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Identification of androgen receptors in normal human osteoblast-like cells

(steroid hormone/estrogen receptor/bone)

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ABSTRACT **The sex steroids, androgens and estrogens, are major regulators of bone metabolism. However, whether these hormones act on bone cells through direct or indirect mechanisms has remained unclear. A nuclear binding assay recently used to demonstrate estrogen receptors in bone [Eriksen, E. F., Colvard, D. S., Berg, N. J., Graham, M. L., Mann, K. G., Spelsberg, T. C. & Riggs, B. L. (1988) *Science* 241, 84–86] was used to identify specific nuclear binding of a tritiated synthetic androgen, [^{3}H]R1881 (methyltrienolone), in 21 of 25 (84%) human osteoblast-like cell strains and a concentration of bound steroid receptors of 821 ± 140 (mean ± SEM) molecules per cell nucleus. Binding was saturable and steroid-specific. Androgen receptor gene expression in osteoblasts was confirmed by RNA blot analysis. Relative concentrations of androgen and estrogen receptors were compared by measuring specific nuclear estrogen binding. Nuclear binding of [^{3}H]estradiol was observed in 27 of 30 (90%) cell strains; the concentration of bound estradiol receptor was 1537 ± 221 molecules per cell nucleus. The concentrations of nuclear binding sites were similar in males and females for both [^{3}H]R1881 and [^{3}H]estradiol. We conclude that both androgens and estrogens act directly on human bone cells through their respective receptor-mediated mechanisms.**

Androgens and estrogens are major regulators of bone metabolism in males and females, respectively (1). Both hormones interact with growth hormone in control of the adolescent growth spurt (2–4). After growth is completed, androgens and estrogens maintain bone mass in the adult. In women, menopause causes accelerated bone loss that can be prevented by estrogen administration (5, 6). Treatment of postmenopausal women with androgen derivatives, synthetic anabolic steroids, produces beneficial effects on bone metabolism (7, 8). In males, hypogonadism is associated with bone loss, which is stabilized by testosterone administration (9).

Because previous attempts to demonstrate receptors in bone cells of experimental animals (10, 11) or humans (12) have been unsuccessful, the prevailing view has been that the action of sex steroids on skeletal tissue is mediated indirectly. Recent data, however, suggest that estrogen has a direct action. Both specific nuclear binding of estradiol and the presence of estrogen receptor mRNA were demonstrated in normal human osteoblast-like cell strains (13) and in rat and human osteogenic sarcoma cell lines (14). Moreover, biologic effects of estrogen treatment *in vitro* have been demonstrated on proliferation and differentiation of normal mouse osteoblast-like cells (15) and rat osteogenic sarcoma cell lines (16), on induction of progesterone receptors in human osteoblast-like cell strains (13), and on induction of gene transcription for type I procollagen and transforming growth factor β in rat and human osteogenic sarcoma cell lines (14).

We have investigated the possibility that bone cells contain androgen receptors as well as estrogen receptors. These studies were carried out with cultured human osteoblast-like cells by using a sensitive, nuclear binding assay for functional sex steroid receptors (17, 18). In addition, a cDNA probe for the human androgen receptor (19) was used to determine whether human osteoblasts contained androgen receptor mRNA.

METHODS

Cell Culture. Human bone cells were cultured from fresh samples of trabecular bone obtained during orthopedic procedures, by a procedure modified from that developed by Robey and Termine (20). These cultured cells had the typical characteristics of the osteoblast phenotype (13, 21). In brief, residual fibrous tissue was carefully dissected from the bone samples, which were then washed and minced in phosphate-buffered saline and digested with crude bacterial collagenase (GIBCO) at 1 mg/ml in Dulbecco's modified Eagle's medium (DMEM, GIBCO) for 2 hr at 37°C in a shaking water bath. These fragments were then cultured in a phenol red-free medium (GIBCO) approximately equivalent to a Ca^{2+}-free 1:1 (vol/vol) mixture of Ham's F-12 and DMEM supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin. Steroid receptor assays were routinely performed after the second passage of cells in 175-cm^2 culture flasks (20–30 population doublings). Two to 3 months of culture were required to obtain the 4 million cells needed for each assay in replicate.

In experiments to assess nuclear binding of radiolabeled steroids, the medium was replaced for 48 hr by the same medium containing 20% fetal bovine serum and 2 mM Ca^{2+}. Twenty-four hours before receptor assay, the medium was changed to serum-free medium containing 2 mM Ca^{2+}.

Nuclear Binding Assay. The specific nuclear binding of tritiated steroids in these cells was measured by a nuclear binding assay as described in detail previously and modified for tissue culture cells (13, 17, 18). In brief, human osteoblast-like cells at confluence were removed by trypsinization and suspended in MCDB-202 medium (Irvine Scientific). Replicate aliquots of the cell suspension (0.5–1 × 10^6 cells per replicate) were incubated with [17α-*methyl*-^{3}H]R1881 (methyltrienolone, a potent synthetic androgen) (DuPont) or [2, 4, 6, 7(n)-^{3}H]estradiol (DuPont) at a final concentration of 10 nM with or without a 100-fold excess of the respective nonradiolabeled steroid for estimation of nonspecific and total binding for 1 hr at 37°C. When sufficient cells were available, quadruplicate assays were done. After the 1-hr incubation,

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the cells were collected by centrifugation, washed in MCDB-202 medium, and suspended in homogenization buffer [50 mM Tris, pH 7.5/10% (vol/vol) glycerol/10 mM KCl/0.1% Triton X-100/0.1% bovine serum albumin]. The cells were homogenized in a Teflon pestle/glass homogenizer (Thomas type A) and the homogenate was layered over a cushion of 1.4 M sucrose in homogenization buffer and centrifuged for 20 min at 7000 × *g*.

Each nuclear pellet was extracted with ethanol and the radioactivity was determined by liquid scintillation spectrometry in a LS-5801 liquid scintillation counter (Beckman). The DNA in each replicate was quantitated by a micro version (17) of the diphenylamine assay of Burton (22) with calf thymus DNA (Calbiochem) treated similarly as a standard. Control experiments indicated that a minimum of 1 μg of DNA per replicate was required for a reliable diphenylamine assay.

Calculations. The specific nuclear steroid binding was determined by subtracting the average of the replicate assays for the total binding of [^{3}H]steroid (expressed in disintegrations per minute per microgram of DNA) minus the corresponding average of the replicate assays for the nonspecific binding. Since a negative calculated value of specific nuclear binding essentially indicates no specific binding, negative values were reset to zero. For calculation of molecules per cell nucleus, a value of 6 μg of DNA per 10^6 cells was used (23).

RNA Extraction and Northern Blot Hybridization. Total RNA was extracted from single cell strains with guanidinium thiocyanate (24), enriched for mRNA by oligo(dT)-cellulose chromatography (25), and separated electrophoretically in a 1% agarose gel. The RNA was transferred to nylon and hybridized overnight at 43°C with human androgen receptor cDNA labeled with [α-^{32}P]dCTP by means of a multiprime kit (Amersham). The cDNA probe was a 713-base-pair *Hin*dIII–*Eco*RI fragment of ARHFL1 (19). The blot was washed twice at 65°C with 15 mM NaCl/1.5 mM sodium citrate, pH 7/0.1% sodium dodecyl sulfate for 15 min and analyzed by autoradiography.

RESULTS

Specific Nuclear Androgen Binding in Human Osteoblast-like Cells. Specific nuclear binding of the tritiated synthetic androgen [^{3}H]R1881 (methyltrienolone) was found in 11 of 12 (92%) osteoblast-like cell strains derived from male patients and in 10 of 13 (77%) cell strains from female patients (Fig. 1 *Left*). For all osteoblast-like cell strains combined, androgen receptor concentration was 821 ± 140 (mean ± SEM) molecules per cell nucleus. The nuclear androgen receptor concentration in the male strains was 628 ± 124 molecules per nucleus and ranged from 0 to 1550 molecules per nucleus. The nuclear androgen receptor concentration in the female strains was 998 ± 233 molecules per nucleus and ranged from 0 to 2750 molecules per nucleus. There was, however, no statistically significant difference between the two means ($0.5 > P > 0.1$, Student's *t* test).

Measurements of estrogen receptors in osteoblast-like cell strains were extended beyond a previous report (13) in which the mean concentration in seven strains from females was reported to be 1615 ± 410 molecules per cell nucleus. The nuclear estradiol receptor concentration for all 30 cell strains, including the original 7 strains, was 1537 ± 221. The mean concentration for the 15 female strains was 1854 ± 306 and that for the 15 male strains was 1220 ± 297 (Fig. 1 *Right*). Although the mean estrogen receptor concentration in female strains was higher, the difference was not statistically significant ($0.5 > P > 0.1$, Student's *t* test).

Saturation of Nuclear Androgen Binding in Human Osteoblast-like Cells. To confirm that the specific nuclear binding observed above was due to classical androgen receptors, saturation of receptor binding and steroid-specific binding were demonstrated. For saturation of nuclear steroid receptor binding, osteoblast-like cells from single strains, or from several pooled strains, were incubated with various concentrations of [^{3}H]R1881 and processed for nuclear binding.

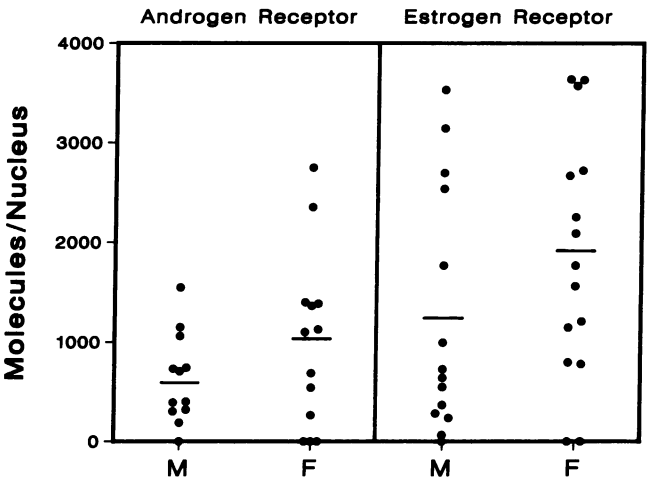

FIG. 1. Nuclear androgen and estrogen receptor binding in normal human osteoblast-like cells. Specific nuclear receptor binding in human osteoblast-like cells was determined as described in *Methods*. Dots represent the mean calculated number of molecules per cell nucleus for each strain. (*Left*) Specific nuclear binding of [^{3}H]R1881 (methyltrienolone, an androgen analog) in 12 strains from normal men and 13 strains from normal women. (*Right*) Specific nuclear [^{3}H]estradiol binding in 15 strains from men and 15 strains from women was determined. The mean receptor concentrations are indicated by the horizontal lines.

Data points on nuclear androgen receptor binding (Fig. 2) each represent a composite of four separate experiments and demonstrate saturable binding of [^{3}H]R1881. The shape of the curve for specific nuclear binding in all four experiments was essentially identical; thus, the SEM bars in Fig. 2 reflect the fact that saturation was achieved at approximately 2300, 2800, 1500, and 600 molecules per nucleus, respectively.

Steroid Specificity of Nuclear Androgen Binding in Human Osteoblast-like Cells. The specificity of nuclear steroid binding was tested to further verify the presence of specific androgen receptors in normal human osteoblast-like cells.

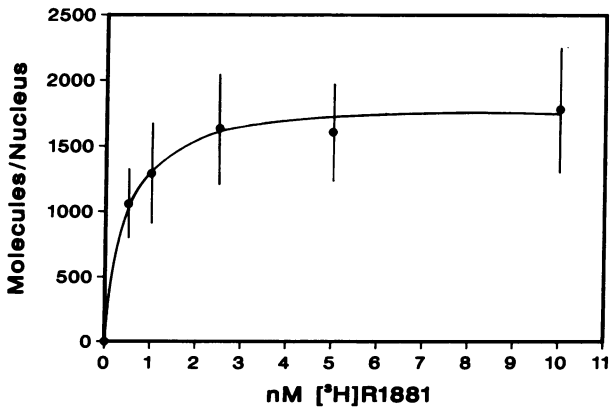

FIG. 2. Saturation of nuclear [^{3}H]R1881 binding in normal human osteoblast-like cells. Osteoblast-like cells at confluence were trypsinized, washed with MCDB-202 medium, incubated with 0.5–10 nM [^{3}H]R1881 alone or with 100-fold excess unlabeled R1881, and processed for specific nuclear binding. Data points present the means for specific binding from four experiments; vertical bars indicate SEM.

When total [^{3}H]R1881 nuclear binding was measured with a variety of homologous or nonhomologous steroids as competitors, only nonradiolabeled R1881 competed significantly with [^{3}H]R1881 for nuclear binding (Fig. 3). Diethylstilbestrol (DES, a synthetic estrogen), as well as dexamethasone (DEX) and triamcinolone acetonide (TAC, a synthetic glucocorticoid), competed only slightly with [^{3}H]R1881 thus demonstrating specific androgen receptor binding. Triamcinolone acetonide was used in several experiments to eliminate [^{3}H]R1881 binding to progesterone and glucocorticoid receptors (26). Competition by triamcinolone acetonide ranged from 7% to 23% with a mean competition of 17%. Since the limited cell numbers available for study precluded using triamcinolone acetonide for each replicate assay point, the concentration of androgen receptors given in Figs. 1 and 2 may be slightly overestimated.

Detection of Androgen Receptor Gene Expression in Normal Osteoblast-like Cells. The results of RNA analysis by Northern blot hybridization support the conclusion that androgen receptor is expressed in human osteoblast-like cells from normal males (Fig. 4). A single band at ≈10 kilobases was observed with mRNA from human osteoblast-like cells and from an androgen receptor-positive tissue, rat ventral prostate (lane 2), when probed with a cDNA for the human androgen receptor (18). mRNA from hen spleen, a tissue devoid of androgen receptor binding activity, did not contain the 10-kilobase species (lane 3). Expression of androgen receptor mRNA was similar in three strains of human osteoblasts tested.

DISCUSSION

Our data provide strong evidence for the presence of androgen receptors in normal human bone cells of the osteoblast lineage. Specific nuclear binding of [^{3}H]R1881 (methyltrienolone, a potent synthetic androgen) was found in the large majority of cell strains studied. The nuclear binding assay employed for these studies provides added specificity because it measures only functional steroid receptors. Functional receptors are defined as those that not only bind the steroid but also become activated and bind to nuclear acceptor sites (18, 27). This event correlates with the biologic response to the steroid (18). Binding of [^{3}H]R1881 was saturable and steroid-specific as is characteristic of steroid target tissues (27). Expression of the androgen receptor gene in these cells was confirmed by RNA blot analysis. Thus, human bone cells contain receptors for both androgen and estrogen.

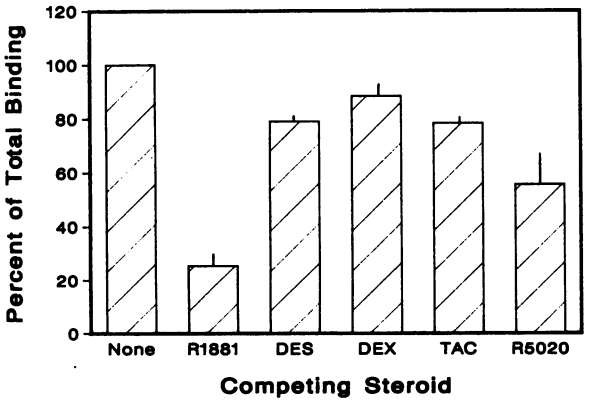

FIG. 3. Steroid specificity of nuclear [^{3}H]R1881 binding in human osteoblast-like cells. Human osteoblast-like cells at confluence were harvested by trypsinization, washed with MCDB-202 medium, and incubated with 10 nM [^{3}H]R1881 alone or with 100-fold excess unlabeled R1881, diethylstilbestrol (DES), dexamethasone (DEX), triamcinolone acetonide (TAC), or R5020 (promegestrone, a progesterone analogue). Nuclear binding was determined by using the assay described in *Methods*. Data presented is expressed as the mean percent of binding relative to the nuclear binding in the absence of competing steroid for each competing steroid from six separate experiments; vertical bars indicate SEM.

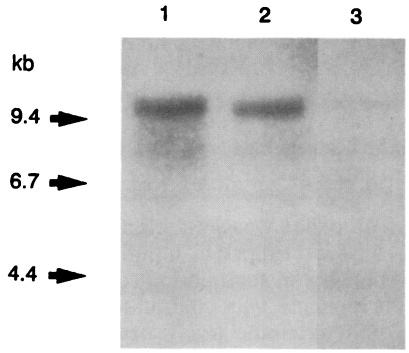

FIG. 4. Northern blot analysis of androgen receptor gene expression in normal human osteoblast-like cells. mRNA (10 μg per lane) isolated from normal human osteoblast-like cells and control tissues was analyzed by blot hybridization with a cDNA to the human androgen receptor (19). Lanes 1, normal male osteoblast-like cells; 2, rat ventral prostate; 3, adult hen spleen. Positions of size markers are indicated at left (kb, kilobases).

Failure of earlier studies to detect sex-steroid receptors in bone cells likely resulted from methodological problems. Earlier studies were performed before it was recognized that assays using dextran-coated charcoal require the addition of molybdate and/or protease inhibitors for stabilization of low-abundance receptors and to protect against proteolytic degradation (28). Moreover, previous studies utilized mixed populations of bone cells, whereas the present study was made on nearly homogeneous populations of cells that exhibited the complete osteoblast phenotype (21).

The androgen receptor concentration of 821 ± 140 molecules per cell nucleus found in the cultured human bone cells was comparable to the concentration of 1270 ± 483 molecules per cell nucleus found in 16 samples of normal human prostate (that is, tissue adjacent to prostatic cancer) and 1611 ± 970 molecules per cell nucleus found in benign hypertrophic prostate obtained at the time of prostatic surgery (unpublished data). Nuclear androgen receptor binding in excess of 500 molecules per nucleus in prostatic cancer tissue correlates significantly with increased survival of the patient and with the duration of response to hormonal therapy (29). The concentration of androgen receptors found in normal human osteoblast-like cells should, therefore, be sufficient for biological activity.

The average concentration of estrogen receptors in the cultured human osteoblast-like cells was about twice the average concentration of androgen receptors. Studies in animal systems have shown that concentrations of 500–1000 estrogen receptors per cell nucleus are needed to induce transcriptional activity (18, 30, 31).

Whether the observed concentrations in cultured cell strains are lower than concentrations in bone cells *in vivo* is unknown. It is possible, however, that prolonged culture of these cells in the absence of sex steroids influenced receptor concentration or encouraged the clonal selection of cells deficient in sex-steroid receptors.

Even though the predominant circulating sex steroid in men is testosterone and the predominant circulating sex steroids in premenopausal women are estradiol and estrone, androgens and estrogens circulate in both sexes. Our findings that bone cells contain both androgen and estrogen receptors and that their concentrations in cells from male and female donors were similar for each receptor suggest that both sex

steroids contribute to the maintenance of bone mass in both sexes. This might be particularly true in postmenopausal women, whose serum estrogens fall to low levels but whose serum testosterone declines only slightly (32).

In summary, our data suggest that both androgens and estrogens act directly on human bone cells through their respective receptor-mediated mechanisms. These findings underscore the importance of sex steroids in bone biology. Both androgen and estrogen deficiency may have important implications in the causation of osteoporosis, an enormously important public health problem.

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