vivo assays are required to detect chemical carcinogens which act specifically in late stages of carcinogenesis but are not tumor promoters or are weak tumor promoters.

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22 April 1988; accepted 25 April 1988

Estrogen Binding, Receptor mRNA, and Biologic Response in Osteoblast-Like Osteosarcoma Cells

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High specific activity estradiol labeled with iodine-125 was used to detect approximately 200 saturable, high-affinity (dissociation constant ≅ 1.0 n*M*) nuclear binding sites in rat (ROS 17/2.8) and human (HOS TE85) clonal osteoblast-like osteosarcoma cells. Of the steroids tested, only testosterone exhibited significant cross-reactivity with estrogen binding. RNA blot analysis with a complementary DNA probe to the human estrogen receptor revealed putative receptor transcripts of 6 to 6.2 kilobases in both rat and human osteosarcoma cells. Type I procollagen and transforming growth factor–β messenger RNA levels were enhanced in cultured human osteoblast-like cells treated with 1 n*M* estradiol. Thus, estrogen can act directly on osteoblasts by a receptor-mediated mechanism and thereby modulate the extracellular matrix and other proteins involved in the maintenance of skeletal mineralization and remodeling.

OSTEOPOROSIS IS A BONE DISEASE characterized by a significant reduction in bone mass leading to increased susceptibility to fractures. The greatest loss is seen in trabecular bone, the cross-linking structure crucial in maintaining the integrity of the vertebral and hip bones. Bone mass is homeostatically maintained by a process described as coupling (*1*), which involves the interaction and activities of two bone cell types participating in formation and resorption, namely, osteoblasts and osteoclasts, respectively. The balance between bone formation and resorption is regulated by the action of hormones and growth factors on these cells. Approximately 25% of postmenopausal women suffer from an accelerated form of osteoporosis probably caused by a reduction in circulating estrogens. Estrogen is presumably necessary to maintain normal osteoblast function or to attenuate the activity of osteoclasts, or both (*2*). Administration of exogenous estrogens to postmenopausal women retards further reduction of bone mass, although it does not stimulate a net accumulation (*3*).

For a steroid hormone such as estrogen to have a direct effect on a cell, the presence of a high-affinity receptor is required. Steroid hormone–receptor complexes interact with specific genes and regulate their transcriptional activity (*4*). However, the presence of estrogen receptors (ERs) in bone or bone-related cells has not been demonstrated (*5*).

We have increased the sensitivity of the receptor assay by utilizing radiolabeled estradiol with a higher specific activity (*6*). Homogeneous populations of the well-characterized rat (ROS 17/2.8) and human (HOS TE85) osteosarcoma cell lines, both of which are phenotypically osteoblast-like, were used to reevaluate the existence of nuclear ERs in bone cells. A binding site that appears to saturate at an estradiol concentration of approximately 1.0 n*M* was identified in both cell lines (Fig. 1, A and B). This high-affinity binding site in the ROS (Fig. 1A, inset) and HOS (Fig. 1B, inset) cells is characterized by a dissociation constant (K_d) of 0.5 n*M* and 1.1 n*M*, respectively, consistent with the calculated value for the ER in typical estrogen target tissues, such as the uterus or human breast tumor cells (*7*). Unlike the uterus, which contains several thousand high-affinity ERs per cell, the osteosarcoma cells possess ~200 detectable high-affinity binding sites per nucleus (corresponding to approximately 10 fmol of receptor per milligram of protein). This value is not significantly altered when ERs are assayed under exchange conditions (1 hour at 37°C) and does not depend on estrogen treatment of the osteosarcoma cells. Our result suggests that the detection of ERs in bone cells was previously limited by less sensitive assay procedures (*5*). The higher capacity specific binding site seen in Fig. 1, A and B, at estradiol concentrations above 2.5 n*M* has been reported as a type II binding site in estrogen target tissues, although the function of this lower affinity site has not been clearly defined (*8*).

With the exception of testosterone, other steroid hormones such as dexamethasone (a glucocorticoid), progesterone, and dihydrotestosterone do not compete with labeled estradiol for binding to the receptor-like sites (Fig. 1, C and D). The synthetic estrogen, diethylstilbestrol, is the most effective competitor, which is consistent with the occurrence of a traditional ER in these osteoblast-like cells. Significant competition by testosterone could result from either the presence of a second sex-steroid binding protein or the existence of a unique ER in bone that also binds certain androgens (*9*). Alternatively, the presence of aromatase in the cell extracts could account for the testosterone competition by conversion of testosterone to estradiol.

Although the low ER number in bone cells precludes immunodetection, a human ER cDNA (*4, 10*) was used as an independent method for indirectly documenting the existence of the estrogen-binding protein.

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The mRNA populations isolated from the ROS and HOS cell lines were analyzed to determine if the transcript that corresponds to the ER could be detected. When mRNA was evaluated by RNA blot analysis (Fig. 2), a transcript of 6.2 kb was observed in the human cell line and a slightly smaller transcript (6.0 kb) appeared in the rat cell line. These sizes correspond to the transcripts for the ER mRNA in MCF-7 cells (*4*) and rat uterus (*11*), respectively (Fig. 2, inset). Although the presence of the ER mRNA does not insure that it is being efficiently translated into protein, when considered with the ligand-binding results in Fig. 1, the detection of the receptor transcript (Fig. 2) supports the conclusion that the ER exists in bone cells.

If there are high-affinity estrogen binding sites in osteoblast-like cells, then it should be possible to demonstrate a biologic response to a physiologic concentration of estradiol. Two mRNA species coding for proteins that are associated with osteoblast function were utilized as markers (*12, 13*). The two probes used to evaluate the RNA blots were a rat type I procollagen cDNA (*14*) and a human transforming growth factor (TGF)–β cDNA (*15*). Type I collagen, the predominant collagen synthesized by osteoblasts, is affected by estrogen in estrogen target tissues and also in tissues not considered to be primary sites of estrogen action (*16*). Osteoblasts are also a rich source of TGF-β, which increases collagen synthesis in a variety of osteosarcoma cell lines and calvaria (*17*). We isolated mRNA from untreated and estradiol-treated ($10^{-9}M$ estradiol, added to culture 48 and 24 hours before the experiments) HOS TE85 cells. The mRNA was then separated by electrophoresis and transferred to nylon membranes that were probed with the type I procollagen, TGF-β, and actin cDNA probes. The concentration of hybridizing type I procollagen mRNA doubled in the estrogen-treated cells (Fig. 3). The pattern of collagen transcripts (5.0 and 5.8 kb) is characteristic of type $I_{\alpha 1}$ procollagen mRNA, and an analogous effect of estrogen on collagen mRNA has been described in the rat uterus (*14*). Similarly, a 2.5-fold increase in the concentration of TGF-β mRNA is apparent in estrogen-treated human osteosarcoma cells (Fig. 4). We also hybridized HOS TE85 cell mRNA with a β-actin cDNA after the removal of the TGF-β cDNA probe by a high-tempera-

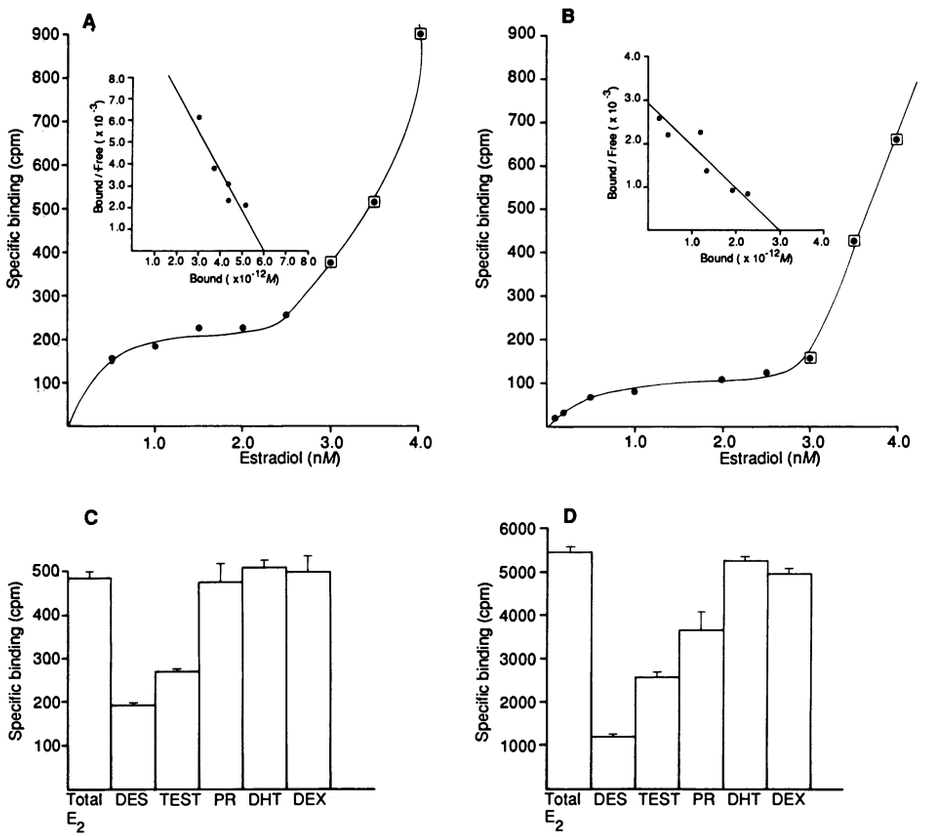

Fig. 1. Saturation, Scatchard, and competition analysis of estrogen binding in nuclear extracts of rat osteosarcoma (ROS 17/2.8) and human osteosarcoma (HOS TE85) cells (*20, 21*). (**A**) Saturation and Scatchard analysis (inset) of estrogen binding in nuclear extracts of ROS 17/2.8 cells cultured in media containing gelding serum and treated with 1 n*M* estradiol for 2 days before harvesting. Boxed circles on the saturation curve are not included in the Scatchard analysis. Data are representative of three independent experiments. $K_d \cong 5.3 \pm 1.0 \times 10^{-10}M$ and $N_{max} \cong 206 \pm 43$ sites per nucleus (± SEM). (**B**) Saturation and Scatchard analysis (inset) of estrogen binding in nuclear extracts of HOS TE85 cells as described for (A). Data are representative of three independent experiments. $K_d \cong 11.0 \pm 2.2 \times 10^{-10}M$ and $N_{max} \cong 200 \pm 114$ sites per nucleus (± SEM). Saturation and Scatchard analysis without estradiol treatment of the cells yielded results comparable to those presented. This fact, together with results in Fig. 2, suggests that estradiol does not regulate its receptor in osteoblast-like cells. (**C**) Competition by various steroids for [^{125}I]estradiol binding to nuclear extracts from ROS 17/2.8 cells grown in media containing 10% fetal bovine serum (FBS). FBS was included in these experiments to determine its effects on the saturation and competition values compared to gelding serum. No detectable variations in binding were measured and only the competition data are presented. E_2, the total estradiol binding in nuclear extracts. Diethylstilbestrol (DES), testosterone (TEST), progesterone (PR), dihydrotestosterone (DHT), and dexamethasone (DEX). (**D**) Competition by various steroids for [^{125}I]estradiol binding in nuclear extracts of HOS TE85 cells grown in media containing 10% gelding serum. The single-concentration competition histograms demonstrate binding specificity but should not be used for quantitation of receptor number; the difference in radioactivity in (C) and (D) is caused by differences in cell number, volume of the nuclear extract counted, and specific activity of the [^{125}I]estradiol. Error bars in (C) and (D) represent SEMs of triplicate samples.

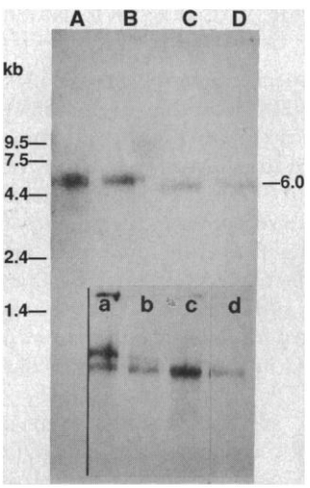

Fig. 2. RNA blot analysis of mRNA from ROS 17/2.8 and HOS TE85 cells probed with a human estrogen receptor (hER) cDNA (*22–25*). Lane A, HOS TE85, no treatment; lane B, HOS TE85, estradiol ($10^{-9}M$)-treated for 48 hours; lane C, ROS 17/2.8, no treatment; lane D, ROS 17/2.8, estradiol ($10^{-9}M$)-treated for 48 hours. The transcript size for the HOS ER mRNA is ~6.2 kb and for the ROS ER mRNA is ~6.0 kb and there is little, if any, effect of estradiol treatment on the amount of hER transcript in either cell line (*26*). (Inset) RNA blot of MCF-7 (lane a), rat uterine (lane b), HOS TE85 (lane c), and ROS 17/2.8 (lane d) mRNA. The upper band in lane a corresponds to the well location in the agarose gel and is probably material that did not enter the gel during electrophoresis. Because the concentrations of mRNA were not normalized in the inset, there were variations in hybridization intensity.

ture, low-salt wash (*18*). Thus, the 2.5-fold increase in TGF-β mRNA elicited by estrogen is not the result of loading unequal amounts of RNA onto the gels; the doubling of collagen transcript concentration by estrogen is also validated by these data, because the same RNA blot was originally probed with the collagen cDNA (Fig. 3). Thus, estrogen regulates the level of two mRNAs important in the synthesis and maintenance of the bone matrix. Because the ER complex acts on DNA to modulate mRNA synthesis, these data support the presence of high-affinity estrogen binding sites (Fig. 1) in osteoblast-like cells.

It is tempting to speculate about the role that estrogen plays in the regulation of collagen and TGF-β. If collagen synthesis is slightly impaired in estrogen deficiency, a significant loss of bone mass could result. We suggest that estrogen may govern the transcriptional activity of the TGF-β gene in osteoblasts. In turn, TGF-β may positively control the transcriptional activity of the type I collagen gene in osteoblast-like cells as demonstrated in NRK-49 rat fibroblasts (*13*). Moreover, TGF-β appears to be a pivotal signal in bone remodeling in that it can stimulate osteoblast proliferation and function, while at the same time diminishing osteoclast activity and osteoclastogenesis.

Estrogen binding and an estrogen-induced response have also been described in human osteoblast cells in primary culture by Eriksen *et al.* (*19*). The lower number of estrogen binding sites in our study compared to that of Eriksen *et al.* (*19*) could result from the different cells used, methodological variations, or the presence of additional lower affinity–higher capacity sites in osteoblasts in primary culture. Yet, as few as 200 binding sites per osteoblast appear to be adequate for a biological response to estrogen (Figs. 3 and 4). Unlike classical estrogen target tissues where there is a relatively rapid and dramatic response to sex steroids, osteoblast regulation by sex steroids may be characterized by a muted response over a longer time frame. Bone is constantly being remodeled, and a small shift in the balance between formation and resorption is sufficient to create significant loss or increased mass. The exacerbated bone mass loss in postmenopausal women that is blunted by estrogen therapy indicates an important role for estrogen in bone maintenance. The present studies lend support to the proposal that estrogen may affect bone directly by a receptor-mediated mechanism in osteoblasts.

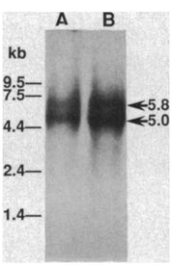

Fig. 3. RNA blot analysis of mRNA from untreated HOS TE85 cells (**A**), and from estradiol-treated ($10^{-9}M$ for 48 hours) HOS TE85 cells (**B**) probed with a rat type I procollagen cDNA. RNA isolation and blotting procedures are as in Fig. 2 (*22–25*). There is a twofold increase in the procollagen transcripts at 5.0 and 5.8 kb in the estradiol-treated cells. Actin mRNA hybridization was used as an internal standard (as in Fig. 2) to normalize the amount of mRNA loaded per lane (~3.0 μg per lane). The results shown here and in Fig. 4 are representative of four separate RNA isolations.

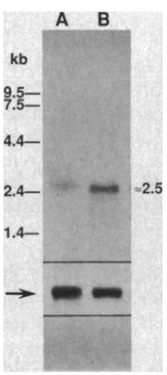

Fig. 4. RNA blot analysis of mRNA from untreated HOS TE85 cells (lane A) and from estradiol-treated ($10^{-9}M$ for 48 hours) HOS TE85 cells (lane B) probed with a human TGF-β cDNA. RNA isolation and blotting procedures are as in Fig. 2 (*22–25*). The transcript hybridizing at 2.5 kb (*15*) is increased ~2.5-fold in the estrogen-treated cells. The arrow points to a portion of the gel that illustrates β-actin cDNA hybridization to the identical membrane utilized for hybridization with the type I procollagen (Fig. 3) and TGF-β cDNA. This portion is not included to demonstrate molecular weight but only to show that the concentration of mRNA in both lanes is comparable.

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22. RNA was isolated with guanidine isothiocyanate (*23*) and mRNA selected by oligo(dT) chromatography (*24*). The mRNA (10 μg) was separated on 1% agarose–formaldehyde gels in 20 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MOPS), 5 m*M* sodium acetate, and 0.1 m*M* EDTA, *p*H 7.0, electrotransferred to a nylon membrane (Schleicher & Schuell, Nytran), and hybridized to a nick-translated, full-length hER cDNA (2000 nucleotides). The

membranes were hybridized and washed as described by Thomas (*25*).
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26. The concentration of mRNA loaded into each lane was determined first by electrophoresis of a calculated amount of mRNA, determined by ultraviolet spectrophotometry. We then hybridized the resulting blot with a β-actin cDNA probe (β-actin is not regulated by estradiol in ROS or HOS cells). The resulting autoradiograph was scanned by laser densitometry, and the amount of mRNA loaded per lane in Fig. 2 was normalized against the actin mRNA concentration in each sample.
27. Supported by NIH grants to B.S.K., M.K., G.L.G., B.W.O., and M.R.H. The human TGF-β cDNA was provided by R. Derynck. A preliminary announcement of these findings has appeared [B. S. Komm *et al.*, *J. Bone Miner. Res.* **2** (suppl. 1) (abstr. 237) (1987)].

30 October 1987; accepted 27 April 1988

Evidence of Estrogen Receptors in Normal Human Osteoblast-Like Cells

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In seven strains of cultured normal human osteoblast-like cells, a mean of 1615 molecules of tritium-labeled 17β-estradiol per cell nucleus could be bound to specific nuclear sites. The nuclear binding of the labeled steroid was temperature-dependent, steroid-specific, saturable, and cell type–specific. These are characteristics of biologically active estrogen receptors. Pretreatment with 10 nanomolar estradiol in vitro increased the specific nuclear binding of progesterone in four of six cell strains, indicating an induction of functional progesterone receptors. RNA blot analysis demonstrated the presence of messenger RNA for the human estrogen receptor. The data suggest that estrogen acts directly on human bone cells through a classical estrogen receptor–mediated mechanism.

ALTHOUGH ESTROGEN DEFICIENCY is a recognized cause of postmenopausal bone loss and estrogen treatment has potent effects on bone (*1*), previous attempts to demonstrate estrogen receptors (ERs) in skeletal tissue of experimental animals (*2*) or humans (*3*), or to show an effect of estrogen on decreasing parathyroid hormone–induced bone resorption in organ culture (*4*), have been unsuccessful. Therefore, the prevailing view is that the effect of estrogen on bone is mediated indirectly. However, the assays for ERs used in the earlier studies may have been insufficiently sensitive or allowed too much degradation to detect low concentrations of ERs in extracts of bone tissue or in heterologous mixtures of bone cells. Recently, however, Gray *et al.* (*5*) reported that 17β-estradiol has direct effects on proliferation and differentiation of a rat osteogenic sarcoma (UMR-106) cell line. We have reinvestigated this issue by using a sensitive new receptor assay (*6*) to measure functional sex-steroid receptors and RNA blot analyses to measure receptor mRNAs in intact cells from a relatively homogeneous population of cultured normal human bone cells.

Human osteoblast-like cells were cultured from explants of human trabecular bone obtained as surgical waste during orthopedic procedures (*7*). The cells were grown in a low Ca^{2+} (0.2 m*M*), phenol red–free medium composed of 45% Ham's F12, 45% Dulbecco's modified Eagle's medium, and 10% fetal bovine serum with penicillin (100 IU/ml) and streptomycin (100 μg/ml). Nuclear ER binding assays were performed after two passages (20 to 30 population doublings). Two to 3 months of culture were required to obtain the 4 million cells needed for each assay in quadruplicate. Twenty-four hours before functional receptor or mRNA analysis, the medium was changed to serum-free medium. These cultured cells had the typical characteristics of the osteoblast phenotype (*8*). They contained 5 to 20 times as much alkaline phosphatase as fibroblasts and responded to parathyroid hormone stimulation by increasing production of adenosine 3′,5′-monophosphate (cAMP). Immunofluorescence studies indicated that 90 to 95% of the cells contained the bone-related proteins bone Gla-protein (BGP, osteocalcin) and osteonectin. After a 48-hour treatment with 1 n*M* 1,25-dihydroxyvitamin D, there was a tenfold increase in bone Gla-protein and a 50% increase in alkaline phosphatase in the culture medium. As assessed by SDS–polyacrylamide gel electrophoresis, more than 90% of the collagen produced by the cells was type I. By immunofluorescence, 95% of the cells contained type I collagen but only 5% contained type III collagen. When β-glycerophosphate was added to the medium, the cells were able to mineralize the newly formed matrix. Human skin fibroblasts cultured by the same method did not have any of these phenotypic characteristics.

A steroid receptor nuclear binding assay (NB assay) was performed as described in (*6*). This assay measures receptor-dependent, receptor-specific nuclear binding of steroids in animal and human tissues (*6*). All seven osteoblast cell strains assayed individually had high-affinity uptake and nuclear binding of [^{3}H]estradiol. The mean (± SEM) concentration of the nuclear-bound receptor was 1615 ± 411 molecules per cell nucleus (Fig. 1). Six of the seven cell strains had functional ER concentrations greater than 500 per cell nucleus, the concentration required to initiate detectable ovalbumin gene transcription in the immature chick oviduct (*6, 9*).

The nuclear binding of [^{3}H]estradiol in

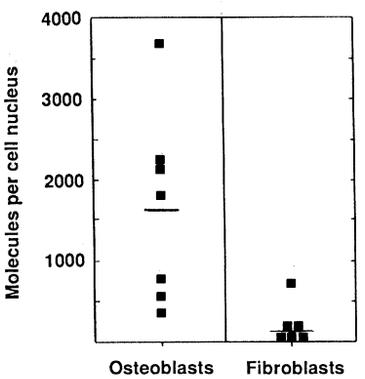

Fig. 1. NB assay for specific nuclear binding of ER in human fibroblasts and in seven strains of human osteoblast-like cells from normal women. Human osteoblast-like cells or human fibroblasts at confluency were trypsinized and incubated with 10 n*M* [^{3}H]17β-estradiol (97.4 Ci/mmol, New England Nuclear) for 45 to 60 min at 37°C alone and in the presence of 100-fold excess of unlabeled DES for total and nonspecific binding, respectively. The remaining steps for the nuclear binding assay were as described in (*6*). Specific binding was determined by subtracting the nonspecific binding (expressed as the mean disintegrations per minute per milligram of DNA for the replicates) from the total binding (in mean disintegrations per minute per milligram of DNA). Control experiments on the limits of the diphenylamine assay for DNA quantitation determined that a minimum of 2 μg of DNA per assay tube was needed for reliability.

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