Characterization of the progestin receptors in the human TE85 and murine MC3T3-E1 osteoblast-like cell lines

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Abstract

Progestins are believed to exert positive effects on bone density through receptors located in osteoblasts. In the present studies, the binding characteristics and regulation of the progestin receptors in two osteoblast-like cell lines were compared with those in human breast lines. Human TE85 and murine MC3T3-E1 osteoblast-like cells contain a single, high-affinity progestin binding site whose affinity and concentration are lower than in human breast cells. The osteoblastic progestin binding sites showed the expected steroid specificity and associated with the cell nuclei when occupied by ligand. The progestin receptors in osteoblastic cells also had sedimentation coefficients similar to those receptors in breast cells. The regulation of the progestin receptor in the osteoblast-like cells was explored by treating them with estradiol. In contrast to the large, rapid change seen in the breast cells, the progestin receptor levels in the MC3T3-E1 cells showed only a

small, delayed up-regulation with estradiol treatment. The progestin receptor number in the TE85 cells was unaffected by estradiol. Down-regulation of the progestin receptors was explored by treating the cells with the progestin, norethindrone (NET). NET administration produced a rapid drop in progestin binding sites in the breast cells and a smaller, more gradual decline in MC3T3-E1 progestin binding. While the maximal decrease in receptor number occurred within 24 h in the breast cells, the receptor number was still continuing to fall after 72 h in the MC3T3-E1 cells. The data presented here demonstrate that both human and murine osteoblast-like cells contain a functional progestin receptor whose binding characteristics and regulation are similar, but not identical, to those receptors in other progestin target tissues such as the breast.

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Introduction

Progestins are frequently prescribed to postmenopausal women in combination with an estrogen for treating osteoporosis (Prior 1990, Sturdee 1997). The estrogenic component in hormone replacement therapy (HRT) exerts a well-known positive effect on bone mass. The primary function of the progestin in HRT has been to counter the unwanted estrogen-induced proliferation of the uterine endometrium. However, progestins can also exert positive effects on bone. In postmenopausal women, the resorption of bone can be reduced (Abdalla *et al.* 1985, Gallagher *et al.* 1991, McNeeley *et al.* 1991) and the formation of new bone increased by progestins (Christiansen *et al.* 1985, Horowitz *et al.* 1987).

The ability of progestins to promote bone formation may be due to their ability to act directly upon osteoblasts. *In vitro*, progestins stimulate the proliferation of human osteosarcoma cells and normal human osteoblasts obtained from bone specimens (Tremollieres *et al.* 1992, Lau *et al.* 1994, Verhaar *et al.* 1994). Progestins can also stimulate the differentiation and the bone-forming activity of osteoblasts (Lau *et al.* 1994, Verhaar *et al.* 1994, Ishida *et al.*

1996). The effects of progestins are believed to be mediated through nuclear steroid receptors. Progestin receptors have been identified in osteoblasts and osteoblast-like osteosarcoma cells with a number of techniques including radioligand binding (Eriksen *et al.* 1988, Etienne *et al.* 1990, Slootweg *et al.* 1992, Wei *et al.* 1993), receptor mRNA detection following reverse transcription PCR (Wei *et al.* 1993, MacNamara *et al.* 1995, MacNamara & Loughrey 1998), immunoassay (Pensler *et al.* 1990, Orwoll *et al.* 1991) and immunocytochemistry (Pensler *et al.* 1990, MacNamara *et al.* 1995). However, neither specific binding nor immunocytochemical staining for the progestin receptor is always detectable in osteoblasts (Colston *et al.* 1989, Pensler *et al.* 1990, Orwoll *et al.* 1991, Wei *et al.* 1993). Common to all these studies is the conclusion that the concentrations of the progestin receptor in osteoblastic cells are low, thus making studies of this receptor difficult. The presence of the progestin receptor in osteoblasts has been well demonstrated but many of the basic characteristics of these receptors are still unclear.

The goal of the present studies was to better describe the characteristics and regulation of the progestin receptor in osteoblastic cells. In these studies, the progestin

receptors in two commonly used osteoblastic cell lines, the human TE85 osteosarcoma cells and the murine MC3T3-E1 osteoblast-like cells were compared with those in human breast cells.

Materials and Methods

Cell culture

Two human breast carcinoma cell lines were used in these studies. T47D breast cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and ZR75 breast cells from Dr Rosalyn Blumenthal, Center for Molecular Medicine and Immunology (Newark, NJ, USA). Both breast cell lines were maintained in RPMI medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% bovine calf serum (BCS, Hyclone Laboratories, Logan, UT, USA). Human osteosarcoma cells (TE85) were obtained from Dr William Lau, J L Pettis Veterans Hospital (Loma Linda, CA, USA). Mouse osteoblast-like cells (MC3T3-E1) were obtained from Dr Tom Einhorn, Mount Sinai Hospital (New York, NY, USA). TE85 and MC3T3-E1 cells were grown in DMEM medium (Gibco Laboratories) supplemented with 10% BCS. All cells were grown under a 5% CO₂ atmosphere at 37 °C with no antibiotics.

In preparation for receptor assays, culture media were replaced with RPMI or DMEM media containing 10% charcoal-stripped fetal calf serum (CSS) 24 h before cell harvest. Cells were harvested by trypsinization and washed with Hanks' balanced salt solution (HBSS; Gibco Laboratories). Cell number was determined following the harvesting of each experimental treatment group. Cell viability were determined by trypan blue exclusion. The cells were homogenized on ice in TEG assay buffer (0.01 M Tris, 1 mM EDTA, 0.01 M molybdic acid, 10% glycerol, pH 7.4) for progesterin receptor assays or TEG with 1 mM dithiothreitol for estrogen receptor assays. The homogenate was centrifuged for 12 min at 541 000 *g* at 4 °C in a Beckman (Fullerton, CA, USA) TL-100 ultracentrifuge. The supernatant containing the cell cytosol and/or the nuclear pellet was diluted in the appropriate buffer according to the number of live cells homogenized.

Progesterin and estrogen receptor binding assays

For saturation and competition progesterin receptor studies, binding was measured in tubes containing the cytosol from 1×10^6 breast (T47D and ZR75) cells, 3×10^6 MC3T3-E1 cells or $4\text{--}5 \times 10^6$ TE85 cells according to the methods of Clark *et al.* (1989). Cell cytosol (200 µl) was incubated overnight at 4 °C with [³H]R5020 (specific activity 86–87 Ci/mmol; NEN, Wilmington, DE, USA). Saturation analyses of progesterin binding in cell cytosols were conducted using 0.1–20 nM concentrations of

[³H]R5020. Non-specific binding was measured in the presence of 100-fold excess non-radioactive R5020. Receptor specificity was determined in competition binding studies using 10 nM [³H]R5020 and 1 µM of various steroids. An aliquot of the [³H]R5020-bound fraction was transferred into vials for scintillation counting. The protein content of the cytosol preparations was determined using a protein measurement kit obtained from Bio-Rad Laboratories (Richmond, VA, USA). Measurement of nuclear-bound progesterin receptors was performed by incubating a known number of live intact cells with 10 nM [³H]R5020 in the presence or absence of 1 µM non-radioactive R5020 for 1 h. The cells were then washed twice with HBSS, homogenized and centrifuged. The nuclear-bound [³H]R5020 was solubilized in 1 ml ethanol and counted. To measure changes in the concentration of progesterin receptors following progesterin treatment, the cytosolic and nuclear fractions of the cells were incubated overnight with 10 nM [³H]R5020 to allow ligand exchange (Clark *et al.* 1989). In some studies, the binding of ¹²⁵I-labelled vinyl 19-nortestosterone (VNT) (NEN; specific activity 367 Ci/mmol) was used to quantitate the number of progesterin binding sites. For these studies, 10 nM ¹²⁵I-VNT was used to determine total binding with 1 µM norethindrone (NET) added for measuring non-specific binding. Bound radioactivity was counted in a gamma counter. Saturation binding studies for estrogen receptors in MC3T3-E1 cells were performed using ¹²⁵I-17β-estradiol (NEN; specific activity 220 Ci/mmol) at concentrations of 0.1–20 nM. Each assay tube contained the cytosol of 12.5×10^6 live cells. Non-specific binding was measured in tubes containing diethylstilbestrol at a concentration 100-fold greater than that of tracer.

The analyses of saturation binding data were conducted using RECEPTORFIT computer software (Lundon Software, Chagrin Falls, OH, USA), which performs weighted non-linear least-squares regression analysis as described by Feldman (1972). Data were graphed according to the method of Scatchard (1949). Saturation binding experiments were replicated three to five times.

Sucrose density gradients

Cytosols from TE85 osteoblastic cells and ZR75 breast cells were fractionated through sucrose density gradients. Cell cytosols were prepared by the standard procedure and 200 µl were layered on a 4.7 ml sucrose density gradient. Gradients of 10–30% sucrose were poured into Beckman Optiseal polyallomer tubes using a gradient maker. Tubes were chilled to 4 °C before the sample was loaded. The tubes were sealed, placed in a Beckman VTi 90 vertical tube rotor and run at 509 650 *g* for approximately 1 h 45 min in a Beckman XL-90 ultracentrifuge. Thirty-six fractions of approximately 200 µl each (3 drops/fraction) were collected from each gradient tube. Fractions

were collected into glass test tubes containing 1 nM ^{125}I -VNT and either assay buffer or NET (100 nM). The tubes were then incubated overnight at 4 °C. Bound and free radioligand were separated by dextran-coated charcoal absorption and bound radioligand counted in a gamma counter. The migration of BSA and catalase was measured in parallel gradients in order to determine sedimentation coefficients of the ^{125}I -VNT binding sites.

Measurement of in vitro alkaline phosphatase activity

TE85 cells were grown in 24-well plates (Corning, Corning, NY, USA) under standard culture conditions. Twenty-four hours after seeding the plates (24 000 cells/well), the medium was replaced with DMEM-CSS. The following day the medium was again replaced with DMEM-0.2% fetal bovine serum containing vehicle (0.1% ethanol) or rest steroid. Each treatment group consisted of six wells. After incubation for 72 h, the cells were washed with HBSS and each well received 0.5 ml Triton-X (1% in distilled water) to lyse the cells. The alkaline phosphatase activity in the lysate was quantitated by measuring the hydrolysis of paranitrophenyl phosphate (10 nM) with reagents from Sigma Chemical Co. (St Louis, MO, USA). Alkaline phosphatase activity was normalized for the protein content in the cell lysates. Statistical comparisons were performed with Dunnett's one-tailed *t*-test.

Results

Saturation analyses with [^3H]R5020

The binding of [^3H]R5020 was measured in cytosol preparations from murine MC3T3-E1 osteoblast-like cells and human TE85 osteoblast-like cells. As a comparator, similar data were generated with the human T47D breast cells, a line in which much of the physical characterization of the human progestin receptor was originally done (Horwitz *et al.* 1985). The cytosols of the osteoblast-like cell lines were found to contain a single, high-affinity, saturable binding site for R5020. Representative Scatchard plots and saturation binding graphs are shown in Fig. 1. The dissociation constants (K_d) and binding site concentrations derived from saturation studies are in Table 1.

While both the MC3T3-E1 and TE85 osteoblast-like cells show high-affinity binding sites for R5020, the affinity of these sites appears lower than for those sites in the T47D breast cells. The R5020 K_d reported here for the T47D progestin receptor is similar to the binding constant for the progestin receptor in human uterus, prostate and breast (Namkung & Petra 1981, Schneider *et al.* 1984, Van der Walt *et al.* 1986). These binding affinities and receptor concentrations for the TE85 and MC3T3-E1 cells are similar to those reported for other human osteosarcoma lines (Etienne *et al.* 1990, Sloatweg

Figure 1 Scatchard plots and saturation graphs of [^3H]R5020 binding in T47D breast cells, TE85 osteoblast-like cells and MC3T3-E1 osteoblast-like cells.

Table 1 Dissociation constants (K_d) and concentrations of [3 H]R5020 binding sites in the cytosol of breast and osteoblast-like cells. Values are means $\pm$ S.E.M. of three to five replicate studies

Cell type	Binding site K_d (nM)	Binding site concentration (fmol/mg protein)	Number of binding sites per cell
T47D breast cells	0.20 $\pm$ 0.05	3296 $\pm$ 947	32 947 $\pm$ 14 517
MC3T3-E1 osteoblast-like cells	3.40 $\pm$ 1.01	241 $\pm$ 50	11 133 $\pm$ 2439
TE85 osteoblast-like cells	4.60 $\pm$ 1.59	244 $\pm$ 59	8546 $\pm$ 1669

et al. 1992, Wei *et al.* 1993). When R5020 binding is expressed in terms of cytosolic protein concentration, the numbers of TE85 and MC3T3-E1 R5020 binding sites are less than one-tenth of that found in the T47D breast cells. However, when expressed on a per cell basis, the osteoblast-like cells contain approximately one-fourth to one-third as many receptors as the T47D cells. The osteoblastic cells are much larger than the T47D cells and so comparing their relative receptor numbers based on cytosolic protein may be less informative than comparing their receptor concentrations on a per cell basis.

Progesterin receptor binding specificity

The steroid specificity of the TE85 and MC3T3-E1 progesterin binding sites was tested with several steroids. The two synthetic progestins, NET and levonorgestrel, inhibited all specific R5020 binding as shown in Table 2. Neither 17 β -estradiol nor dexamethasone competed for any R5020 binding. The androgen, dihydrotestosterone showed some competition for the R5020 binding sites in both osteoblast-like cell lines.

[3 H]R5020 binding to the nuclei of TE85 osteoblast-like cells

The binding of a progesterin to its receptor within the cell induces receptor activation and gives the receptor the capability to bind to DNA (Truss & Beato 1993). To further demonstrate the presence of progesterin receptors in TE85 cells, the ability of the receptor to bind to the cell

nucleus was tested. After incubation with [3 H]R5020 (10 nM) for 1 h at either 0 $^{\circ}$ C or 37 $^{\circ}$ C, TE85 cells were homogenized and the nuclei collected. In those cells incubated at 0 $^{\circ}$ C, the amount of [3 H]R5020 associated with the nuclear pellet was equivalent to approximately 202 receptors per nucleus. When incubated at 37 $^{\circ}$ C the quantity of [3 H]R5020 bound to the nuclear pellet increased to an amount equivalent to 1043 receptors per nucleus.

Identification of the progesterin receptor in sucrose density gradients

One method of comparing steroid receptors has been to study their migration through sucrose density gradients. The migration of the progesterin binding sites in cytosol from TE85 cells was determined by measuring progesterin binding in sucrose gradient fractions. Parallel studies were performed using cytosol from ZR75 cells. Like the T47D cells, the ZR75 cells are another human breast line containing well-characterized progesterin receptors (Allegra *et al.* 1981, Van den Berg *et al.* 1990). The ZR75 cells, however, contain a lower concentration of progesterin receptors making them the better comparator for the osteoblastic cells in these gradient studies.

To more easily quantitate progesterin binding in the gradient fractions, 125 I-VNT was used as the radioligand in place of [3 H]R5020. [3 H]R5020 is the most commonly used radioligand but does not have sufficient specific activity for the present studies. 125 I-VNT is a specific, high-affinity ligand for the progesterin receptor (Hochberg *et al.* 1985) with a specific activity more than four times greater than that of [3 H]R5020. As shown in Fig. 2, the progesterin binding sites in the cytosol of the ZR75 and TE85 cells show similar migration in sucrose density gradients. The sedimentation coefficients were calculated to be 8.8 $\pm$ 0.4 (mean $\pm$ S.E.M. of 5 gradients) and 8.9 $\pm$ 0.2 (mean $\pm$ S.E.M. of 6 gradients) for the ZR75 and TE85 receptors respectively. These values agree with other reports in which the uterine progesterin receptor was found to have sedimentation coefficients ranging between 7 and 9.5 (Faber *et al.* 1973, Tai *et al.* 1986, Renoir *et al.* 1990). NET displaced the majority of the bound 125 I-VNT from the gradient fractions of both cell lines.

Table 2 Inhibition (%) of total [3 H]R5020 binding (10 nM) in the cytosol of MC3T3-E1 and TE85 cells by various steroids (1 mM)

Steroid	MC3T3-E1 cells	TE85 cells
R5020	100	100
NET	100	100
Levonorgestrel	100	100
17 β -estradiol	0	0
Dexamethasone	0	0
Dihydrotestosterone	60	24

Figure 2 Binding of ^{125}I -VNT to sucrose gradient fractions of cytosol from ZR75 and TE85 cells. Gradient fractions were incubated overnight with 1 nM ^{125}I -VNT and assay buffer or NET (100 nM) before separation of bound and free tracer.

Steroid regulation of progesterin receptors in osteoblast-like cells

In progesterin-responsive tissues, progesterin receptor number is under positive regulation by estradiol. To explore the regulation of progesterin receptors in osteoblasts, the effects of estradiol on progesterin receptor number in the TE85 and MC3T3-E1 cells were determined. The effects of estradiol in the osteoblast-like cells were compared with that in human ZR75 human breast cells. ZR75 breast cells contain functional estrogen receptors which can regulate progesterin receptors (Allegra *et al.* 1981, Van den Berg *et al.* 1990). Cytosolic progesterin receptors in the ZR75 cells were increased by 17β -estradiol within 24 h and reached their maximum by 72 h (Fig. 3). In contrast, estradiol treatment had no effect on cytosolic progesterin receptor numbers in TE85 cells and a relatively modest effect on MC3T3-E1 receptor number. Only after 72 h of treatment did estradiol affect MC3T3-E1 receptor number, increasing it to 147% of control.

When under constant exposure to ligand, progesterin receptors undergo down-regulation and their number drops accordingly. The effects of NET (1 nM) on progesterin binding were followed over the course of 72 h in ZR75 and MC3T3-E1 cells (Fig. 4). NET caused a dramatic decline in ^{125}I -VNT binding in the ZR75 cells, dropping to 10% of control levels within 24 h. The number of progesterin binding sites remained at these low levels through 72 h. Progesterin binding also decreased in MC3T3-E1 cells during NET treatment but the decline was more gradual and reached a minimum of 16% of control levels only after 72 h.

When compared with the effects seen in the ZR75 cells, the small osteoblastic cell responses following estradiol treatment may reflect cell-specific differences in progesterin receptor regulation or may simply result from a low number of estrogen receptors within the osteoblast-like cells. Estrogen receptors have been found in MC3T3-E1 cells and 17β -estradiol will stimulate the proliferation of these cells (Kanatani *et al.* 1991, Masuyama *et al.* 1992). To confirm the presence of and quantitate the estrogen receptors in the MC3T3-E1 cells used in the present study, binding studies were performed using [^{125}I] 17β -estradiol. Saturation analyses showed that the MC3T3-E1 cells contained a single, high-affinity binding site for 17β -estradiol numbering 3976 ± 1837 per cell with a K_d of 0.72 ± 0.09 nM (mean $\pm$ s.e.m. of three assays). The receptor binding affinity and receptor concentration agree with previous studies with the MC3T3-E1 cells (Masuyama *et al.* 1992) and are similar to those reported for other osteoblast-like lines including the TE85 cell line (Komm *et al.* 1988, Benz *et al.* 1991).

Progesterin stimulation of osteoblast differentiation

Alkaline phosphatase is a marker of osteoblast differentiation and an index of osteoblast activity. The ability of progestins to affect TE85 osteoblast activity was tested by measuring cellular alkaline phosphatase activity after treatment with progestational, androgenic and estrogenic steroids (Table 3). Only treatment with the three progestins increased alkaline phosphatase activity in the TE85 cells while neither cyproterone acetate, dihydrotestosterone nor estradiol had any effect.

Figure 3 Change in progesterin receptor number in ZR75, TE85 and MC3T3-E1 cells after 24–96 h of treatment with 17 β -oestradiol (1 nM). Columns represent the mean number of ¹²⁵I-VNT binding sites from three measurements ($\pm$ S.E.M.) normalized for cell number. * P <0.05 versus vehicle-treated cells.

Discussion

The goal of the present studies was to better characterize the progesterin receptors in osteoblasts. There exists sufficient evidence from other laboratories to prove the existence of such receptors but little has been done to study and compare the regulation of these receptors. Progesterin receptors have been localized to osteoblasts by RIA, immunocytochemistry and radioligand binding. The

presence of the progesterin receptor in osteoblastic cells has been validated through the use of PCR and Northern blot analysis (Wei *et al.* 1993, MacNamara *et al.* 1995, MacNamara & Loughrey 1998). However, quantitating changes in the concentrations of the receptor or its message have been difficult due to their low number per cell. In fact, in some experiments the receptor was not detected in certain osteoblast cell lines (Colston *et al.* 1989, Pensler *et al.* 1990, Orwoll *et al.* 1991, Wei *et al.* 1993). To perform the present studies, we employed large numbers of cells from two different osteoblastic lines to ensure a reasonable signal-to-noise ratio for the binding assays and also used a radioligand with a higher specific activity than has been

Figure 4 Change in progesterin receptor number in ZR75 and MC3T3-E1 cells after 24–72 h of treatment with NET (1 nM). Columns represent the mean number of ¹²⁵I-VNT binding sites from three measurements ($\pm$ S.E.M.) expressed as a percent of the number of binding sites found in vehicle-treated cells. * P <0.05 versus vehicle-treated cells.

Table 3 Alkaline phosphatase activity in TE85 cells after 72 h of treatment with various steroids (100 nM). Values are means alkaline phosphatase activity measured in six wells ($\pm$ S.E.M.) expressed as a percent of the enzyme activity measured in vehicle-treated cells

	Alkaline phosphatase activity (% vehicle control)
Vehicle	100 $\pm$ 4
NET	147 $\pm$ 3*
Levonorgestrel	121 $\pm$ 2*
Medroxyprogesterone acetate	138 $\pm$ 7*
Cyproterone acetate	102 $\pm$ 4
Dihydrotestosterone	114 $\pm$ 9
17 β -oestradiol	106 $\pm$ 10

* P <0.05 versus vehicle-treated cells.

used in the past. The data presented here demonstrate that osteoblast-like cells contain a functional progestin receptor. These receptors are like progestin receptors in all tissues in that they selectively bind progestins, become associated with the cell nuclei when occupied and have a sedimentation coefficient identical to that of breast cell receptors. However, the progestin receptors in osteoblastic cells differ from that in breast cells in terms of their binding affinity and regulation.

In our initial experiments, the binding of R5020 was quantitated in T47D breast cells and the two osteoblast-like lines, the TE85 and MC3T3-E1 cells. A single, high-affinity binding site was found in each cell type. Interestingly, the affinity of the receptors within the T47D breast cells was greater than the affinities in the osteoblastic cells. The values reported here for R5020 binding in the T47D, TE85 and MC3T3-E1 cells all fall within the general range of reported progestin receptor affinities. However, given that the present values were generated in concurrent experiments using the same procedures in the same laboratory, these differences are meaningful and do suggest differences in progestin binding in breast and osteoblastic cells. Tissue differences in the binding affinities of progestin and glucocorticoid receptors have been previously reported (Al-Khouri & Greenstein 1985, Ojasoo *et al.* 1988). The difference in progestin receptor affinity in breast and osteoblastic cells does not help explain how some progestins can stimulate the proliferation and activity of osteoblast-like cells at concentrations below those necessary to elicit a response in other progestin target tissues (Tremollieres *et al.* 1992, Lau *et al.* 1994, Verhaar *et al.* 1994). It is counter intuitive to imagine that a receptor with a higher K_d would allow a cell to respond to lower concentrations of hormone than other cells containing a receptor whose ligand affinity is greater. In addition to binding affinity, another notable difference between the T47D cells and the two osteoblastic cell lines is the concentration of progestin binding sites. This comparison had been made in earlier studies that had found even greater differences between the number of R5020 binding sites in breast and osteoblastic cells (Wei *et al.* 1993).

As anticipated, estrogen treatment increased the number of progestin binding sites in the breast and osteoblast-like cells. However, the increases in progestin binding sites in the ZR75 breast cells occurred more quickly and were of greater magnitude than that seen in the TE85 or MC3T3-E1 cells. The inability of estradiol to affect progestin binding sites in the TE85 cells could be due to a species difference between these cells and the murine MC3T3-E1 cells. This difference is minor, however, compared with that observed between breast and osteoblastic cell types. The apparent tissue differences may be due to several factors. M3T3-E1 cells contain fewer estrogen receptors than have been found in the ZR75 breast cells (Lippman *et al.* 1981) and this may make these osteoblastic cells less responsive to estradiol. The culture

conditions of the MC3T3-E1 cells may also have reduced their estrogen responsiveness. Osteosarcoma cells transfected with the estrogen receptor will express a greater number of those receptors when the cells are maintained in sub-confluent culture conditions (Migliaccio *et al.* 1993). In the present experiments, both the TE85 and the MC3T3-E1 cells reached confluency at some time during the estradiol treatments. The confluency of these cells could have not only reduced their number of estrogen receptors but may also have blunted the stimulatory effects of estradiol on progestin receptor levels. Osteoblasts and osteoblast-like cells have shown a wide range of responsiveness to estrogens, which has not always correlated with the estrogen receptor number. Human fetal osteoblastic cells transfected with the estrogen receptor show a 2.5-fold increase in progestin binding sites after estradiol addition (Harris *et al.* 1995). Yet, in similar experiments, human osteosarcoma HTB-96 cells transfected with the human estrogen receptor show no progestin receptor induction or increase in alkaline phosphatase activity after estradiol treatment (Watts *et al.* 1989).

Cell differences in progestin receptor regulation were also apparent when NET-induced receptor down-regulation was measured. Within the first 24 h of treatment, NET induced a rapid loss in cytosolic progestin binding sites in the ZR75 cells. Only after 72 h of treatment was a loss of similar magnitude seen in the MC3T3-E1 cells. It is not known if the reduction in binding sites represents a decline in total cellular binding sites or a loss of cytosolic binding sites, the remainder being associated with the cell nuclei. In either case, the ligand-occupied progestin receptors are processed differently within the two cell types.

In the present studies, the steroid-induced changes in the progestin binding site concentrations suggest that while osteoblasts do respond to estrogens and progestins, the responses are smaller and slower to occur than commonly observed in other steroid-responsive tissues. During the reproductive cycle, the normal variations in estrogen and progestin levels have pronounced acute effects on many steroid target tissues such as the uterus and breast. Bone formation is also under the influence of estrogen and progesterone as shown by cyclical changes in biochemical markers of bone formation during the menstrual cycle (Nielson *et al.* 1990, Gorai *et al.* 1998). Unlike reproductive tissues, an exceedingly rapid response to changes in circulating steroid concentrations may not be necessary for the osteoblasts to optimally serve their function.

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