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Laboratory Investigations

Androgen Receptors in Osteoblast-Like Cell Lines

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Summary. Although androgens exert major effects on bone remodeling, the mechanisms by which they exert their effects remain unclear. Recently, it has become apparent that receptors for sex steroids may be present in osteoblastic cells. We have examined several cell lines with osteoblastic phenotypes to determine if specific, high affinity androgen receptors are present. Two cell lines of human origin (Saos-2 and U2-OS) and one of rat origin (UMR-106.01) were studied. Androgen binding sites were present in all cell lines. Binding affinities were high (K_D = 1.6 – 2.5 × 10^{-10} M), and similar to those in classical androgen target tissues (prostate, kidney). Concentrations were greater in the human cell lines (1277 and 1605 sites/cell) than in the rodent line (74 sites/cell). In the human cell lines androgen binding was also specific and typical of androgen receptors in other tissues. Specific estrogen binding was not present in the UMR-106.01 cells, and no estrogen receptors were detectable in the human cell lines using an enzyme-linked receptor immunoassay. Specific binding for progesterone was also absent in the UMR-106.01 cells, but progesterone receptors were detected immunologically in the Saos-2 (119 sites/cell) and U2-OS (118 sites/cell) lines. These findings indicate the presence of androgen receptors that are of similar character to those in classical androgen target tissues, and suggest that the study of these cell lines may be useful in the study of the regulation of androgen effects in osteoblasts.

Key words: Androgen – Estrogen – Receptors – Osteoblast.

In clinical situations, estrogens have long been noted to exert profound effects on indices of bone and mineral metabolism as well as on bone mass [1–5]. More recently, estrogen receptors have been described in bone cells, and estrogenic effects have been described on a variety of functions in osteoblastic cell populations [6–11]. Although the effects of androgens are less well characterized, androgen receptors have also been reported in primary cultures of human osteoblastic cells [12]. Androgen effects have been noted on the rate of bone cell proliferation *in vitro* [13, 14], and the parathyroid hormone responsiveness of at least one osteoblast-like cell line (Saos-2) is apparently altered by androgen exposure [15]. Androgen insufficiency in men is clearly related to the development of osteopenia [16–18], and androgen replacement appears to partially reverse the osteopenia present in hypogonadal men [19]. Similarly, in postmenopausal women, androgen therapy results in a reduction in the rate of bone loss or an actual augmentation of bone mass [20–22]. Hence, androgens seemingly play an important role in skeletal physiology.

In these studies we have examined several osteoblast-like cell lines of human and nonhuman origin for the presence of specific, high-affinity androgen binding, and have found the characteristics of this binding activity to be similar to androgen receptors localized in classical androgen responsive tissues such as the prostate and kidney.

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Methods

Cell Cultures

Three cell lines of osteoblast phenotype have been utilized in these studies: Saos-2 (passage 34) and U2-OS (passage 34) are both of human (female) origin and were obtained from the American Type Tissue Collection; UMR-106.01 cells (passage 19), of rat origin, were kindly supplied by Nicola Partridge (Pediatric Research Institute, St. Louis MO). UMR-106.01 cells were grown in Eagle's minimal essential medium (MEM) with Earl's salts with 10% newborn calf serum, and the U2-OS and Saos-2 cells were grown in McCoy's 5a with 10% newborn calf serum. Radioimmunoassays revealed a pool of newborn calf serum to contain less than 20 pg/ml estradiol and 20 ng/dl testosterone. All cells were grown to 90–95% confluence in 75 or 175 cm^2 plastic flasks before being harvested (1 mM EDTA, 0.25% trypsin in Hank's salt solution), washed (Hank's), frozen, and stored at −70°C for subsequent androgen, estrogen, and progesterone binding analysis, as well as immunoassay of estrogen and progesterone receptors in the human cell lines.

Androgen Receptor Quantitation

Frozen cell pellets consisting of approximately 5.0 × 10^8 cells were thawed on ice and resuspended in 6.0 ml of 0.05 M Tris buffer containing 1.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 20 mM sodium molybdate, and 1 mM phenylmethylsulphonylfluoride at 0–4°C. Suspensions were homogenized using two bursts of a Brinkman polytron (probe = 13 mm). Homogenates were centrifuged at 800 x *g* for 15 minutes. Cytosol fractions were separated from crude nuclear pellets, and in order to remove any endogenous sex steroids, cytosols were poured onto dextran-coated charcoal pellets and vortexed, resulting in a 0.5% charcoal suspension. Suspensions were incubated at 0–4°C for 30 minutes with periodic vortexing. Cytosol-charcoal suspensions were centrifuged at 40,000 x *g* for 60 minutes at 0–4°C, and cytosols were removed from charcoal

pellets. Nuclear pellets were extracted in 3.0 ml of 0.6 M KCl, 0.05 M Tris, 1.5 mM EDTA, 0.05 mM dithiothreitol, 10% glycerol buffer for 30 minutes at 0°C. Following incubation, the extracts were diluted 1:1 with Tris buffer (0.1 M Tris, 3.0 mM EDTA, 1.0 mM dithiothreitol, 20% glycerol, 40 mM sodium molybdate, and 2 mM phenylmethylsulphonylfluoride). Nuclear extracts were centrifuged at 40,000 x *g* for 60 minutes (0–4°C). After removal of salt extracts, the residual nuclear pellets were processed for DNA quantitation according to the method of Burton [23].

Androgen receptors were quantitated in the cytosolic and nuclear salt extracts of all cell lines by titration analysis using 0.25–5.0 nM ^{3}H-R1881 (New England Nuclear, Claremont, CA, specific activity 70–87 Ci/mM) at concentrations of 0.25, 0.5, 1.0, 2.0, and 5.0 nM. The degree of nonspecific ^{3}H-R1881 binding was established by competitive displacement using an excess of unlabeled R1881 (5.0×10^{-7} M). Nonspecific ^{3}H-R1881 binding was subtracted from total ^{3}H-R1881 binding to determine the specific ^{3}H-R1881 bound. Triamcinolone acetonide (2.5×10^{-6} M) was added to all binding tubes to prevent the possibility of ^{3}H-R1881 binding to progestin receptors. Binding equilibrium was established by incubating for 20 hours at 0–4°C.

Following equilibration, bound ^{3}H-R1881 was separated from free radioligand in the cytosolic fractions by the addition of a 0.05% dextran, 0.5% charcoal slurry in assay buffer. The addition of a 50% slurry of hydroxyappatite (HAP) was used to separate bound and free radioligand in the nuclear extracts. Radioactivity in aliquots of the cytosol and of alcohol extracts of the washed HAP pellets was quantitated by scintillation spectrometry. Specific ^{3}H-R1881 binding capacity was estimated by Scatchard plot analysis [24]. The high affinity K_D = 1-5.0 $\times 10^{-10}$ M), specific binding of ^{3}H-R1881 was expressed in fmol/mg DNA. The number of binding sites per cell was calculated from the specific binding, the number of cells analyzed, and the cellular DNA content. The sensitivity for the detection of specific androgen binding using these methods was 5 fmol/ml, representing 15 sites/UMR-106.01 cell and 90 sites/U2-OS or Saos-2 cell.

The androgen binding assays were controlled by including standardized pools of rat and human prostate tissues containing known concentrations of androgen receptor. Androgen receptor concentrations in normal (noncastrate) rat prostate and kidney, and in human prostatic carcinomas (freshly obtained at the time of prostatectomy) were determined using identical methodology.

Estrogen and Progesterone Receptor Quantitation

An essentially identical approach was used to prepare UMR-106.01 cells for estrogen and progesterone binding assays, except the cell pellets (approximately 5.0×10^8 cells) were thawed on ice and resuspended in 4.0 ml homogenization buffer. Steroid binding assays in cytosol fractions and nuclear salt extracts were performed as described above by titration analysis using ^{3}H-estradiol (New England Nuclear, specific activity 90–110 Ci/mM) at concentrations of 0.2, 0.4, 0.8, 1.0, and 2.0 nM, or ^{3}H-R5020 (New England Nuclear, specific activity 80–100 Ci/mM), a synthetic progestin receptor radioligand, at concentrations of 0.5, 1.0, 2.0, 5.0, and 10.0 nM. The extent of nonspecific ^{3}H-estradiol and ^{3}H-R5020 binding was established by competitive displacement using excess unlabeled diethylstilbestrol (DES) (2.0×10^{-7} M) and R-5020 (1×10^{-6} M), respectively. Nonspecific ^{3}H-estradiol and ^{3}H-R5020 binding was subtracted from total radioligand binding to determine specific binding of ^{3}H-estradiol and ^{3}H-R-5020 to estrogen and progestin receptors, respectively. The sensitivity for the detection of specific estrogen or progestin binding using this technique is 5 fmol/ml, representing 90 sites/UMR-106.01 cell.

The estrogen and progesterone receptors in cytosolic and nuclear fractions of human-derived cell lines (U2-OS and Saos-2) were quantitated by enzyme-linked immunoassay. Human estrogen and progesterone receptor immunoassay kits were obtained from Abbott Laboratories (North Chicago, IL) and the assays were conducted according to the manufacturer's specifications. Cytosol or nuclear extracts were incubated with plastic beads coated with either antiestrogen receptor or antiprogesterone receptor monoclonal antibodies for 18 hours at 2–8°C. Immobilized steroid receptors were separated from unbound substances by washing and aspiration. A second specific estrogen receptor or progesterone receptor antibody conjugated to horseradish peroxidase was added and a complex of layered antibodies and receptor was formed. After a 1 hour incubation at 37°C for estrogen receptor or at 2–8°C for progesterone receptor, the beads were washed. Enzyme substrate (hydrogen peroxide and o-phenylenediamine-HCl) was added producing a colorometric reaction which proceeded for 30 minutes (25°C) and was terminated by the addition of sulfuric acid (1 N). The color intensity was proportional to the concentration of steroid receptor and was quantitated spectrophotometrically at 492 nM. Receptor concentrations were determined from standard curves of estrogen or progesterone receptor run simultaneously. The sensitivity for the detection of the estrogen and the progesterone receptor using this method is 5 fmol/ml, representing 90 sites/U2-OS or Saos-2 cell.

Results

Specific androgen binding sites were found in all three cell lines (Table 1) (Fig. 1a, b). The affinities (K_D) of these sites for androgen binding were very similar in the three osteoblastic lines, and in turn were similar to those of sites in human and rat prostatic and renal tissue (Fig. 1c, d), classical androgen responsive tissues. Specific androgen binding was saturable in the cell lines (Fig. 2a, b) and in normal tissues (Fig. 2c, d). The number of sites per cell in the two human cell lines were consistently greater than in the UMR-106.01 cells of rat origin. In all three osteoblastic lines the total number of sites (cytoplasmic and nuclear) was lower than in rat and human prostate, which contain abundant concentrations of receptors. However, the number of sites in these osteoblastic cells is very similar to that seen in the renal tissue.

Characterization of the binding specificities of the putative androgen receptors in the two human cell lines (U2-OS and Saos-2) is shown in Figure 3. In both cell lines, high concentrations of DES (1.0×10^{-7} M) did not compete for ^{3}H-R1881 binding, whereas excess concentrations of unlabeled dihydrotestosterone (1×10^{-7} M) and R1881 (1×10^{-7} M) produced 72 and 76% displacement of ^{3}H-R1881. Of interest, progesterone (1.0×10^{-7} M) resulted in considerable inhibition of ^{3}H-R1881 binding (51%).

In the UMR-106.01 cell line, no estrogen binding could be detected, and in the Saos-2 and U2-OS lines no estrogen receptor immunoreactivity was detected. In the two human cell lines (Saos-2, U2-OS) progesterone receptor immunoreactivity was clearly detectable, but no high affinity, specific progestin binding was observed in the UMR-106.01 cells.

Discussion

The structures of human and rat androgen receptors have recently been characterized. They are similar proteins with an identical cysteine-rich DNA binding region, which is in large part shared with other steroid hormone receptors [25, 26]. Colvard et al. [12] found specific androgen binding and evidence of androgen receptor expression (specific mRNA) in primary cultures of human osteoblastic cells. The presence of specific, high affinity androgen binding in the human and rat cell lines studied here indicates the presence of androgen receptor in these cells as well.

The character of the putative androgen receptor in these cell lines is similar to those previously described in other androgen-responsive tissues [27, 28]. Receptor affinity was high in all cell lines (K_D = 1–3 $\times 10^{-10}$ M). Studies of binding specificity revealed significant competition by an-

Table 1. Sex steroid receptor quantitation in osteoblastic cell lines

	Androgen receptor[a]							
	Cytosol		Nucleus		Estrogen receptor[b]		Progesterone receptor[b]	
	Sites/Cell	$K_D \times 10^{-10}$	Sites/Cell	$K_D \times 10^{-10}$	Cytosol	Nucleus	Cytosol	Nucleus
Cell line								
UMR-106.01	74 ± 10	1.6 ± 0.5	20 ± 1	5.1 ± 1.1	ND	ND	ND	ND
U2-OS	1277 ± 239	1.7 ± 0.3	ND	—	ND	ND	ND	118 ± 28
Saos-2	1605	2.5	ND	—	ND	ND	ND	119
Prostate carcinoma (human)	1855 ± 206	2.8 ± 0.3	1239 ± 145	3.4 ± 0.4	738 ± 124	216 ± 46	2295 ± 423	483 ± 72
Ventral prostate (intact rat)	2456 ± 453	5.9 ± 1.4	4297 ± 250	6.3 ± 0.2	NA	NA	NA	NA
Kidney (intact rat)	791 ± 85	3.1 ± 0.4	534 ± 49	4.4 ± 0.4	NA	NA	NA	NA

ND = none detected; NA = not analyzed
All studies were performed at least in duplicate, and results are reported as the mean of the two experiments. In cases in which the studies were performed on three or more occasions, the mean and standard errors of the mean are reported
[a] Quantitated by steroid binding assay
[b] Quantitated by enzyme-linked immunoassay in U2-OS and Saos-2, and by steroid binding assay in UMR-106.01

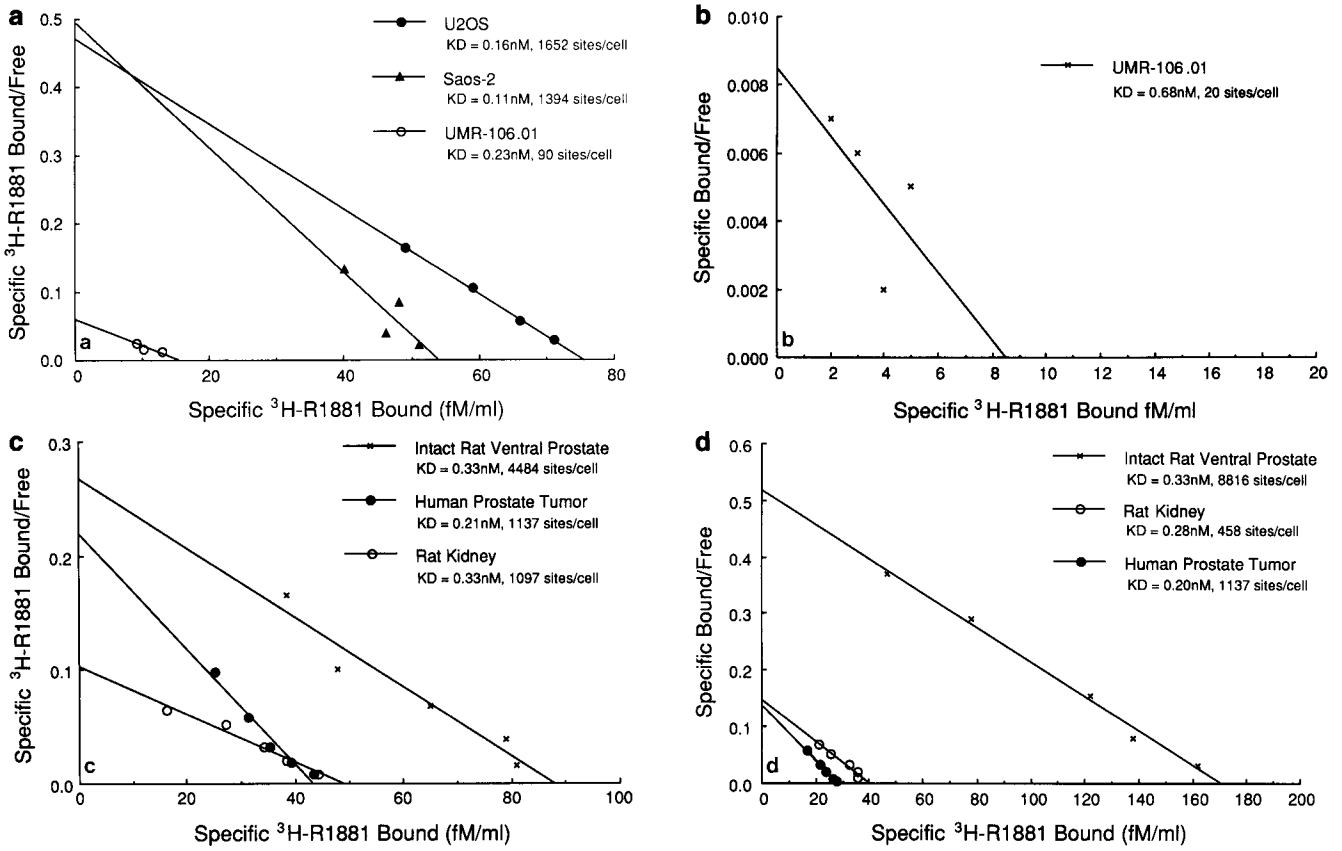

Fig. 1. Scatchard plot analysis of specific ^{3}H-R1881 binding in cytosols of osteosarcoma cell lines **(a)**, in nuclear extracts of UMR-106.01 cell line **(b)**, in cytosols of prostate glands and kidney **(c)**, and in nuclear extracts of prostate glands and kidney **(d)**.

drogens, but competition for ^{3}H-R1881 binding by estrogen was not observed. Progesterone exhibited a moderate degree of cross-reactivity. The density of binding sites in the two human osteoblastic cell lines was similar to that reported in primary human osteoblast cultures [12]. Although clearly present in lower concentrations than in the prostate, binding sites in osteoblastic cells are similar to those found in the kidney, an androgen-responsive tissue [29, 30]. Hence, these concentrations are consistent with the potential for androgen sensitivity. Androgen binding sites were reproducibly detected in the rodent cell line (UMR-106.01) but in lower amounts than in the two human lines. Receptors were presumed to be most abundant in the cytosolic fraction because in the absence of high androgen concentrations *in vitro* most

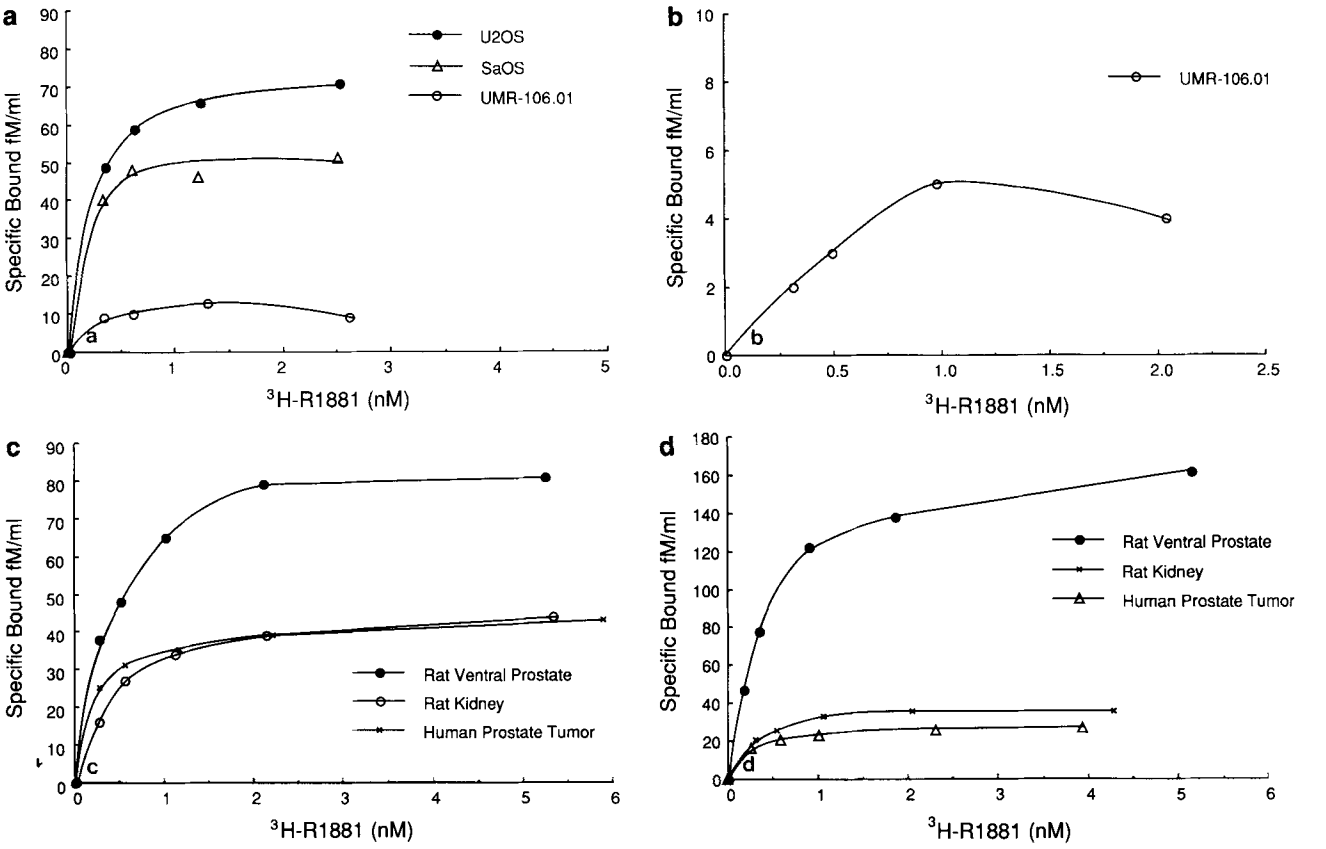

Fig. 2. Saturable binding of ^{3}H-R1881 in cytosols of osteosarcoma cell lines (**a**), in nuclear extracts of UMR-106.01 cell line (**b**), in cytosols of prostates and kidney (**c**), and in nuclear extracts of prostates and kidney (**d**).

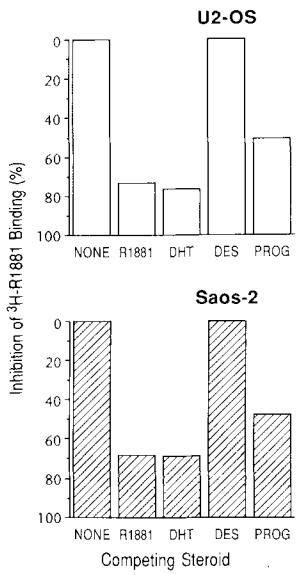

Fig. 3. Specificity of androgen biding in the U2-OS and Saos-2 cell lines (DHT = dihydrotestosterone, DES = diethylstilbestrol, Prog = progesterone).

receptors are not tightly bound to nuclear elements, and are removed by the extraction buffer, thus appearing in the cytosol of tissue homogenates. It is also possible that nuclear receptors may have been lost due to protease digestion, but the presence of a protease inhibitor (PMSF) in the extraction buffer makes this less likely.

In contrast to the consistent presence of specific androgen binding in each of the cell lines examined, we were unable to detect either specific estrogen binding in the UMR-106.01 cells or estrogen receptor immunoreactivity in the human cell lines. Although the steroid binding assay used for the detection of estrogen receptors in the UMR-106.01 cells may have lacked sensitivity, the immunoassay utilized for the measurement of estrogen receptors in the human lines is both very sensitive and specific. There have been no previous reports of the presence of estrogen receptors in any of these cell lines, despite reports of estrogen-induced effects in both the UMR-106.01 [8, 11] and Saos-2 lines [10]. Colston et al. [31] have recently reported the inability to detect estrogen receptors in other osteoblast-like lines using immunocytochemical techniques, despite the presence of specific staining for Ag 29, an estrogen receptor-associated protein. These findings raise the question of whether the detection of the estrogen receptor is being masked by other factors, or whether estrogen receptor is strongly associated with the nuclear compartment and is not extracted by hypertonic buffers during *in vitro* receptor analysis. Although estrogen receptor mRNA has been reported in one human osteosarcoma cell line (TE85), estrogen receptor immunoreactivity was absent [6], as reported here in two other human cell lines (Saos-2, U2-OS). The presence of estrogen binding in TE85 cells [6] was demonstrated with ^{125}I-estradiol, a high specific activity label which yields greater sensitivity then do the tritiated radioligands used in the present studies. The presence of a more readily detectable estrogen receptor in normal human osteoblasts [7] may indicate a higher level of receptor expression in normal cells.

As yet, there have been no reports of progesterone-mediated effects on bone cells. Nevertheless, clinical studies suggest the presence of an effect of progesterone or related steroids on bone remodeling [32–34]. In the two human lines studied here, progesterone receptors were detected immunologically in high-salt extracts of the nuclear fraction, and progesterone receptors have been characterized in primary cultures of human osteoblast-like cells [7]. In view of the considerable affinity of progesterone for the androgen receptor, progesterone bioactivity in osteoblastic cells could be mediated via interactions of progestins with either androgen or progesterone receptors.

The presence of androgen and progesterone receptors in these cell lines, particularly the human lines examined, suggests that these cells may be useful in the elucidation of the mechanism by which androgens and/or progestins affect osteoblastic function, and how androgens interact with other modulators of skeletal physiology.

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