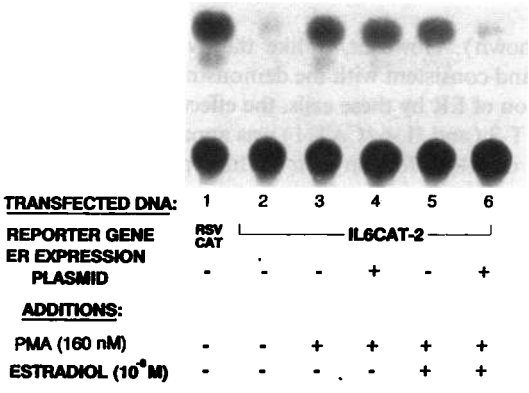

Figure 4. 17β-estradiol inhibits stimulated transcription from the proximal 225 bp of the human IL-6 promoter in transfected HeLa cells. HeLa cells were transfected with the IL-6/CAT 2 construct alone or with both the IL-6/CAT 2 and an hER expression plasmid, using the calcium phosphate precipitation method. After transfection, the cells were incubated as in Fig. 3 in the absence or presence of 10^{-8} M E_2, stimulated with 160 nM PMA for 24 h, and cytosolic extracts were prepared for the CAT assay. Addition of PMA resulted in an eightfold increase in CAT activity (lane *3*) compared to unstimulated cells (lane *2*). Cotransfection of HeLa cells with the hER plasmid in the absence of E_2 or addition of E_2 in the absence of hER did not significantly effect PMA-induced IL-6/CAT 2 activations (lanes *4* and *5*). Addition of E_2 to HeLa cells cotransfected with the hER blocked the PMA-induced increase in IL-6/CAT 2 activation (lane *6*).

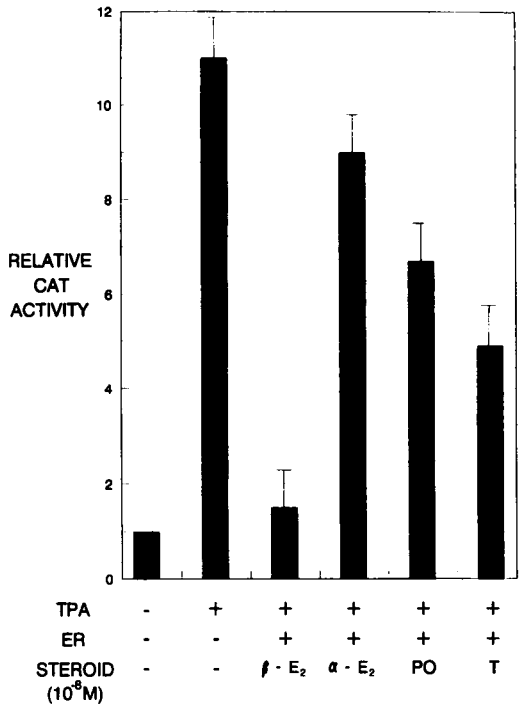

Figure 5. Specificity of the inhibition of phorbol ester–induced IL-6/CAT 2 expression by 17β-estradiol in HeLa cells. HeLa cells were cotransfected with both the IL-6/CAT 2 plasmid and the hER expression plasmid as previously described. PMA-induced stimulation of CAT activity was significantly inhibited by 17β-estradiol (β-E_2) but was not affected by equimolar concentrations (10^{-8} M) of 17α-estradiol (α-E_2). Progesterone (*PO*) at 10^{-8} M and testosterone (*T*) at 10^{-8} M, partially inhibited the PMA-induced stimulation of CAT activity in a manner corresponding to their respective affinities for the estrogen receptor.

(data not shown). However, unlike the evidence from the HeLa cells, and consistent with the demonstration of constitutive expression of ER by these cells, the effect of 17β-estradiol on IL-6/CAT 2 (and IL-6/CAT 1) was apparent without the requirement of cotransfection with the hER plasmid. Furthermore, and in agreement with our previous results at the protein level, IL-1 (1.6 nM) + TNF (0.3 nM) caused a sixfold increase in expression of the IL-6/CAT 2 construct in the MBA 13.2 cells (Fig. 7). This effect was completely inhibited by preincubation of the MBA 13.2 cells with 17β-estradiol.

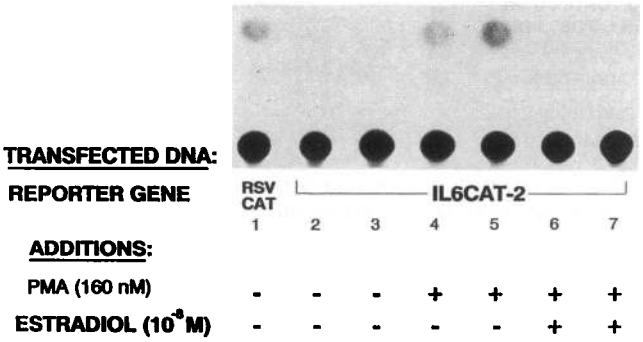

Figure 6. 17β-estradiol inhibits phorbol ester–induced IL-6/CAT 2 expression in the murine bone marrow–derived stromal cell line MBA 13.2. MBA 13.2 cells were transfected with the IL-6/CAT 2 plasmid and stimulated with PMA in the presence or absence of 10^{-8} M E_2. PMA induced a 15-fold increase in the expression of CAT activity in the MBA 13.2 cells (lanes *4* and *5*) compared to unstimulated cells (lanes *2* and *3*). Addition of E_2 completely eliminated the PMA-induced increase in CAT activity (lanes *6* and *7*).

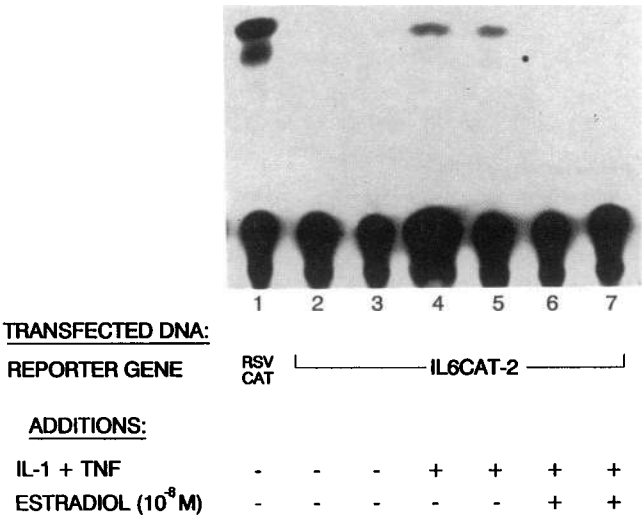

Figure 7. 17β-estradiol inhibits IL-1 + TNF-induced IL-6/CAT 2 expression in MBA 13.2 cells. MBA 13.2 cells were transfected with the IL-6/CAT 2 plasmid and stimulated with the cytokines IL-1 (1.6 nM) and TNF (0.3 nM) in the presence or absence of 10^{-8} M E_2. IL-1 + TNF induced a sixfold increase in the expression of CAT activity in the MBA 13.2 cells (lanes *4* and *5*) compared to unstimulated cells (lanes *2* and *3*). Addition of E_2 completely eliminated the IL-1 + TNF–induced increase in CAT activity (lanes *6* and *7*).

Finally, to investigate whether the ER-mediated effect of 17β-estradiol was caused by direct interaction of the receptor ligand complex with the IL-6 promoter sequence, we studied the ability of the 225-bp fragment to compete for binding of the yeast-derived hER to a labeled ERE. As shown in Fig. 8, the control yeast extract did not contain factors that specifically shifted the ERE. Extracts of yeast expressing hER retarded the ERE oligonucleotide, and this binding could be competed by excess unlabeled ERE. The unlabeled 225-bp fragment of the IL-6 promoter, however, failed to compete with the ERE (Fig. 8). Gel retardation assays were also performed using the 225-bp fragment as the labeled probe (Fig. 9). Nuclear extracts from either HeLa or MBA 13.2 cells formed three specific protein DNA complexes that were readily competed by the unlabeled probe. However, formation of these complexes could not be prevented by incubation with the consensus estrogen response element {GATCTCAGGTCACAGTGACCTGA}. Finally, incubation of the labeled 225-bp probe with cytosol preparations from yeast transfected with recombinant hER expression plasmid failed to form a retarded band in DNA gel shift assays (data not shown), suggesting that the hER does not bind to this DNA segment, although it inhibits the expression of the promoter–reporter construct.

Discussion

The expression of the pleiotropic cytokine IL-6 is regulated by many agents, including phorbol esters (PMA), cytokines such as IL-1 and TNF (20), as well as systemic hormones such as

parathyroid hormone and the parathyroid hormone related peptide (21). Although the expression of IL-6 is tightly controlled, upregulation of its production is now recognized to play a critical role in the pathogenesis of several disease states (6, 7).

The data presented in this paper represent an extension of our earlier observations that cytokine-stimulated IL-6 production by bone marrow and bone-derived cells from rodents and humans is inhibited by 17β-estradiol (8). Specifically, the present results establish that 17β-estradiol inhibits IL-6 production through a potent effect of the hormone on the activity of the IL-6 promoter in transfection assays. The demonstration of this effect using the human IL-6 promoter–reporter constructs in human cells (HeLa) indicates that the molecular mechanism elucidated in our studies is relevant to human biology. In addition, the confirmation of the findings in a bone marrow–derived cell line indicates that these observations are relevant to the role of IL-6 in bone homeostasis. Furthermore, taken together with the in vivo evidence for the mediating role of IL-6 in the osteoclastogenesis that ensues upon ovariectomy in mice (9), the observations of this paper strongly support the contention that the regulation of IL-6 by 17β-estradiol is indeed relevant to the bone changes associated with the loss of ovarian function in humans.

The 1.2-kb fragment of the 5′ flanking region of the IL-6 gene contains three transcription start sites and all the necessary elements for induction of the IL-6 gene by a variety of stimuli (22). Deletion analysis previously performed by others indicates that the proximal 225-bp segment of the promoter is involved in the inducibility of the IL-6 gene by phorbol esters and cytokines in transient transfection assays in HeLa cells (10). Based on this evidence, we have examined the effect of

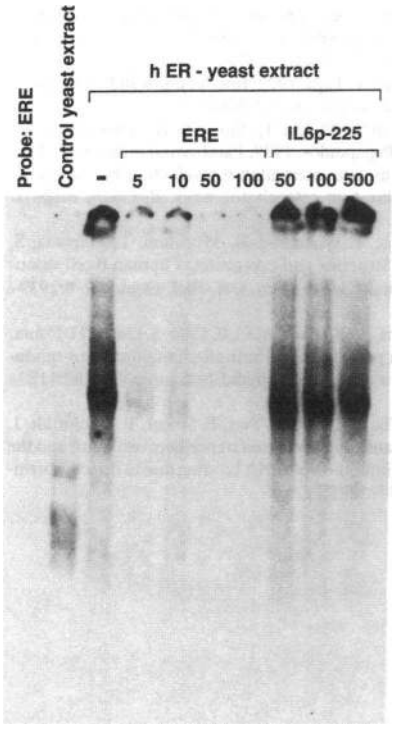

Figure 8. ER binding to an ERE is not inhibited by the human IL-6 promoter. Cytosolic extracts prepared from yeast expressing a transfected hER were incubated with a ^{32}P-labeled ERE {GATCT-CAGGTCACAGT-GACCTGA} in the presence or absence of 5, 10, 50, or 100 × molar excess of unlabeled ERE or in the presence or absence of 50, 100, or 500 × molar excess of the proximal 225 bp of the IL-6 promoter (IL-6 p-225). Cytosolic extracts from nontransfected yeast were used as a control. The hER-yeast extract produced two specific bands when incubated with the ERE. These bands were effectively competed by unlabeled ERE, but were not affected by up to a 500-fold excess of IL-6 p-225.

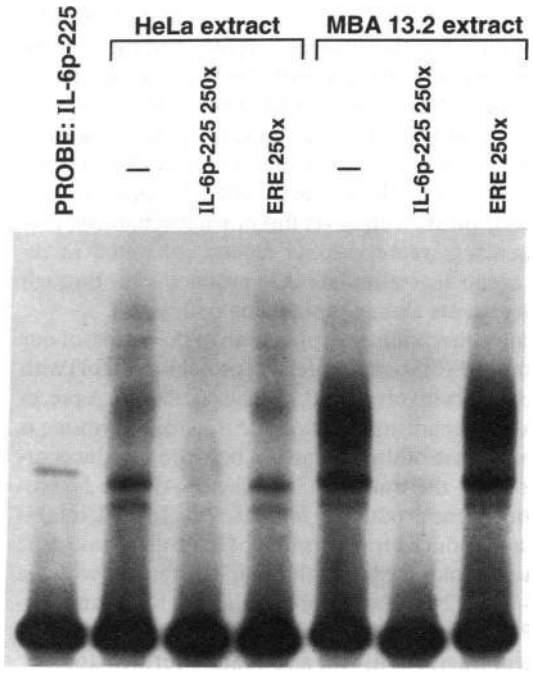

Figure 9. Lack of an effect of an ERE oligonucleotide on the binding of HeLa and MBA 13.2 cell nuclear extracts to the proximal 225 bp of the human IL-6 promoter. Nuclear extracts prepared from HeLa and MBA 13.2 cells were incubated with ^{32}P-labeled IL-6 p-225 and separated on a nondenaturing polyacrylamide gel. Binding of the IL-6 p-225 to the HeLa and MBA 13.2 extracts was competed off by addition of 250 × molar excess of unlabeled IL-6 p-225, but not by 250 × molar excess of a consensus ERE oligonucleotide.

17β-estradiol on the induced expression of the IL-6 promoter–CAT constructs containing either the entire promoter (IL-6/CAT 1) or its proximal 225 bp (IL-6/CAT 2). We found that 17β-estradiol had no effect on the activity of either construct in HeLa cells, unless these cells were cotransfected with an hER expression plasmid. This finding indicates that the effect of the hormone requires the presence of the estrogen receptor (ER). Consistent with this, we found that 17β-estradiol inhibited the expression of the IL-6/CAT 2 plasmid in MBA 13.2 cells that express the ER constitutively, without the requirement of cotransfection with an hER expression plasmid. Additional support for the contention that the effect of estradiol on the expression of these constructs is mediated by the estrogen receptor was provided by the results of the specificity experiments, which indicated that the relative potency of progesterone and testosterone in inhibiting expression corresponds with their respective affinity for the estrogen receptor. Intriguingly, in spite of the substantial difference in the level of expression of the hER in the HeLa cells transfected with the hER expression plasmid (17 fmol/mg protein), as compared to the MBA 13.2 cells that express the ER constitutively (1 fmol/mg), the magnitude of the effect of 17β-estradiol on the expression of the IL-6/CAT constructs was indistinguishable in these two cell types. This finding raises the possibility that the relatively low levels of estrogen receptor observed in bone and bone marrow cells, as compared to the classical target tissues for estradiol (breast or uterus), are adequate for estrogen responsiveness and, therefore, consistent with the great sensitivity of bone to estrogens.

In these experiments, we were unable to demonstrate a direct interaction between the estrogen receptor and the human IL-6 promoter. This is consistent with the fact that the proximal 225 bp of the IL-6 promoter, which drives transcription of the IL-6/CAT 2 construct, lacks a classical estrogen response element. Therefore, the inhibition of expression of IL-6 promoter–CAT fusion genes by the activated ER appears to be exerted indirectly through an as yet unknown mechanism, perhaps by influencing transcriptional factors generated in response to the agents that stimulate IL-6 production or through effects on other events along the signaling pathway.

An alternative mechanistic explanation of the results of our studies is that the hER interacts directly (protein–protein) with other *trans* activators involved in IL-6 gene regulation. A precedent for such a mechanism of action for a steroid hormone is established in the case of the interaction between the glucocorticoid receptor and the transcriptional factor AP-1, a heterodimer of the oncogene products c-*fos* and c-*jun*. Indeed, inhibition of basal and induced transcription of the collagenase gene by glucocorticosteroids is exerted through interference with the action of AP-1 (23, 24). The possibility that the hER can interfere with AP-1 or other transcription factor activity through protein–protein interactions will also require further studies.

In conclusion, the results presented here indicate that 17β-estradiol inhibits IL-6 production via a receptor-mediated action that results in the inhibition of expression of a reporter construct driven by the IL-6 promoter. It seems likely, but is yet unproven, that this effect occurs at the level of gene transcription. However, this effect does not appear to be mediated by direct interaction of the hER with the IL-6 promoter, but perhaps through an interference with events along the signaling pathways initiated by the IL-6–stimulating agents.

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