Journal of Cellular Biochemistry 83:448–462 (2001)

Estrogen Regulation of Human Osteoblast Function is Determined by the Stage of Differentiation and the Estrogen Receptor Isoform

Katrina M. Waters,[1] David J. Rickard,[1]* B. Lawrence Riggs,[2] Sundeep Khosla,[2] John A. Katzenellenbogen,[3] Benita S. Katzenellenbogen,[4] John Moore,[5] and Thomas C. Spelsberg[1]

[1]Department of Biochemistry and Molecular Biology, Division of Endocrinology, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55905
[2]Department of Internal Medicine, Division of Endocrinology, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55905
[3]Department of Chemistry, University of Illinois, Urbana, Illinois 61801
[4]Department of Molecular and Integrative Physiology, University of Illinois, Urbana, Illinois 61801
[5]Glaxo Wellcome Research, Research Triangle Park, North Carolina 27709

Abstract Although osteoblasts have been shown to respond to estrogens and express both isoforms of the estrogen receptor (ERα and ERβ), the role each isoform plays in osteoblast cell function and differentiation is unknown. The two ER isoforms are known to differentially regulate estrogen-inducible promoter-reporter gene constructs, but their individual effects on endogenous gene expression in osteoblasts have not been reported. We compared the effects of 17β-estradiol (E) and tamoxifen (TAM) on gene expression and matrix formation during the differentiation of human osteoblast cell lines stably expressing either ERα (hFOB/ERα9) or ERβ (hFOB/ERβ6). Expression of the appropriate ER isoform in these cells was confirmed by northern and western blotting and the responses to E in the hFOB/ERβ6 line were abolished by an ERβ-specific inhibitor. The data demonstrate that (1) in both the hFOB/ER cell lines, certain responses to E or TAM (including alkaline phosphatase, IL-6 and IL-11 production) are more pronounced at the late mineralization stage of differentiation compared to earlier stages, (2) E exerted a greater regulation of bone nodule formation and matrix protein/cytokine production in the ERα cells than in ERβ cells, and (3) the regulated expression of select genes differed between the ERα and ERβ cells. TAM had no effect on nodule formation in either cell line and was a less potent regulator of gene/protein expression than E. Thus, both the ER isoform and the stage of differentiation appear to influence the response of osteoblast cells to E and TAM. J. Cell. Biochem. 83: 448–462, 2001.

Key words: ER alpha and beta; bone cells; maturation; mineralization; cytokine synthesis; osteoporosis

Estrogen (E) deficiency is the principal cause of postmenopausal osteoporosis and although increased bone resorption by osteoclasts is the major mechanism responsible for this bone loss, direct effects of E on bone forming osteoblasts may also be important [Spelsberg et al., 1999]. For example, the action of numerous cytokines and hormones, including E, on osteoclast formation and activity are mediated in part via the osteoblast. Although estrogen receptors (ER) and E responses have been reported in primary osteoblasts and osteoblast cell lines, the generally small and often conflicting responses observed have been attributed to the low and variable ER concentrations in the model systems used [Ernst et al., 1991; Davis et al., 1994]. The discovery of a second ER isoform, ERβ, in addition to the classical ER (ERα) has further added to the complexity of E responses and may

K.M.W. and D.J.R. are co-first authors on this paper

Grant sponsor: NIH ; Grant number: AG04875; Grant sponsor: NIH (to K.M.W., D.J.R.); Grant number: CA09441; Grant sponsor: NIH (to J.A.K.); Grant number: DK15563; Grant sponsor: NIH (to B.S.K.); Grant number: CA18119; Grant sponsor: Mayo Foundation.

Katrina M. Waters's present address is Division of Endocrine, Reproductive and Developmental Toxicology, CIIT Centers for Health Research, Research Triangle Park, NC 27709.

*Correspondence to: David J. Rickard, Ph.D., Department of Biochemistry and Molecular Biology, Mayo Clinic and Mayo Medical School, 200 First Street, S.W, Rochester, MN 55905.

Received 15 June 2001; Accepted 20 June 2001

DOI 10.1002/jcb.1242

explain the reports in the literature regarding the variable effects of E treatment on osteoblasts [Rickard et al., 1999].

The ERα and ERβ isoforms are products of different genes and are most divergent in their N-terminal domains which possess transactivating activity and interact with other factors of the transcriptional apparatus [Kuiper and Gustafsson, 1997]. The two isoforms have been shown to exhibit different ligand binding affinities and tissue distribution [Kuiper et al., 1997], and, acting as homodimers, exert differential transcriptional regulation at E-responsive DNA elements and promoters [Paech et al., 1997; Saville et al., 2000]. In addition, one report has shown that under some circumstances ERβ inhibits the transcriptional activity of ERα, possibly through ERα/β heterodimer formation [Hall and McDonnell, 1999]. Taken together, these findings suggest that the response of a cell to E is dependent, in part, upon the relative ratios of ERα and ERβ and that the isoforms may perform different functions in cells.

In bone, ERα and ERβ have been localized primarily to osteoblasts and growth plate chondrocytes, with lower levels in osteocytes and osteoclasts [Kusec et al., 1998; Vidal et al., 1999; Braidman et al., 2001]. However, despite their mostly overlapping pattern of expression in bone, adult mice with null mutations in either the ERα or ERβ genes possess distinct skeletal abnormalities [Korach et al., 1997; Windahl et al., 1999], thereby confirming that both ER isoforms play important roles in the skeleton and are not functionally equivalent. In this regard, in osteoblasts it is unknown whether E elicits the same or different endogenous gene responses depending upon the ER isoform.

The expression levels for ERα and ERβ have both been reported to increase during rat and human osteoblast differentiation in culture [Arts et al., 1997; Bodine et al., 1998]. That the strongest mRNA and protein expression for the ERs in bone tissue has been detected in differentiated osteoblasts opposed to the bone surface [Kusec et al., 1998; Vidal et al., 1999; Braidman et al., 2001], is therefore in agreement with findings in vitro. Moreover, differential gene regulation by E has been associated with the differentiation state in both rat and human osteoblast cells expressing ERα [Rao et al., 1994; Bodine et al., 1998]. However, it is unknown whether similar changes in E responsiveness occur during the differentiation of osteoblasts expressing ERβ.

The aims of this investigation were, therefore, to determine the effects of the differentiation state and the ligand (E vs. the selective estrogen receptor modulator tamoxifen [TAM]), on endogenous cellular and gene responses in human osteoblast cell lines stably transfected with ERα or ERβ. Additionally, we sought to determine whether the ERα and ERβ-mediated responses differ within the same stage of differentiation. The hFOB/ER cell lines used in this study are capable of complete differentiation from active cell proliferation, matrix synthesis following cessation of proliferation, and finally matrix mineralization [Robinson et al., 1997]. In this paper we have identified differentiation-dependent effects of E on several parameters in both ERα-expressing (hFOB/ERα9) and ERβ-expressing (hFOB/ERβ6) cells, including alkaline phosphatase activity and the production of interleukin (IL)-6 and IL-11. Additionally, differential responses to E and TAM were noted between osteoblasts expressing ERβ compared with cells expressing ERα, particularly in the rate of formation of a mineralized matrix. Our data suggest that the response of human osteoblasts to E is affected both by the state of cell differentiation and the particular ER isoform present, as well as by species of the ligand and promoter composition.

MATERIALS AND METHODS

Materials

Cell culture reagents and steroids were obtained from Sigma Chemical Co. (St. Louis, MO), with the exception of the charcoal-stripped fetal bovine serum (FBS), which was obtained from HyClone Laboratories (Logan, UT). Neomycin G418 (geneticin) was purchased from Gibco Laboratories (Gaithersburg, MD), hygromycin B was purchased from Boehringer Mannheim (Indianapolis, IN), and zeocin was purchased from Invitrogen (Carlsbad, MA). The pure E antagonist, ICI 182,780, was a kind gift from Zeneca Pharmaceuticals (Macclesfield, Cheshire, England). The BCA kit for protein determination was obtained from Pierce (Rockford, IL), and the alkaline phosphatase enzyme assay kit was purchased from Sigma Chemical Co. The C-terminal propeptide of type

I collagen kit was obtained from Metra Biosystems (Mountain View, CA), and the human interleukin-6 (IL-6), IL-11, and macrophage-colony stimulating factor (M-CSF) kits were purchased from R&D Systems (Minneapolis, MN). The ERβ-selective antagonist R,R-tetrahydrochrysene (THC) has been described previously [Sun et al., 1999]. Recombinant human ERα and ERβ were purchased from Panvera (Madison, WI). The monoclonal antibody to the N-terminal domain of ERα was from Immunotech (Marseilles, France), and the polyclonal antibody to an N-terminal peptide of human ERβ was from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Sigma Chemical Co., and the ECL chemiluminescence detection reagent, and [α^{32}P]-radiolabeled dCTP and dTTP were purchased from Amersham Pharmacia (Piscataway, NJ).

Cell Culture

hFOB 1.19 cells were maintained in DMEM-F12 medium supplemented with 10% (v/v) FBS and 300 μg/ml geneticin [Harris et al., 1995a]. hFOB/ERα9 cells, derived from the ER-negative hFOB 1.19 cells, contain a stably transfected ERα expression vector with the hygromycin B resistance gene, and were cultured as previously described [Harris et al., 1995b]. The hFOB/ERβ6 cell line was derived from the hFOB 1.19 line by stable transfection with the ERβ expression construct, pZeoERβ. The vector pZeoERβ was generated by inserting the human $ER\beta_1$ truncated transcript [Moore et al., 1998] into pZeoSV2(+) (Invitrogen). hFOB 1.19 cells were transfected with pZeoERβ or pZeoSV2 by electroporation. After 48 h, selection of positive transfectants was initiated by culturing in medium supplemented with zeocin (500 μg/ml). Selection was maintained for 2 weeks, at which time individual colonies were obtained by ring-cloning and maintained in medium containing, alternately, either 250 μg/ml zeocin or 300 μg/ml geneticin. For all experiments, the hFOB/ER cells were grown in medium containing 10% (v/v) charcoal-stripped FBS with either 100 μg/ml hygromycin (hFOB/ERα9 cells) or 250 μg/ml zeocin (hFOB/ERβ6) on an alternating feeding schedule with 300 μg/ml geneticin, with medium changes every other day. Experiments with the parental hFOB cells were performed in medium supplemented with 10% (v/v) charcoal-stripped FBS and 300 μg/ml geneticin.

Northern Analysis

Cells were plated at 2×10^6 cells in 6 cm dishes and grown to confluence. Upon confluence, cells were harvested and total RNA was isolated using a phenol/guanidine isothiocyanate extraction method. Ten microgram of total RNA was denatured in glyoxal/dimethyl sulfoxide buffer and separated on a 1% (w/w) agarose-glyoxal gel. Northern blots were prepared and probed as described previously [Harris et al., 1995b]. ERβ mRNA levels were assessed using a cDNA fragment isolated from the pZeoERβ vector. Northern blots were exposed to phosphoimager screens for 12–24 h and then scanned on a Storm 840 phosphoimager (Molecular Dynamics, Sunnyvale, CA). Loading and integrity of RNA was confirmed by hybridization with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe.

Western Blotting for ERα and ERβ

Whole cell lysates were prepared from confluent cells in RIPA buffer [1X phosphate buffered saline, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS] containing protease inhibitors. Protein aliquots (50 μg for ERα detection, 100 μg for ERβ detection) were separated by SDS–PAGE under reducing conditions and transferred to nitrocellulose by electroblotting. Membranes were blocked in blotto [1X Tris-buffered saline (TBS), 0.05% (v/v) Tween-20, 5% (w/v) nonfat dried milk] overnight at 4°C. Detection of ERα was performed using a monoclonal antibody specific for the ERα N-terminal domain, diluted to 5 μg/ml in blotto. ERβ was detected with a polyclonal antibody to an N-terminal peptide of human ERβ, diluted to 2 μg/ml in TBS, 0.05% (v/v) Tween-20, and 2% (w/v) nonfat dried milk, for 2 h at room temperature. After incubation with the appropriate HRP-conjugated secondary antibody, the reactive proteins were visualized by chemiluminescence. Antibody specificity was confirmed by including purified recombinant ERα and ERβ proteins.

Mineralized Matrix Staining

Cells were plated at 8.6×10^4 cells per well in 12-well dishes and cultured to confluence. Upon

confluence (day 1), cells were treated with one of the following: ethanol vehicle, 10^{-8}M 17β-estradiol (E), 10^{-8}M tamoxifen (TAM), or 10^{-8}M ICI 182,780 (ICI). Each treatment was performed in triplicate. At the indicated time in postconfluent culture, cells were fixed with 1% (w/v) paraformaldehyde in TBS (20 mM Tris, pH 7.4, and 0.15M NaCl) for 10 min, and stained by the Von Kossa procedure by a modified method of Schenk et al. [1984], as described previously [Robinson et al., 1997]. Mineralized nodules were photographed using a Nikon Diaphot 300 inverted microscope (Nikon Corp., Melville, NY). For Alizarin red staining, cells were fixed with 70% (v/v) ethanol for 1 h, rinsed with water, and then stained with 40 mM alizarin red S, pH 4.2, for 10 min. Following several rinses with water, the stain was then solubilized with 10% cetylpyridinium chloride in 10 mM sodium phosphate, pH 7.0. Relative quantitation of staining was performed spectrophotometrically by measuring absorbance at 562 nm.

Bone Matrix Protein and Cytokine Assays

Cells were plated at 8.6×10^4 cells per well in 12-well dishes and cultured to confluence. Upon confluence (day 1), cells in triplicate wells were treated with ethanol vehicle, 10^{-8} M 17β-estradiol, 10^{-8} M tamoxifen or 10^{-8} M ICI 182,780 for up to 21 days. At the given time point in postconfluent culture, indicated in the figure legends, cells were harvested, and whole cell lysates were assayed for total protein using the BCA method (Pierce) and for alkaline phosphatase using the reagents from Sigma. Media were collected and assayed by ELISA for human IL-6, IL-11, TNFα, and M-CSF. Media from duplicate plates were also collected and concentrated for analysis of the C-terminal propeptide of type I collagen. All protein kit analyses were performed according to manufacturers' instructions using the standards provided.

Data Analysis and Statistics

All data are expressed as the mean±SEM, and are generally presented as the percent of ICI control value. Statistical significance was analyzed using the non-paired Students' *t*-test. A value of $P < 0.05$ was considered to be statistically significant. In the figures statistical significance is indicated by: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

RESULTS

Analysis of Estrogen Receptor Expression in hFOB/ER Cell Lines

To confirm the expression of the transfected receptors, northern analysis was performed on all clones surviving antibiotic selection and continued culturing. Figure 1 shows representative gels displaying the relative mRNA expression of ERα and ERβ in the two hFOB/ER cell lines selected for these studies. The hFOB/ERα9 cells represent the ERα-positive cell line, and have been described previously as the hFOB/ER9 cell line [Harris et al., 1995b]. This cell line exhibits numerous responses to E [Kassem et al., 1996; Robinson et al., 1997]. As described in the Methods Section, the hFOB/ERβ6 cell line was developed from the resistant clones following transfection with the ERβ expression vector and was selected for its relatively high expression of ERβ mRNA.

The presence of receptor proteins in the two hFOB/ER lines was determined by Western blot analysis of whole cell lysates using ER isoform-specific antibodies. As shown in Figure 2A, a single band of approximately 65 kDa that comigrates with recombinant ERα is readily detected in hFOB/ERα9 cells, but not in the

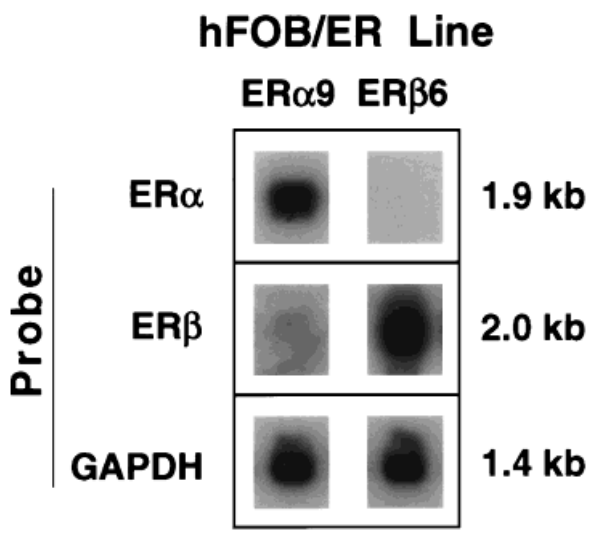

Fig. 1. Northern blot analysis of ERα and ERβ mRNA levels in hFOB/ERα9 and hFOB/ERβ6 cell lines. Total RNA (10 μg) from hFOB/ER cells was subjected to northern analysis and membranes probed for expression of ERα, ERβ, and GAPDH as described in the Materials and Methods. The size of each transcript in kilobases (kb) is indicated on the right. Representative gels are shown.

hFOB/ERβ6 cells. Figure 2B shows that an anti-ERβ antibody recognizes a major protein band of approximately 60 kDa in hFOB/ERβ6 cells, vaginal tissue, and LnCAP prostate carcinoma cells. This band is also detected in the parent hFOB cell line, although at lower intensity. Similar results are obtained with several other ERβ antibodies, both commercial and recently prepared by our laboratory, and which generally possess low affinity and poor specificity for ERβ.

The hFOB/ERβ6 Cells Exhibit Functional ERβ Receptors

In addition to detecting transcripts and protein for ERβ in the hFOB/ERβ6 cells, the ERβ receptors were also demonstrated to be functional using the ERβ-specific antagonist, R,R-tetrahydrochrysene (THC) [Sun et al., 1999]. THC is an agonist with ERα, acting like estradiol, but acts as an antagonist with ERβ. Treatment of both hFOB/ERα9 and hFOB/ERβ6 cells with E inhibited the constitutive production of IL-6 (Fig. 3). An E-mediated inhibition of IL-6 synthesis has previously been demonstrated in the hFOB/ERα9 cell line [Kassem et al., 1996] and in stromal/osteoblast cells [Girasole et al., 1992]. Cotreatment of hFOB/ERα9 cells with E and THC had no effect on the E-mediated inhibition of IL-6 levels and, as expected, THC alone inhibited IL-6 production similar to the effect of E (Fig. 3). In contrast, cotreatment of the hFOB/ERβ6 cell line with E and a 300-fold molar excess of THC significantly reversed the inhibition of IL-6 induced by E, whereas THC added alone to these cells had little effect on IL-6 production. These results demonstrate that the effect of E on IL-6 production by hFOB/ERβ6 cells is mediated through ERβ and not through ERα and that the

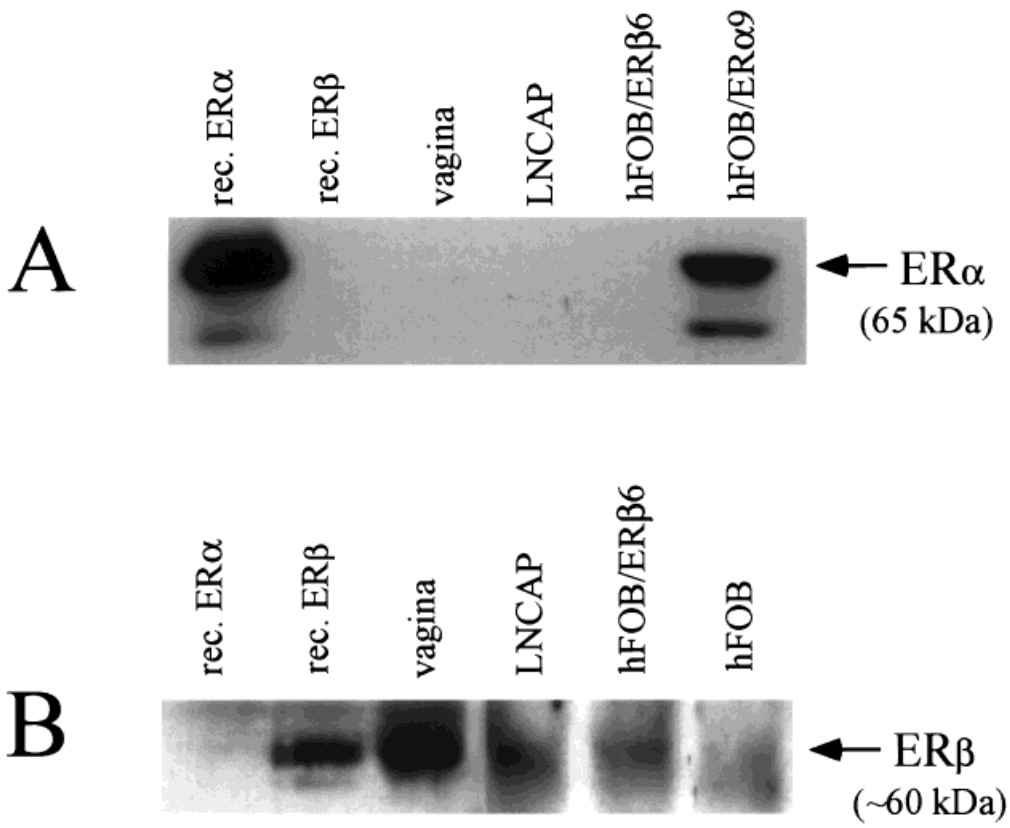

Fig. 2. Western blot detection of ERα and β proteins in hFOB/ER cell lines. Proteins in whole cell lysates were separated by SDS–PAGE and transferred to nitrocellulose. ERα was detected in 50 μg of lysate using a specific monoclonal antibody **(A)** and ERβ was identified in 100 μg of lysate using a polyclonal antibody **(B)**. Lysates prepared from LNCAP cells and human vaginal tissue were used as positive controls for the expression of ERβ. Antibody specificity was verified with recombinant human ERα (50 ng) and ERβ (540 ng) proteins.

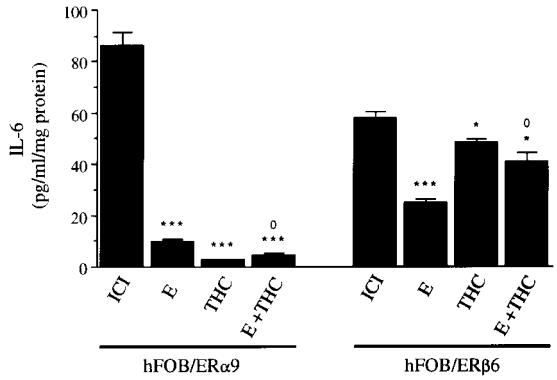

Fig. 3. An ERβ-selective antagonist reverses the E-mediated inhibition of IL-6 production in hFOB/ERβ6 cells but not in hFOB/ERα9 cells. Cells were cultured to confluency in 12-well plates and then treated for 4 days with either ICI 182,780 (ICI, 10^{-8} M), 17β-estradiol (E, 10^{-9} M), R,R-tetrahydrochrysene (THC, 3×10^{-7} M) or E+THC in medium containing charcoal-stripped FBS. The medium was changed and fresh treatments added after 2 days of stimulation. At the end of the treatment period IL-6 and total protein were assayed in the conditioned medium and cell layers, respectively. Values for IL-6 are given as the mean±SEM of triplicate treatments and are normalised for cell protein. Statistically significant differences between each treatment and the ICI control level is indicated by asterisks whereas a significant difference between E+THC and E alone is indicated by '0', where $P < 0.05$. Data are representative of two (hFOB/ERα9) and three (hFOB/ERβ6) separate experiments.

expressed ERβ in this cell line is biologically active.

Mineralized Nodule Formation in hFOB and hFOB/ER Cell Lines Treated With Estrogen, Tamoxifen and ICI 182,780

The ability of the hFOB, hFOB/ERα9, and hFOB/ERβ6 cells to produce mineralized nodules in culture was then determined. As described previously to promote mineralized matrix deposition, cultures were maintained for several days past confluency to suppress the synthesis of the SV40 large T antigen immortalization protein and induce a mature osteoblast phenotype [Harris et al., 1995b; Robinson et al., 1997]. As shown by the presence of nodules that are intensely stained by the Von Kossa technique (Fig. 4), the hFOB/ERβ6 cells deposit a mineralized extracellular matrix by 12 days postconfluency (Fig. 4), as do the parent hFOB cells (not shown). However, the hFOB/ERα9 cells required between 18 and 21 days of postconfluent culture for mineralization to occur. Continuous treatment of hFOB/ERα9 cells with E significantly inhibits nodule formation so that only minimal mineralization is evident even by day 21 of postconfluent culture. Interestingly, treatment of hFOB/ERα9 cells with the E antagonist ICI increases the rate of nodule formation, resulting in mineralization by day 12, comparable to that in the parent hFOB cultures. These results suggest that residual E in charcoal-stripped FBS and/or ligand-independent activation of ER results in some degree of ERα signaling in hFOB/ERα9 cells. TAM also reduces mineralized matrix deposition in hFOB/ERα9 cells but is considerably less potent than E (Fig. 4), consistent with the weak agonist character of TAM in bone. An inhibition of mineralized matrix deposition with E treatment was also observed in other hFOB/ERα cell lines possessing slightly different levels of ERα protein (data not shown). Neither E nor TAM has any affect on mineralized matrix formation in the parent hFOB cell line (data not shown) or in hFOB/ERβ6 cells (Fig. 4).

In addition, cell lines containing the corresponding empty expression vectors for ERα and ERβ, hFOB/pHyg and hFOB/pZeo, respectively, which serve as controls for the presence of vector, display the same chronology of mineralization as the parent hFOB cells. Furthermore, E, TAM, and ICI treatment of the vector control cells does not alter the rate of matrix mineralization (data not shown), demonstrating that the regulation observed in the ER-expressing lines is not due to the vector transfection.

Quantitation Time Course Of Mineralized Matrix Formation in hFOB and hFOB/ER Cell Lines Treated With Estrogen, Tamoxifen and ICI 182,780

To quantitate the differences between the cell lines in their ability to synthesize a mineralized matrix, the above experiments were repeated with alizarin red staining and quantitation of the mineralization by solubilization of the cell layers and matrix. Figure 5 shows the effects of E, TAM, and ICI on the deposition of a mineralized matrix in the parent hFOB line and in the two hFOB/ER cell lines by measurement of the Alizarin red staining. Using this technique, treatment with E, TAM or ICI shows no significant effect in the hFOB and hFOB/ERβ6 cells (Fig. 5A, B), in agreement with the results of Von Kossa staining. However, in the hFOB/ERα9 cells, treatment with E and TAM significantly inhibits mineralization prior to day 14 of culture when compared with ICI treatment

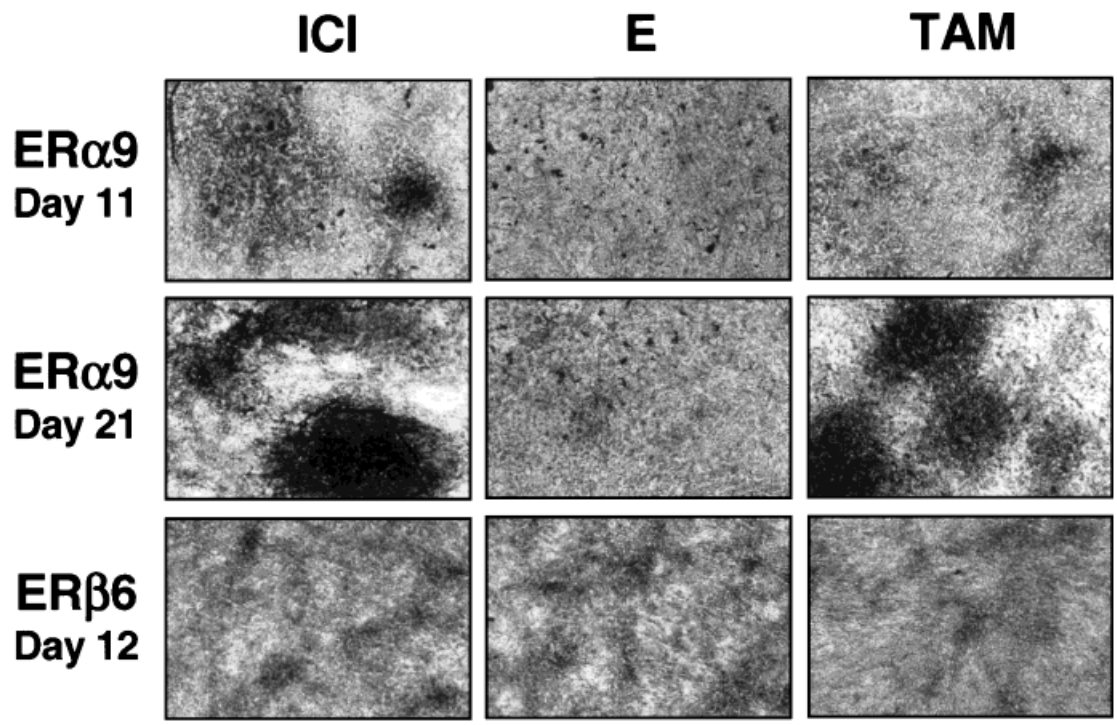

Fig. 4. Effect of estrogen, tamoxifen and ICI 182,780 on mineralized nodule formation in the hFOB/ER cell lines. Cells were plated at 8.6×10^4 cells per well in 12-well dishes and grown to confluence in medium containing charcoal-stripped FBS. At confluence (day 1), the medium was changed and cells treated with ICI 182,780 (ICI, 10^{-8} M), 17β-estradiol (E, 10^{-8} M) or tamoxifen (TAM, 10^{-8} M). At the indicated time, cells were fixed and stained for mineralized matrix by the Von Kossa procedure and visualized by light microscopy. **(A)** hFOB/ERα9 cells at day 12, **(B)** hFOB/ERα9 cells at day 21, **(C)** hFOB/ERβ6 cells at day 12. Magnification × 25.

(Fig. 5C). These data further support that E agonists prevent the formation of mineralized nodules in hFOB/ERα9 cells but not in hFOB/ERβ6 cells.

Since the major goal of this investigation was to examine the effect of differentiation on E responsiveness in the two hFOB/ER cell lines, it was necessary to identify the different differentiation stages in these cells. Based on the results presented in Figures 4 and 5, and on findings from a previous investigation performed in this laboratory [Harris et al., 1995b], mineralization is first detected in control-treated hFOB and hFOB/ERβ6 cultures between 2 and 4 days postconfluency. Matrix mineralization subsequently increases and by days 10–12 in postconfluent culture there is intense mineral deposition (Fig. 5A, B). In contrast, mineralization in control (ICI-treated) hFOB/ERα9 cells begins at approximately day 6 of postconfluent culture and the matrix does not become heavily mineralized until around day 18 (Figs. 4 and 5C). Therefore, the stages of osteoblast differentiation represented by these cultures [namely, matrix synthesis/premineralization (early), matrix maturation/early mineralization (middle), and late mineralization (late)] occur at different periods of postconfluent culture in the two hFOB/ER cell lines. Consequently, and as shown in Figure 5, time points representing these different differentiation stages (henceforward referred to as early, middle and late differentiation stages) were chosen as days 4, 11, and 21 for the hFOB/ERα9 cells and days 3, 6, and 12 for the hFOB/ERβ6 cells. In addition, because of the suspected activation of ERα occurring in the hFOB/ERα9 cells in the absence of added hormone, in all subsequent experiments the effect of E and TAM treatment was compared to cells treated with ICI. Since treatment of hFOB/ER cell lines with ICI generally had only minor effects on the various parameters measured compared with the effects of E and TAM, the same responses are observed when expressed relative to the vehicle treated or ICI treated value, except for the regulation of alkaline phosphatase activity (see below).

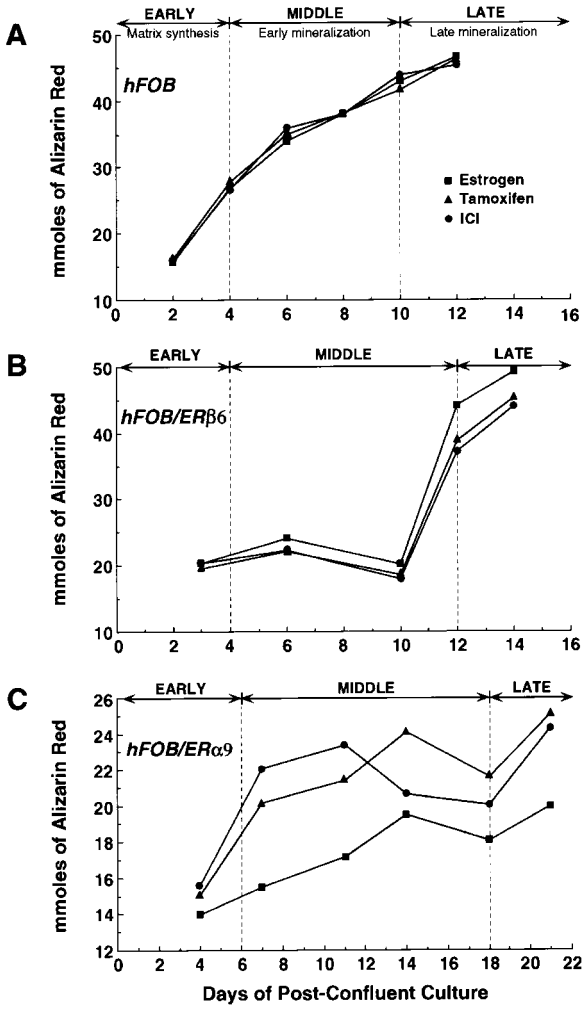

Fig. 5. Quantitation of matrix mineralization during differentiation of hFOB and hFOB/ER cell lines. Cells were plated and treated from confluence (day 1) with either 17β-estradiol, tamoxifen or ICI 182,780 (10^{-8} M) as described in Figure 4. At the indicated time, the amount of mineralized matrix deposited was determined by measurement of Alizarin Red staining. Based upon these time-courses, the matrix synthesis/premineralization (early), early mineralization (middle) and late mineralization (late) stages of differentiation were identified for the two hFOB/ER cell lines under ICI-treated conditions. **(A)** hFOB, **(B)** hFOB/ERβ6, and **(C)** hFOB/ERα9.

Regulation of Alkaline Phosphatase Activity by Estrogen and Tamoxifen in hFOB/ER Cells

Alkaline phosphatase (AP) is a widely used marker of osteoblast differentiation whose expression is induced after cessation of proliferation and increases during further differentiation and the early stages of mineralization [Owen et al., 1990; Robinson et al., 1997]. In premineralizing cultures, the AP activity in hFOB/ERα9 cells is approximately 50% of that measured in hFOB/ERβ6 cells. However, the AP activity increases in both cell lines to reach a comparable level of around 0.65 μmoles/mg protein in late mineralizing cultures. AP activity has previously been shown to be induced by E in hFOB/ERα9 cells and in certain other osteoblast lines [Majeska et al., 1994; Robinson et al., 1997]. As shown in Figure 6A, E causes a dramatic stimulation of AP activity in hFOB/ERα9 cells at each differentiation stage. When expressed relative to the activity in ICI-treated cultures, treatment with E produces a 3-fold greater induction at the late differentiation stage (late mineralization) compared to the early (matrix synthesis/premineralization) and middle (matrix maturation/early mineralization) stages. However, this effect of differentia-

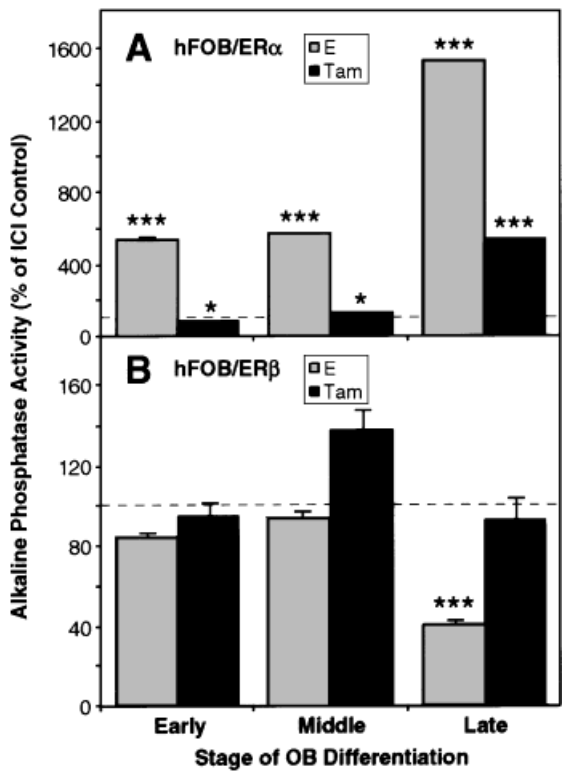

Fig. 6. Regulation of alkaline phosphatase activity by estrogen and tamoxifen in hFOB/ER cells. The cells were cultured and treated with either 17β-estradiol, tamoxifen or ICI 182,780 (10^{-8} M) as described in Figure 4. At the early, middle and late differentiation stages identified in Figure 5 (corresponding to days 4, 11, and 21 of postconfluent culture in hFOB/ERα9 cells and to days 3, 6, and 12 of postconfluent culture in hFOB/ERβ6 cells), the cell layers were harvested and assayed for alkaline phosphatase activity and total protein. The data are expressed as the percent of ICI-treated control cultures after normalizing for total protein content, and are presented as the mean±SEM of triplicate determinations. **(A)** hFOB/ERα9 cells, **(B)** hFOB/ERβ6 cells.

tion is not apparent when the AP activity is expressed relative to vehicle-treated cells. TAM shows little effect on AP activity in the early and middle stages of differentiation but increases AP 5-fold over that in ICI-treated cells at the late stage, consistent with TAM acting as a weak E agonist in bone. In the hFOB/ERβ6 cells, while no increase in AP activity with E treatment is observed in the early and middle stages, E actually decreases AP levels by 60% during the late mineralization stage compared with either ICI or vehicle treated cultures (Fig. 6B). TAM has no effect on AP activity in hFOB/ERβ6 cells.

Regulation of Type I Collagen Production by Estrogen and Tamoxifen in hFOB/ER Cells

Type I collagen (Col I) is the major collagen produced by osteoblasts and deposited in the bone matrix. Synthesis of Col I has been shown to be regulated by E in certain osteoblastic cells in vitro including hFOB/ERα9 cells [Ernst et al., 1989; Robinson et al., 1997]. In our studies, the basal level of Col I production in the hFOB/ERβ6 cells is 2-fold greater than in the hFOB/ERα9 cells at all stages (data not shown). As shown in Figure 7A, treatment with E significantly increases Col I production in hFOB/ERα9 cells, but only during the middle stage of differentiation. A trend towards increased Col I production in the middle stage is also seen with TAM but this effect is not statistically significant. Neither E nor TAM modulates Col I production in hFOB/ERβ6 cells during early and middle stages of differentiation, and only a slight, nonsignificant, inhibition is observed at the late stage (Fig. 7B).

Regulation of Cytokine Production by Estrogen and Tamoxifen in hFOB/ER Cells

Similar studies were performed with interleukin (IL)-6, IL-11, tumor necrosis factor-α (TNFα), and macrophage-colony stimulating factor (M-CSF). These cytokines have been implicated in mediating the increased bone resorption caused by E deficiency via increased osteoclast formation and activity [Manolagas and Jilka, 1995]. As shown in Figure 8, the regulation of the production of these cytokines by E in the hFOB/ER cell lines is differentiation stage-dependent. In hFOB/ERα9 cells, both E and TAM significantly inhibit IL-6 production at all stages of differentiation although the inhibition is greater at the middle and late

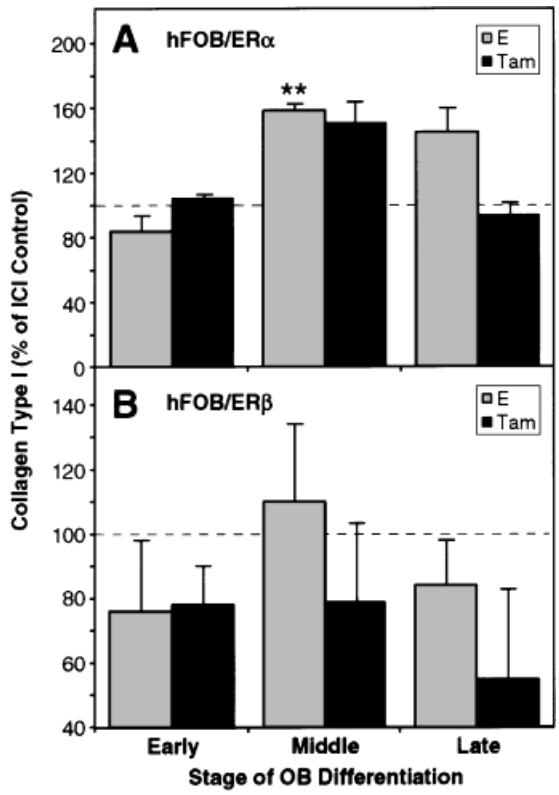

Fig. 7. Regulation of type I collagen production by estrogen and tamoxifen in hFOB/ER cells. The cells were cultured and treated with either 17β-estradiol, tamoxifen or ICI 182,780 (10^{-8} M) as described in Figure 4. At the early, middle and late differentiation stages (corresponding to days 4, 11, and 21 of postconfluent culture in hFOB/ERα9 cells and to days 3, 6, and 12 of postconfluent culture in hFOB/ERβ6 cells), the conditioned medium and cell layers were harvested and assayed for the C-terminal propeptide of type I collagen and total proteins, respectively. The data are expressed as the percent of ICI-treated control cultures after normalizing for total protein content, and are presented as the mean±SEM of triplicate determinations. **(A)** hFOB/ERα9 cells, **(B)** hFOB/ERβ6 cells.

stages compared to the early stage (Fig. 8A, top panel). However, at all stages of differentiation E is a more potent inhibitor of IL-6 production than TAM. E also inhibits the production of IL-6 in hFOB/ERβ6 cells throughout differentiation, although the degree of inhibition is lower than that measured in the hFOB/ERα9 cells (Figure 8A, lower panel). In the hFOB/ERβ6 cells, TAM modestly inhibits IL-6 production only in the late mineralization (late) stage.

The effects of E and TAM on the production of IL-11 in both hFOB/ER lines parallels, in general, their effects on IL-6. As shown in Figure 8B (top panel), IL-11 production is inhibited by E and TAM in hFOB/ERα9 cells with a greater inhibition observed in more differentiated cells. Again, the inhibitory effect

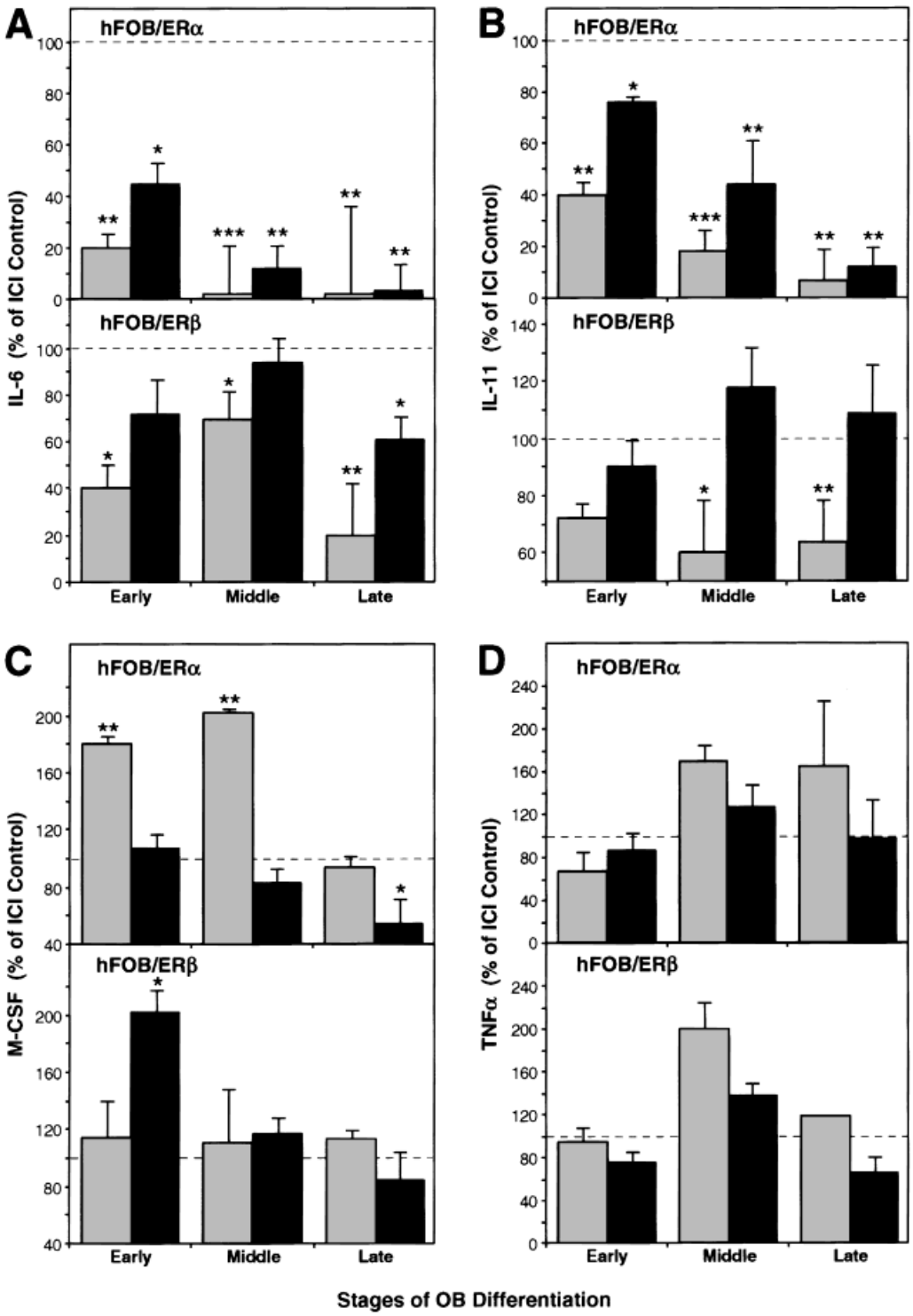

Fig. 8. Effect of estrogen and tamoxifen on cytokine production by hFOB/ER cells. The cells were cultured and treated with either 17β-estradiol, tamoxifen or ICI 182,780 (10^{-8} M) as described in Figure 4. At the early, middle and late differentiation stages (corresponding to days 4, 11, and 21 of postconfluent culture in hFOB/ERα9 cells and to days 3, 6, and 12 of postconfluent culture in hFOB/ERβ6 cells), the conditioned medium and cell layers were harvested and assayed for cytokine concentrations by ELISA and total proteins, respectively. The effects of E (gray bars) and TAM (black bars) treatment are presented as the percent of ICI-treated control cultures normalized for total protein content, and are the mean±SEM of triplicate determinations. For each cytokine, results for hFOB/ERα9 cells are shown in the upper panel, and for hFOB/ERβ6 cells in the lower panel. **(A)** IL-6, **(B)** IL-11, **(C)** M-CSF, and **(D)** TNFα.

of TAM is weaker than that of E at all differentiation stages. In hFOB/ERβ6 cells, IL-11 production is inhibited by E only in the more differentiated cells, and TAM is ineffective at all differentiation stages (Fig. 8B, lower panel). These data on the actions of E and TAM on IL-6 and IL-11 production by osteoblasts again demonstrates the partial agonist activity of TAM in these cells when acting through ERα but not ERβ. Identical responses in IL-6 and IL-11 production are observed when the effects of E and TAM are expressed relative to vehicle treated cultures.

In contrast to the inhibitory effect of E on IL-6 and IL-11 production by hFOB/ERα9 cells, E stimulates the production of M-CSF in these cells at early and middle stages of differentiation (Fig. 8C, top panel). TAM inhibits M-CSF production by hFOB/ERα9 cells only at the late stage. In hFOB/ERβ6 cells, M-CSF levels are unaffected by E but are stimulated 2-fold by TAM during the early stage of differentiation (Fig. 8C, lower panel). The effects of E and TAM on M-CSF production are the same when compared to either ICI or vehicle treatment.

TNFα production is not significantly affected by E or TAM at all stages of differentiation in both hFOB/ERα9 and hFOB/ERβ6 cells (Fig. 8D). These latter findings demonstrate specificity in the response of both cell lines to the treatments.

DISCUSSION

The purpose of this investigation was to compare the ERα- and ERβ-mediated effects of E and TAM on endogenous cellular and gene responses in osteoblasts, measured both within the same stage of differentiation and at different differentiation stages. Although the two ER isoforms produce differential regulation of both synthetic and native inducible promoters, as shown by transient transfection of nonosteoblastic cells with reporter gene constructs and ER expression vectors [Paech et al., 1997; Hall and McDonnell, 1999; Zou et al., 1999; Saville et al., 2000], their relative importance to endogenous target gene regulation and osteoblast function remains unknown. Using human fetal osteoblast cell lines stably transfected with either ERα or ERβ we have been able to demonstrate that (1) the response of each cell line to E and TAM is dependent on the stage of differentiation, implying modulation of the ER signaling pathway(s) by secondary factors, for e.g., the steroid receptor coregulators, and (2) during the same differentiation stage, the regulation of select genes/protein expression by E and TAM differed between the two lines, and thus exhibited an ER isoform specificity.

Each hFOB/ER cell line was shown to express only one or other of the ER isoforms at both mRNA and protein levels and, moreover, the response to E in the hFOB/ERβ6 cell line could be abolished by cotreatment with the ERβ-selective antagonist, THC. Thus, the actions of E are mediated by a single ER isoform in each cell line, allowing the assessment of unique activities of ERα and β in human osteoblasts. This is in contrast to cells expressing both ER isoforms whose responses to E may differ from cells expressing either isoform alone because ERα and β are able to function as α/β heterodimers [Pace et al., 1997] and because ERβ is capable of antagonizing the activity of ERα [Hall and McDonnell, 1999].

The regulation of expression of several proteins by E, e.g., IL-6, IL-11, and possibly AP, was more pronounced in both hFOB/ERα9 and hFOB/ERβ6 cell lines at the late mineralization stage of differentiation. These findings support previous observations that E responsiveness in osteoblastic cells is differentiation-dependent [Rao et al., 1994; Bodine et al., 1998]. For example, Bodine et al. reported divergent actions of E on osteoblast marker genes (including AP) between the matrix synthesis and late mineralization stages of differentiation in cultures of fetal rat calvarial cells, when the ERα mRNA levels were moderately high and low, respectively. Also, SaOS-2 human osteosarcoma cells became responsive to E (indicated by increased AP activity) only after exposure to glucocorticoid [Rao et al., 1994], which induces both differentiation and ERα protein levels in these cells [Sutherland et al., 1996]. It should be noted that osteoblast responses to other calciotropic hormones, including 1,25-dihydroxyvitamin D_3 and parathyroid hormone, have also been shown to be differentiation stage-dependent [Owen et al., 1991; Rao et al., 1994].

There are several possible explanations for the altered responsiveness to E during osteoblast differentiation in our cell lines. First, ER activity may be modulated by differentiation-dependent changes in the levels of steroid receptor coregulators [McKenna et al., 1999]. An alteration in the ratio of coactivators to

corepressors has been demonstrated to affect ligand-dependent transactivation by the ER [Smith et al., 1997], and although changes in steroid receptor coregulator levels during osteoblastic differentiation have not been reported, it is possible that the coactivator/corepressor ratio is altered by differentiation thereby enhancing or repressing E action. Second, the ER activity may be modulated by cross-talk with other signaling pathways which in turn are affected by the stage of differentiation. Signaling pathways for E/ER and growth factors/cytokines are known to be integrated because the transcriptional activity of ERα- and possibly of ERβ- has been demonstrated to be enhanced by protein kinases A and C-mediated phosphorylation of the receptor [Aronica and Katzenellenbogen, 1993; Migliaccio et al., 1993], and because some cytokines, such as epidermal growth factor, elicit ER-dependent actions [Kato et al., 1995; Curtis et al., 1996]. Third, changes in cell-matrix interactions as a result of differentiation-induced changes in matrix composition, may be transduced to the cell and modify cellular responses to E. In this regard, it is well established that integrins are capable of transducing signals across the plasma membrane [Juliano and Haskill, 1993], and that the type of extracellular matrix affects osteoblast function [Lynch et al., 1995]. Whether any of these processes are responsible for conferring the differentiation-dependent effects of E in the hFOB/ER cell lines will require further investigation.

In addition to differentiation-dependent regulation of ER-mediated responses, ER isoform-specific effects within the same differentiation stage were also observed. In particular, AP activity, type I collagen synthesis, and matrix mineralization were regulated by E in hFOB/ERα9 cells but not in hFOB/ERβ6 cells. The differential regulation of native gene promoters for endogenously expressed proteins that we observed in the ERα and ERβ-expressing osteoblasts is therefore consistent with numerous reports of ER isoform-specific effects on E-sensitive reporter gene constructs [Paech et al., 1997; Zou et al., 1999; Saville et al., 2000]. However, because the relative level of ERα and ERβ proteins in the two hFOB/ER cell lines is unknown, we cannot exclude the possibility that some of the differential responses to E described here are due to differences in ER content.

Many of the effects of E on the hFOB/ERα9 cells were also produced by TAM, including stimulation of AP and inhibition of IL-6 and IL-11, although the effect of TAM in these cells was generally weaker than that of E. These findings are in agreement with the well-recognized ability of TAM to act as a weak E agonist in bone. In contrast to E, TAM had virtually no effect on the hFOB/ERβ6 cell line. The lack of effect of TAM via ERβ is consistent with the agonist activity of TAM being mediated through the AF-1 domain of ERα [Berry et al., 1990], an activity which appears to be absent or nonfunctional in ERβ [Hall and McDonnell, 1999].

The inhibition of mineralized matrix formation by E in the hFOB/ERα9 cells is perhaps unexpected since E increased both AP activity (considered to be necessary for biomineralization) and synthesis of type I collagen (the major component of osteoblast-derived matrix). One possible explanation for this apparent discrepancy is that E inhibits mineralization (as detected by the Von Kossa and Alizarin red staining procedures) but does not inhibit matrix deposition. An E-mediated inhibition of mineralized matrix deposition is not an artifact of the hFOB/ERα9 clone, because similar inhibitory effects were observed in several other hFOB lines stably transfected with ERα. Interestingly, although the increases in AP and type I collagen by E treatment in hFOB/ERα9 cells are in agreement with studies utilizing other osteoblast culture systems [Ernst et al., 1989; Majeska et al., 1994], they are opposite to the effect of E on the expression of these genes in periosteal osteoblasts examined ex vivo [Turner et al., 1990]. In any case, our studies indicate that the levels of AP and type I collagen in osteoblasts, as well as the rate of matrix mineralization, are regulated by E signaling predominantly through the ERα isoform.

Our studies also demonstrate that E inhibited the production of IL-6 and IL-11 in both hFOB/ERα9 and hFOB/ERβ6 cell lines, whereas the production of M-CSF was increased by E only in hFOB/ERα9 cells, while the production of TNFα was unaffected. Our results for the agonist action of E and TAM on the regulation of IL-6 synthesis therefore support and extend previous observations showing that E inhibits IL-6 production in hFOB/ERα9 cells [Kassem et al., 1996], normal osteoblasts and bone marrow stromal cells [Girasole et al., 1992], and in a rat decidual (endometrial) cell line that expresses

only ERβ [Deb et al., 1999]. Our findings further suggest that E agonists may down-regulate IL-6 and IL-11 production in all osteoblastic cells expressing ER. Since in our study cytokine concentrations in conditioned medium were assayed, we are unable to state whether the regulation by E and TAM is transcriptional, post-transcriptional, or both.

The regulation of bone-resorptive cytokine synthesis, including IL-1, IL-6, TNFα, M-CSF, and GM-CSF, by cells of either the stromal/osteoblast or monocyte/macrophage lineages is believed to be one of the principal mechanisms for the anti-resorptive activity of E [Manolagas and Jilka, 1995]. However, it remains controversial which cytokine(s) is/are primarily responsible for the increased bone resorption in E deficient states. Both IL-6 and M-CSF have been strongly implicated in mediating the effects of E deficiency on bone resorption [Jilka et al., 1992; Kimble et al., 1996]. The level of M-CSF production in bone marrow stroma has been shown to rise indirectly via loss of an E-mediated inhibition of IL-1 and TNF synthesis by monocyte/macrophages [Kimble et al., 1996; Srivastava et al., 1998]. However, the direct action of E on M-CSF production by osteoblasts was not reported. Therefore, the discrepancy between our findings of an E-mediated stimulation of M-CSF production and the inhibitory effects of E previously reported, may be explained by the fact that we have analyzed direct effects of E on M-CSF production in osteoblasts.

Our findings demonstrating a marked inhibition of IL-11 production by E, via both ERα and ERβ, suggest that E regulation of IL-11 may also play a role in the enhanced bone resorption following menopause or ovariectomy. IL-11 is closely related to IL-6, utilizes the same signal transducing gp130 receptor subunit, and consequently possesses overlapping activities with IL-6 including stimulation of osteoclast formation [Girasole et al., 1994]. The contribution of IL-11 to the accelerated bone loss in conditions of E deficiency is currently unknown. Previous investigations by other laboratories have shown E to either inhibit [Cheleuitte et al., 1998] or have no effect on [Kim et al., 1997] IL-11 secretion by human bone marrow stromal cells. Moreover, IL-11 supports osteoclast formation in bone marrow of mice independently of E status [Girasole et al., 1994]. Therefore, our results reinforce earlier studies suggesting that E may inhibit basal IL-11 secretion from osteoblast lineage cells. It should be mentioned that the effect of E on cytokine/hormone-stimulated IL-11 secretion in the hFOB/ER cell lines has not been examined.

In summary, using osteoblast cell lines stably expressing either ERα or ERβ, we have been able to identify responses to estrogen that are both ER isoform-specific and differentiation stage-dependent. Thus, shifts in the overall differentiation state of the osteoblast cell pool, along with environmentally-imposed changes in the ER isoform concentration or ratio in osteoblasts, could result in differential regulation of gene expression in response to estrogens, possibly leading to deleterious effects on osteoblast activity, bone formation, and bone remodeling. Further investigation is currently underway in our laboratory to elucidate the mechanisms responsible for the changes in ER action during osteoblast differentiation as well as to analyze differential regulation by the two ER isoforms of the various target genes identified.

ACKNOWLEDGMENTS

We thank Larry Pederson and Paula Gibson for excellent technical assistance and Ms. Jacquelyn House for preparation of the manuscript.

REFERENCES

Aronica SM, Katzenellenbogen BS. 1993. Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-1. Mol Endocrinol 7:743–752.

Arts J, Kuiper GGJM, Janssen JMMF, Gustafson JA, Lowik CWGM, Pols HAP, Van Leeuwen PTM. 1997. Differential expression of estrogen receptors α and β mRNA during differentiation of human osteoblast SV-HFO cells. Endocrinology 138:5067–5070.

Berry M, Metzger D, Chambon P. 1990. Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. EMBO J 9:2811–2818.

Bodine PVN, Henderson RA, Green J, Aronow M, Owen T, Stein GS, Lian JB, Komm BS. 1998. Estrogen receptor-α is developmentally regulated during osteoblast differentiation and contributes to selective responsiveness of gene expression. Endocrinology 139:2048–2057.

Braidman IP, Hainey L, Batra G, Selby PL, Saunders PTK, Hoyland JA. 2001. Localization of estrogen receptor β protein expression in adult human bone. J Bone Miner Res 16:214–220.

Cheleuitte D, Mizuno S, Glowacki J. 1998. In vitro secretion of cytokines by human bone marrow: effects of age and estrogen status. J Clin Endocrinol Metab 83:2043–2051.

Curtis SW, Washburn T, Sewall C, DiAugustine R, Lindzey J, Couse JF, Korach KS. 1996. Physiological coupling of growth factor and steroid receptor signaling pathways: estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. Proc Natl Acad Sci USA 93:12626–12630.

Davis VL, Couse JF, Gray TK, Korack KS. 1994. Correlation between low levels of estrogen receptors and estrogen responsiveness in two rat osteoblast-like cell lines. J Bone Miner Res 7:983–991.

Deb S, Tessier C, Prigent-Tessier A, Barkai U, Ferguson-Gottschall S, Srivastava RK, Faliszek J, Gibori G. 1999. The expression of interleukin-6 (IL-6), IL-6 receptor, and gp130-Kilodalton glycoprotein in the rat decidua and a decidual cell line: regulation by 17β-estradiol and prolactin. Endocrinology 140:4442–4450.

Ernst M, Heath JK, Rodan GA. 1989. Estradiol effects on proliferation, messenger ribonucleic acid for collagen and insulin-like growth factor-1, and parathyroid hormone-stimulated adenylate cyclase activity in osteoblastic cells from calvariae and long bones. Endocrinology 125:825–833.

Ernst M, Parker MG, Rodan GA. 1991. Functional estrogen receptors in osteoblastic cells demonstrated by transfection with a reporter gene containing an estrogen response element. Mol Endocrinol 5:1597–1606.

Girasole G, Jilka RL, Passeri G, Boswell S, Boder G, Williams DC, Manolagas SC. 1992. 17β-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effects of estrogens. J Clin Invest 89:883–891.

Girasole G, Passeri G, Jilka RL, Manolagas SC. 1994. Interleukin-11: a new cytokine critical for osteoclast development. J Clin Invest 93:1516–1524.

Hall JM, McDonnell DP. 1999. The estrogen receptor β isoform (ERβ) of the human estrogen receptor modulates ERα transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 140:5566–5578.

Harris SA, Enger RJ, Riggs BL, Spelsberg TC. 1995a. Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. J Bone Miner Res 10:178–186.

Harris SA, Tau KR, Enger RJ, Toft DO, Riggs BL, Spelsberg TC. 1995b. Estrogen response in hFOB1.19 human fetal osteoblastic cell line stably transfected with the human estrogen receptor gene. J Cell Biochem 59:193–201.

Jilka RL, Hangoc G, Girasole G, Passeri G, Williams DC, Abrams JS, Boyce B, Broxmeyer H, Manolagas SC. 1992. Increased osteoclast development after estrogen loss: mediation by interleukin-6. Science 257:88–91.

Juliano RL, Haskill S. 1993. Signal transduction from the extracellular matrix. J Cell Biol 120:577–585.

Kassem M, Harris SA, Spelsberg TC, Riggs BL. 1996. Estrogen inhibits interleukin-6 production and gene expression in a human osteoblastic cell line with high levels of estrogen receptors. J Bone Miner Res 11:193–199.

Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P. 1995. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270:1491–1494.

Kim GS, Kim CH, Choi CS, Park JY, Lee K-U. 1997. Involvement of different second messengers in parathyroid hormone- and interleukin-1-induced interleukin-6 and interleukin-11 production in human bone marrow stromal cells. J Bone Miner Res 12:896–902.

Kimble RB, Srivastava S, Ross FP, Matayoshi A, Pacifici R. 1996. Estrogen deficiency increases the ability of stromal cells to support murine osteoclastogenesis via an interleukin-1- and tumor necrosis factor-mediated stimulation of macrophage colony-stimulating factor production. J Biol Chem 271:28890–28897.

Korach KS, Taki M, Kimbro KS. 1997. The effects of estrogen receptor gene disruption on bone. In: Paoletti R, editor. Women's Health and Menopause. Amsterdam, The Netherlands: Kluwer Academic Publishers and Fondazione Giovanni Lorenzini. p 69–73.

Kuiper GGJM, Gustafsson J-A. 1997. The novel estrogen receptor-β subtype: potential role in the cell- and promoter-specific actions of estrogen and anti-estrogens. FEBS Letters 410:87–90.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138:863–870.

Kusec V, Virdi AS, Prince R, Triffitt JT. 1998. Localization of estrogen receptor-alpha in human and rabbit skeletal tissues. J Clin Endocrinol Metab 83:2421–2428.

Lynch MP, Stein JL, Stein GS, Lian JB. 1995. The influence of type I collagen on the development and maintenance of the osteoblast phenotype in primary and passaged rat calvarial osteoblasts. Exp Cell Res 216:35–45.

Majeska RJ, Ryaby JT, Einhorn TA. 1994. Direct modulation of osteoblastic activity with estrogen. J Bone Joint Surg 76:713–721.

Manolagas SC, Jilka RL. 1995. Bone marrow, cytokines, and bone remodeling. N Engl J Med 332:305–311.

McKenna NJ, Lanz RB, O'Malley BW. 1999. Nuclear receptor coregulators: Cellular and molecular biology. Endocrine Rev 20:321–344.

Migliaccio S, Wetsel WC, Fox WM, Washburn TF, Korach KS. 1993. Endogenous protein kinase-C activation in osteoblast-like cells modulates responsiveness to estrogen and estrogen receptor levels. Mol Endocrinol 7:1133–1143.

Moore JT, McKee DD, Slentz-Kesler K, Moore LB, Jones SA, Horne EL, Su JL, Kliewer SA, Lehmann JM, Willson TM. 1998. Cloning and characterization of human estrogen receptor β isoforms. Biochem Biophys Res Comm 247:75–78.

Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS. 1990. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 143:420–430.

Owen TA, Aronow MS, Barone LM, Bettencourt B, Stein GS, Lian JB. 1991. Pleiotropic effects of vitamin D on

osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in normal rat osteoblast cultures. Endocrinology 128:1496–1504.

Pace P, Taylor J, Suntharalingam S, Coombes RC, Ali S. 1997. Human estrogen receptor β binds DNA in a manner similar to and dimerizes with estrogen receptor α. J Biol Chem 272:25832–25838.

Paech K, Webb P, Kuiper GGJM, Nilsson S, Gustafsson JA, Kushner PJ, Scanlan TS. 1997. Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science 277:1508–1510.

Rao LG, Wylie JN, Kung Sutherland MS, Murrat TM. 1994. 17β-estradiol and parathyroid hormone potentiate each other's stimulatory effects on alkaline phosphatase activity in SaOS-2 cells in a differentiation-dependent manner. Endocrinology 134:614–620.

Rickard DJ, Subramaniam M, Spelsberg TC. 1999. Molecular and cellular mechanisms of estrogen action on the skeleton. J Cell Biochem Suppl 32(33):123–132.

Robinson JA, Harris SA, Riggs BL, Spelsberg TC. 1997. Estrogen regulation of human osteoblastic cell proliferation and differentiation. Endocrinology 138:2919–2927.

Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson J-A, Safe S. 2000. Ligand-, cell-, and estrogen receptor subtype (α/β)-dependent activation at GC-rich (Sp1)promoter elements. J Biol Chem 275:5379–5387.

Schenk RK, Olah AJ, Herrmann W. 1984. Preparation of calcified tissues for light microscopy. In: Dickson GR, editor. Methods of Calcified Tissue Preparation. New York: Elsevier. p 1.

Smith CL, Nawaz Z, O'Malley BW. 1997. Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol 11:657–666.

Spelsberg TC, Subramaniam M, Riggs BL, Khosla S. 1999. The actions and interactions of sex steroids and growth factors/cytokines on the skeleton. Mol Endocrinol 13:819–828.

Srivastava S, Weitzmann MN, Kimble RB, Rizzo M, Zahner M, Milbrandt J, Ross FP, Pacifici R. 1998. Estrogen blocks M-CSF gene expression and osteoclast formation by regulating phosphorylation of Egr-1 and its interaction with Sp-1. J Clin Invest 102:1850–1859.

Sun J, Meyers MJ, Fink BE, Rajendran R, Katzenellenbogen JA, Katzenellenbogen BS. 1999. Novel ligands that function as selective estrogens or antiestrogens for estrogen receptor-α or estrogen receptor-β. Endocrinology 140:800–804.

Sutherland MK, Hui DU, Rao LG, Wylie JN, Murray TM. 1996. Immunohistochemical localization of the estrogen receptor in human osteoblastic SaOS-2 cells: association of receptor levels with alkaline phosphatase activity. Bone 18:361–369.

Turner RT, Colvard DS, Spelsberg TC. 1990. Estrogen inhibition of periosteal bone formation in rat long bones: down-regulation of gene expression for bone matrix proteins. Endocrinology 127:1346–1351.

Vidal O, Kindblom LG, Ohlsson C. 1999. Expression and localization of estrogen receptor-β in murine and human bone. J Bone Miner Res 14:923–929.

Windahl S, Vidal O, Andersson G, Gustafsson J-A, Ohlsson C. 1999. Increased cortical bone mineral content but unchanged trabecular bone mineral density in adult female estrogen receptor-β-/- mice. J Clin Invest 104:895–901.

Zou A, Marschke KB, Arnold KE, Berger EM, Fitzgerald P, Mais DE, Allegretto EA. 1999. Estrogen receptor β activates the human retinoic acid receptor α-1 promoter in response to tamoxifen and other estrogen receptor antagonists, but not in response to estrogen Mol Endocrinol 13:418–430.