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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

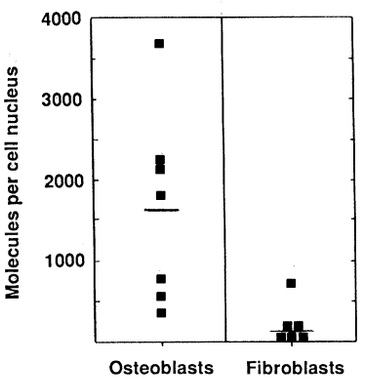

Fig. 1. NB assay for specific nuclear binding of ER in human fibroblasts and in seven strains of human osteoblast-like cells from normal women. Human osteoblast-like cells or human fibroblasts at confluency were trypsinized and incubated with 10 n*M* [^{3}H]17β-estradiol (97.4 Ci/mmol, New England Nuclear) 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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the cultured bone cells have the characteristics of a steroid receptor–mediated system. First, they display tissue- and cell-type specificity for the nuclear binding. In cultured skin fibroblast strains (Fig. 1), the mean specific nuclear binding of [^{3}H]estradiol was 136 ± 110 molecules per nucleus, which is significantly less ($P < 0.01$) than the 1615 ± 411 molecules per nucleus for the cultured bone cells. The nuclear binding of [^{3}H]estradiol found by this assay in nontarget human tissues is equivalent to these fibroblast values (*6*). Second, the nuclear binding is steroid-specific, as in other steroid target tissues (*6, 10*). Human osteoblast-like cells (five strains) were harvested by trypsinization and incubated with 10 n*M* [^{3}H]estradiol alone or with 100-fold excess of unlabeled diethylstilbestrol (DES), dexamethasone, or promegestrone, a synthetic progestin. DES competed for 29.9 ± 8.1% (mean ± SEM) of the [^{3}H]estradiol nuclear binding, whereas promegestrone and dexamethasone competed for 0.0 ± 15.8% and 2.8 ± 1.2%, respectively. Although the mean competition with unlabeled DES is shown as ~30% in these assays, competition as high as ~70% was observed in some experiments. When the ER concentration was low there was a higher background, resulting in lower apparent competition with unlabeled DES. Third, the nuclear binding is saturable. To have sufficient material for reliable analysis of saturation of nuclear binding, we had to pool several cell strains for these sterid titrations. A composite of seven steroid titrations with various cell strains in the NB assay is indicative with saturation of nuclear binding (Fig. 2A). The large standard errors of some of the values may be due to the marked variation in nuclear binding from different strains of cells, as shown in Fig. 1. The demonstration of functional ERs by the NB assay was corroborated by use of a dextran-coated charcoal assay (*11*) on osteoblast-like cell cytosol (Fig. 2B). The specific binding of [^{3}H]estradiol in the cytosol from pooled cell strains by this charcoal assay appears to be saturable. Fourth, the nuclear uptake by these cells in the NB assay is temperature-dependent, with maximal binding at 37°C (1006 ± 115 molecules per nucleus; mean ± SEM) and low binding at 0° and 22°C (160 and 240 molecules per nucleus, respectively). Fifth, the progesterone receptor is inducible, which is one of the most characteristic and specific biologic responses to ER binding in target cells (*12*). Pretreatment of six separate osteoblast-like cell strains with 10 n*M* estradiol for 24 hours showed an overall 2.4-fold increase in specific nuclear binding of [^{3}H]progesterone (Fig. 3) from 554 ± 212 molecules per nucleus to 1340 ± 221 (mean ± SEM). This increase was not statistically significant ($P < 0.15$) because two of the cell strains displayed no induction. However, four of the six strains did show an increase in functional progesterone receptors.

The mean concentration of nuclear-bound ERs in these osteoblast-like cells was 2.5 to 5 times higher than that required to induce gene transcription in experimental animals (*6, 9*) and was in the lower range of that found in benign human endometrium (*6*), an estrogen target tissue. In some strains of cells with a low level of specific nuclear binding, it was not possible to demonstrate saturable nuclear binding by the NB assay or a saturable cytosolic binding by the dextran-coated charcoal assay. The variable levels of ER (Fig. 1) found in different strains of cells may be due to prolonged culture of these cells in the absence of estrogen, which encourages the clonal selection of ER-deficient cells. These cells would not respond to estrogen. Thus, the number of ERs in osteoblasts may be even higher in vivo.

Expression of mRNA for ERs was demonstrated by RNA blot analysis. Hybridiza-

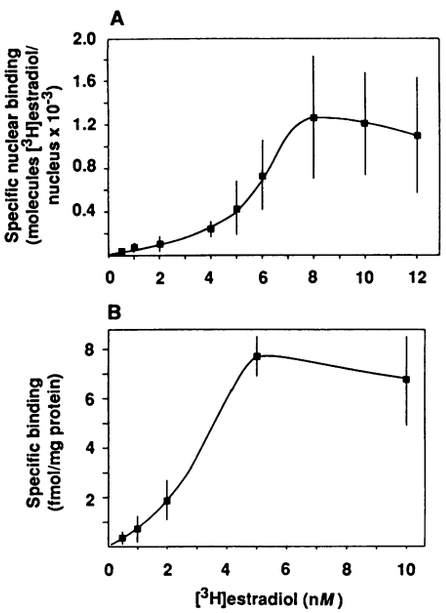

Fig. 2. Saturation of nuclear [^{3}H]estradiol binding in human osteoblast-like cells. (**A**) Osteoblast cells were incubated with 1 to 12 n*M* [^{3}H]estradiol alone or in the presence of 100-fold excess of unlabeled estradiol. The data points represent means of nuclear [^{3}H]estradiol binding from six separate experiments; bars indicate SEM. (**B**) We prepared cytosol from a pool of osteoblast cell strains by sonication in 50 m*M* tris, *p*H 7.4, 10% glycerol, 1 m*M* EDTA, 20 m*M* molybdate, and 0.1 m*M* phenylmethylsulfonyl fluoride, and centrifugation at 105,000*g* for 1 hour at 4°C. Portions of the resultant supernatant were labeled in triplicate for 4 hours at 4°C with [^{3}H]estradiol at 0.5 to 10 n*M*, with and without 100-fold excess of unlabeled estradiol. Specific binding was determined by charcoal adsorption essentially as described (*11*). The data points represent the means of the specific binding at each steroid concentration for a single experiment; bars indicate SD.

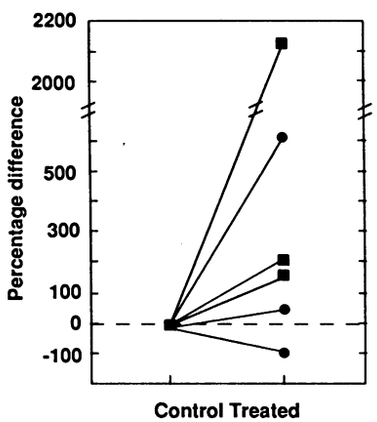

Fig. 3. Induction of nuclear progesterone receptor by treatment of osteoblasts with unlabeled estradiol. Six sets of individual osteoblast-like cell strains [three female strains (■) and three male strains (●) are given] were incubated at near confluency in 40% Ham's F12, 40% Dulbecco's modified Eagle's medium, 20% fetal bovine serum, and 2 m*M* Ca^{2+} for 48 hours, and then in serum-free medium without (control) or with (treated) 10 n*M* estradiol for 24 hours. The cells were incubated with 10 n*M* [^{3}H]R5020 alone (total binding) or in the presence of 100-fold excess of unlabeled progesterone (nonspecific binding). Nuclear progesterone binding was determined by the NB assay (*6*). The results are presented as the percentage difference between the specific [^{3}H]R5020 binding in mean molecules per nucleus in the estrogen-treated cells compared to that in the control cells (that is, not estradiol-treated).

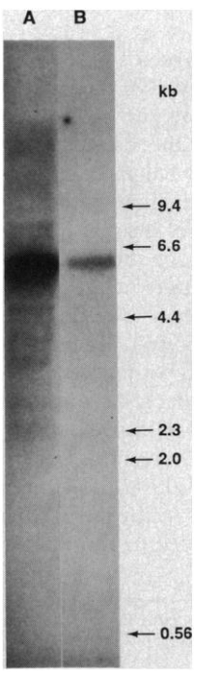

Fig. 4. Expression of estrogen receptor mRNA. Total RNA was extracted from a single cell strain with guanidine thiocyanate (*15*), enriched for mRNA by oligo(dT)-cellulose chromatography (*16*), separated on a 1% agarose-glyoxal gel, and transferred to a nylon membrane. The mRNA on the membrane was hybridized for 18 hours at 43°C with a cDNA to human ER labeled with ^{32}P by random primer extension with a multiprime kit (Amersham). The cDNA probe is a 1.8-kb Tth 111I–Sac I fragment. The blot was washed twice at 65°C in standard saline citrate and 0.1% SDS and then analyzed by autoradiography for 40 hours. Quantities loaded were (lane A) MCF-7 cell mRNA, 2 μg, and (lane B) osteoblast mRNA, 7.5 μg.

tion with a full-length cDNA (*13*) to the human ER revealed a single species of approximately 6.4 kb, identical in size to that found in MCF-7 cells (*13, 14*), an ER-positive human mammary carcinoma cell line (Fig. 4). Expression of ER mRNA was similar in all three strains tested.

In summary, cultured human osteoblast-like cells possess the properties of target cells for estrogen, in that they display a steroid-specific, saturable, temperature-dependent nuclear binding, and an induction of progesterone receptor in response to estrogen treatment. Lastly, they contain mRNA for the ER. Since estrogen is the major hormone responsible for the maintenance of bone mass, the presence of specific ERs in osteoblasts could indicate a direct effect of estrogen on these cells.

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30 October 1987; accepted 4 May 1988

Local Embryonic Matrices Determine Region-Specific Phenotypes in Neural Crest Cells

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Membrane microcarriers were used to determine the ability of regional extracellular matrices to direct neural crest cell differentiation in culture. Neural crest cells from the axolotl embryo responded to extracellular matrix material explanted from the subepidermal migratory pathway by dispersing and by differentiating into pigment cells. In contrast, matrix material from the presumptive site of dorsal root ganglia stimulated pronounced cell-cell association and neurotypic expression. Cell line segregation during ontogeny of the neural crest that leads to diversification into pigment cells of the skin or into elements of the peripheral nervous system appears to be controlled in part by local cell-matrix interactions.

DURING EMBRYONIC DEVELOPMENT, neural crest (NC) cells of the trunk region migrate from their original position along the dorsal neural tube in two principal directions. Cells following the subepidermal migratory pathway give rise to pigment cells of the skin, whereas NC cells proceeding along the medioventral route will form various structures of the peripheral nervous system (*1–3*). Although the initial state of phenotypic commitment is probably heterogeneous within the premigratory NC cell population (*1, 4, 5*), there is evidence that expression of various phenotypes is affected by environmental cues encountered during migration (*1, 4–9*). The extracellular matrix contacted by the moving NC cells has been proposed to constitute a site from which instructive stimuli for both cell differentiation and morphogenetic processes occurring during NC development may emanate (*6, 9–13*).

We showed earlier that by implanting Nuclepore membranes into living axolotl embryos (*Ambystoma mexicanum*) it is possible to explant regional matrix materials that are synthesized and deposited onto the surface of the membranes in situ (*3, 6, 12, 13*). When tested in a defined culture system, matrix material explanted on such membrane microcarriers was found to promote NC cell differentiation into pigment cells (*6*). We have now examined the function of the extracellular matrix in cell line segregation during NC development by use of a novel nitrocellulose-based membrane microcarrier with high adsorbance capacity.

Microcarriers were introduced into the subepidermal space (subepidermal matrix material) or in the presumptive region of dorsal root ganglia (preganglionic matrix material) of living axolotl embryos (Fig. 1). After 10 to 12 hours of conditioning, the microcarriers were removed from the embryo and covered with isolated NC cells or processed for analysis of the explanted matrix materials. Scanning electron microscopy revealed that the material adsorbed onto the surfaces of the microcarriers was organized as sparse patches of short fibrils densely decorated with ruthenium red–precipitated granules, which might represent proteoglycan-based complexes as well as matrix-affiliated growth factors. Although the amounts of material isolated from the two embryonic regions were not quantitated, they appeared comparable. Both matrix materials became adsorbed onto the microcarrier surfaces as fibrillar tufts and smaller fragments, distributed in different proportions (Fig. 2, A and B). Incubation with selected antibodies demonstrated the presence of characteristic matrix components, including fibronectin (*14*), laminin (*15*), collagen types I and III (*16*), and sulfated proteoglycans as revealed by identification of their chondroitin-6-sulfate moieties (*17*). No differences between the two regional matrices were detected after immunohistochemical labeling of matrix-covered microcarriers. The nanogram quantities of subepidermal and preganglionic matrices isolated on microcarriers were also analyzed by polyacrylamide gel electrophoresis with a newly developed technique that permits detection of protein amounts below 10^{-12} g (*18*). Primarily, two-dimensional separation according to this procedure (*19*) revealed differences in the protein contents of the two matrices that were predominantly localized in regions corresponding to molecular sizes of 45 to 50 kD and 70 to 95 kD (Fig. 3).

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