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Steroid Receptors in Human Osteoblast-like Cells

M.C. Etienne, J.L. Fischel, G. Milano, P. Formento, J.L. Formento, M. Francoual, M. Frenay and M. Namer

The presence and functions of steroid receptors were evaluated in three human osteosarcoma cell lines (OS1 = SA OS; OS2 = HOS TE 85, and OS3 = MNNG HOS TE 85). The human breast cancer cell line MCF-7 was used as internal control for oestrogen receptors (E_2R). High and low affinity sites were characterised. The high affinity sites had a similar dissociation constant in all four cell lines. In contrast, the number of sites per cell was higher in MCF-7 cells. E_2 did not significantly modify the number of progesterone receptors (PgR) per cell in any of the osteosarcoma lines. As expected, E_2 increased the number of PgR sites per MCF-7 cell. 4-hydroxytamoxifen decreased the growth of MCF-7 cells only. OS1 and OS2 were sensitive only to the highest concentration tested, which produces only non-specific cytotoxic effects. Thus E_2R and PgR were found in osteoblast-like cells, but the function of E_2R in such cells remains unknown.
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INTRODUCTION

ALTHOUGH BOTH estradiol (E_2) and progesterone (Pg) are involved in the regulation of bone metabolism [1], bone cells were generally not thought to contain steroid receptors [2, 3]. In 1988, Kaplan *et al.* [4] described E_2 receptors (E_2R) in bone from a patient with McCune–Albright syndrome. More convincing evidence was provided by Eriksen *et al.* [5], who reported osteoblast-like cells displaying steroid-specific, saturable, and temperature-dependent nuclear binding. In breast cancer, E_2R and PgR assays predict response to endocrine treatment [6] in addition to having a prognostic value [7]. Our study was designed to evaluate the presence of E_2R and PgR in human osteosarcoma cell lines, and to study the functions of such receptors. We used three cell lines derived from human osteosarcomas. The human breast cancer cell line, MCF-7, known to be E_2R and PgR positive, was used as internal control.

Correspondence to: G. Milano.
M.C. Etienne, J.L. Fischel, G. Milano, P. Formento, J.I. Formento, M. Francoual, M. Frenay and M. Namer are at the Centre Antoine Lacassagne, 36 voie Romaine, 06054 Nice Cedex, France.

MATERIAL AND METHODS

Chemicals

The antioestrogen 4-hydroxytamoxifen (4-OHT) was from ICI. 17 β E_2 was from Sigma and [2,4,6,7-^{3}H]17 β E_2 (specific activity 315–407 × 10^{10} Bq/mmol), the synthetic steroid Organon 2058 and [^{3}H]Organon 2058 (148–222 × 10^{10} Bq/mmol) were from Amersham. Dihydrotestosterone and cortisol were from Sigma. [^{3}H]thymidine (185 × 10^{10} Bq/mmol) was from the C.E.A. (Saclay, France). Bisbenzimide was from Sigma.

Cell cultures

The three human osteosarcoma cell lines (OS1, OS2, OS3) were obtained from the A.T.C.C. (Rockville): OS1 = SAOS, a human osteogenic sarcoma (ATCC HBT 85); OS2 = HOS TE 85, a human osteogenic sarcoma (ATCC CRL 1543); and OS3 = MNNG HOS TE 85, clone F 5 (ATCC CRL 1547). MCF-7 was provided by Prof. H. Rochefort (INSERM U 148, Montpellier). OS1, OS2, and OS3 were grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (FBS) and 5 mmol/l glutamine and supplemented with 50 000 μg/l streptomycin and 50 000 IU/l penicillin (Flow). For MCF-7, the medium was supplemented with 0.1 μmol/l insulin and 0.85 μmol/l transferrin (Sigma). FBS was reduced to 5% for experiments testing the effects of E_2. The concentration of E_2 in FBS was lower than 7.1^{-11} mmol/l; the Pg concentration was 4.1^{-10} mmol/l (measured by radioimmunoassay).

The following 4-OHT concentrations were tested: 10^{-8}, 10^{-7}, 3 × 10^{-7}, 10^{-6}, 3 × 10^{-6}, and 10^{-5} mol/l. The culture medium was changed daily during the 7 days of exposure. Depending on the cell line, 10 000–15 000 cells were initially plated per well.

Evaluation of cell growth

Cell growth was assessed by measuring [^{3}H]thymidine incorporation into DNA. Cells were washed once at 37°C with Eagle's 199 (E199) medium (Gibco), 500 μl per well. 250 μl E199 containing 10% FBS were distributed in each well, followed by 50 μl [^{3}H]thymidine (5.6 × 10^{-15} Bq/ml). Cells were incubated for 16 h at 37°C in 5% CO_2. The reaction was stopped by transferring the plates onto a tray covered with ice; three rinses of 250 μl per well were done with phosphate buffered saline (PBS) at 4°C. Cells were disrupted with 250 μl 10% trichloracetic acid (30 min, 4°C). The supernatant was withdrawn and DNA was resolubilized in 1 mol/l NaOH (300 μl per well) for 15 min at 37°C. Radioactivity was measured by β counting. Final results were expressed as the percentage of [^{3}H]thymidine incorporation compared with controls.

Steroid receptor assays

The assay procedure was derived from the method of Sutherland *et al.* [8]. At 90% confluence, cells grown in 24-well plates were rinsed three times with 500 μl RPMI 1640 (Gibco) containing 0.1% bovine serum albumin (BSA) (Sigma) at 37°C. After rinsing, 150 μl medium plus 50 μl ligand solution in the same vehicle were added. The final concentrations of [^{3}H]E_2 and [^{3}H]Organon 2058 were: 0.03, 0.04, 0.06, 0.1, 0.2, 0.4, 0.6, 1, 3, 8, and 15 nmol/l. To measure non-specific binding, an excess of unlabelled ligand (1500 nmol/l) was added to the highest concentration of labelled ligand. Dihydrotestosterone (200 times more concentrated than labelled E_2) was added for assay of E_2R; cortisol (200 times more concentrated than labelled Organon 2058) was added for measurement of PgR. Cells were incubated for 1.5 h at 37°C in 5% CO_2. Plates were placed on a tray with ice to stop the reaction and the supernatant was removed from each well. Cells were washed three times with PBS containing 5% BSA (4°C, 250 μl per well). The total amount of time required for rinsing was less than 20 min. After removal of the supernatant, cells were solubilized with 1 mol/l NaOH at 37°C (300 μl per well for 15 min). The radioactivity of each well was measured by β counting. Results were expressed in fmol per well. Scatchard analysis [9] was used to assess the number of receptor sites per cell and the dissociation constant (K_d). Each point of every Scatchard plot was obtained in

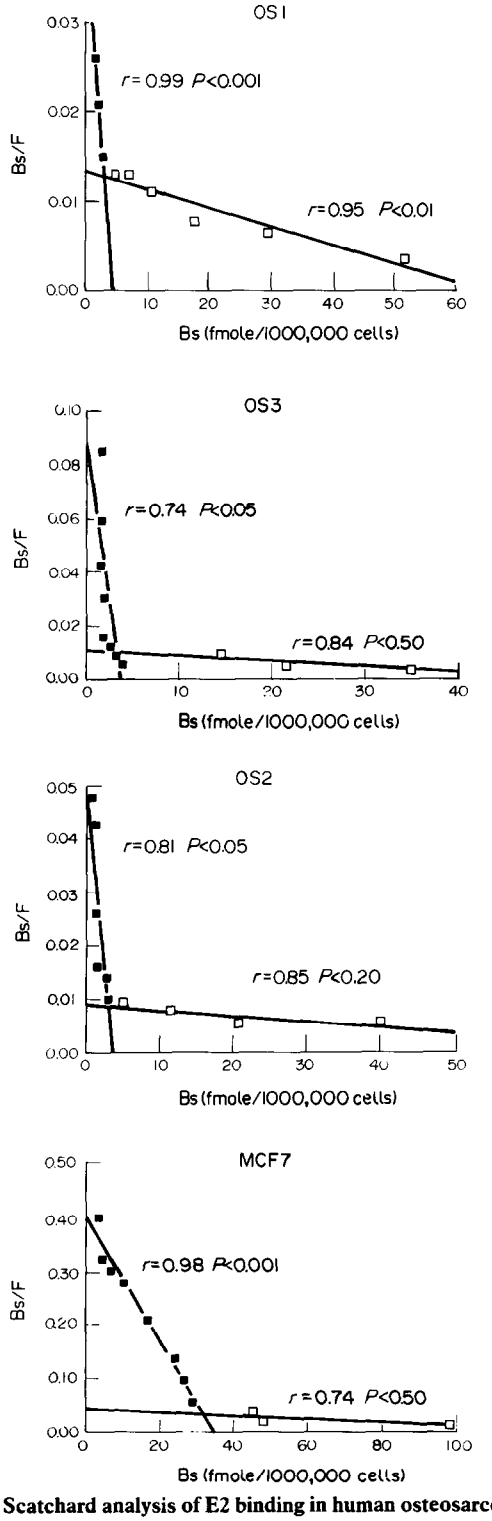

Fig. 1. Scatchard analysis of E2 binding in human osteosarcoma cell lines and in MCF-7 cells. Solid squares = high affinity sites; open squares = low affinity sites; *r* = linear coefficient of correlation.

quadruplicate or sextuplicate; the coefficient of variation was less than 10%. Cells were counted in three wells run in parallel, resuspended in 200 μl PBS at room temperature, and counted with a haemocytometer. The validity of the Scatchard plot was checked by linear regression.

RESULTS

Figures 1 and 2 show the Scatchard plots used to measure E_2R and PgR in OS1, OS2, OS3, and MCF-7. Two categories

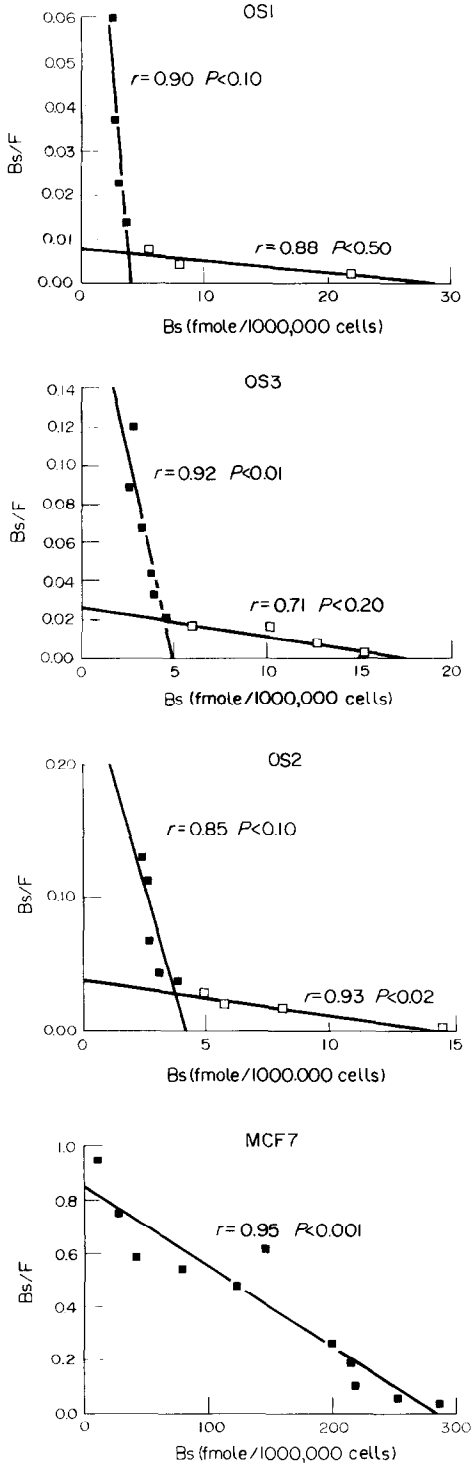

Fig. 2. Scatchard analysis of Pg binding in human osteosarcoma cell lines and in MCF-7 cells. Solid squares = high affinity sites; open squares = low affinity sites; r = linear coefficient of correlation.

Table 1. E_2R characteristics

Cell line	High affinity site K_d (nmol/l)	Sites per cell	Low affinity site K_d (nmol/l)	Sites per cell
OS 1	0.111 (0.006)	2600 (100)	4.7 (0.3)	38 500 (6300)
OS 2	0.140 (0.051)	2200 (500)	17.7 (7.6)	48 600 (6200)
OS 3	0.073 (0.027)	2200 (500)	8.7 (5.6)	31 600 (9800)
MCF-7	0.152 (0.012)	21 000 (800)	5.5 (4.9)	80 800 (37 000)

Mean (S.E.).

of sites were identified for both receptors in all three osteosarcoma cell lines: one with high affinity and one with low affinity. Analysis of high affinity binding sites revealed similar K_d values for E_2R, about 0.1 nmol/l for all cell lines (Table 1). For PgR the K_d values for osteosarcoma were close (0.03 nmol/l) and that for MCF-7 was 0.39 nmol/l (Table 2). The number of high affinity binding sites per cell in MCF-7 was 10 and 70 times higher for E_2R and PgR, respectively, compared with the osteosarcoma cells. These experiments were repeated and gave similar results.

Table 3 shows the effects of various E_2 concentrations (7 days' incubation) on PgR induction. No changes were noted in the number of PgR sites per cell in any of the osteosarcoma cell lines. In contrast, E_2 increased the number of PgR sites per MCF-7 cell: 230% rise between the control and the culture exposed to 10^{-7} mol/l E_2.

Figure 3 shows the dose–response curves of the four cell lines after exposure to 4OHT. Over the concentration range tested, 4-OHT progressively reduced MCF-7 cell growth only. OS1 and OS2 were sensitive to only the highest concentration of 4-OHT (10^{-5} mol/l).

DISCUSSION

After reports of E_2R in human osteoblast-like cells [5, 10], we studied osteoblast-like cells derived from human osteosarcomas and assessed the function of E_2R by pharmacological investigations. We looked at total cellular receptor binding rather than just nuclear binding. Most E_2R is localized in the nuclear compartment [11, 12]. However, as stressed by Jensen [13], this does not rule out the possibility that this nuclear oestrophilin may constitute a pool in equilibrium with extranuclear receptors. This view concurs with the model proposed by Martin and Sheridan [14]. We identified E_2R in all of the osteosarcoma cell lines studied. Interestingly, the K_d values in these cell lines were similar to that of cell line MCF-7, which suggests the presence of similar receptor sites in breast cancer derived cells and these osteoblast-like cells. The receptor sites had a higher affinity (K_d about 0.1 nmol/l) than that reported by others (K_d about

Table 2. PgR characteristics

Cell line	High affinity site K_d (nmol/l)	Sites per cell	Low affinity site K_d (nmol/l)	Sites per cell
OS 1	0.033 (0.011)	2400 (600)	3.6 (1.9)	17 200 (4400)
OS 2	0.032 (0.011)	2500 (600)	0.8 (0.2)	8800 (1300)
OS 3	0.039 (0.008)	2900 (400)	1.1 (0.6)	10 300 (3900)
MCF-7	0.391 (0.043)	171 600 (11 700)	-	-

Mean (S.E.)

Table 3. Effect of E2 on progesterone receptors

Cell line	Control	E_2 (mol/l) 10^{-9}	10^{-8}	10^{-7}
OS1				
K_d (nM)	0.033	0.025	0.055	0.031
N	2400	2600	3300	2800
OS2				
K_d (nM)	0.032	0.046	0.031	0.033
N	2500	3300	1800	1700
OS3				
K_d (nM)	0.039	0.017	0.037	0.038
N	3000	2700	2700	3200
MCF-7				
K_d (nM)	0.391	0.420	0.347	0.355
N	172 000	355 000	226 000	568 000

N = number of sites per cell.

1 nmol/l) [10]. The number of sites per cell (around 2300) concurred with the data of Eriksen *et al.* [5], who found a mean of 1615 sites per cell nucleus in seven strains of normal human osteoblast-like cells. In contrast, in the study of Komm *et al.* [10], sarcoma cell line HOS TE 85 (OS2 in our study) had only 200 detectable high affinity E_2R sites per nucleus. The K_d value (0.15 nmol/l) of our internal control was similar to data reported by Manaway *et al.* [15].

Investigation of the functions of E_2R in our three osteosarcoma cell lines was based on the inducibility of PgR under the influence of E_2. The demonstration by Gray *et al.* [16] that factors such as cell density, cell characteristics, and presence or absence of steroids in the medium influenced the response of a rat osteosarcoma cell line to E_2 contributed to our decision to use an internal control. The presence of PgR was evaluated simultaneously in the MCF-7 cells and in the osteosarcoma cell lines. E_2 concentrations ranging between 10^{-9} and 10^{-7} mol/l induced PgR in MCF-7 cells but not in the osteoblast-like cells. Eriksen *et al.* [5] showed a (non-significant) increase in the specific nuclear binding of progesterone in 4 of 6 osteoblast-like cells, after pretreatment with 10 nmol/l E_2 for 24 h; Komm *et al.* [10] found that type 1 procollagen and transforming growth factor β (TGF β) mRNA levels were enhanced in HOS TE 85 cells (OS2) treated with 1 nmol/l E_2 and concluded that E_2 can act directly on osteoblasts by a receptor-mediated mechanism and modulates the extracellular matrix and other proteins involved in the maintenance of skeletal mineralization and remodeling.

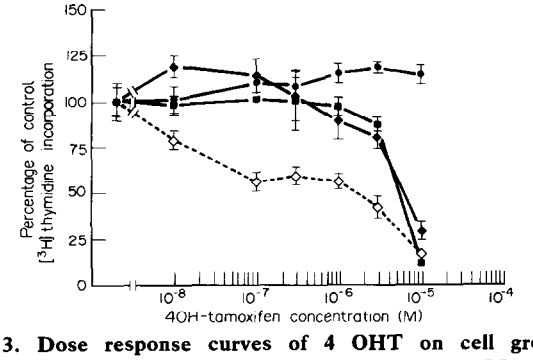

Fig. 3. Dose response curves of 4 OHT on cell growth. Squares = OS1; solid diamonds = OS2; circles = OS3; open diamonds = MCF-7.

This contradicts the report by Dickson *et al.* [17], that E_2 reduced TGF β production by MCF-7 cells.

Our failure to observe any apparent effect of 4-OHT on three osteoblast-like cell lines was not related to the experimental conditions because 4-OHT did have a concentration-related effect on MCF-7 cell growth, as shown previously [18, 19]. Although a 4-OHT concentration of 10^{-5} mol/l inhibited the growth of two osteosarcoma cell lines, 4-OHT is cytotoxic by itself at this concentration, and its effects are no longer mediated by E_2R sites [19]. Our results thus suggest that the use of antiestrogens does not appear promising in the treatment of human osteosarcoma.

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