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Modulation of Vitamin D Receptor and Estrogen Receptor by 1,25(OH)$_2$-Vitamin D$_3$ in T-47D Human Breast Cancer Cells

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1,25(OH)$_2$-Vitamin D$_3$ inhibits breast cancer cell proliferation through interaction with the vitamin D receptor (VDR). Regulation of VDR is under the influence of several factors which include the functional ligand for this receptor (1,25(OH)$_2$-vitamin D$_3$) as well as heterologous steroid hormones. We evaluated the nature of homologous regulation in T-47D human breast cancer cells with a radiolabelled ligand binding assay and a ribonuclease protection assay for VDR. Significant VDR up-regulation, as measured by hormone binding assays, occurred with pre-incubations with 10^{-9} M through 10^{-6} M 1,25(OH)$_2$-vitamin D$_3$ ($P < 0.05$). A 7-fold VDR up-regulation with 10^{-8} M 1,25(OH)$_2$-vitamin D$_3$ occurred at 4 h treatment and was not associated with an increase in VDR mRNA expression on ribonuclease protection assay. This supports the hypothesis that up-regulation of VDR is probably the result of ligand-induced stabilization of pre-existing receptor. All-*trans*-retinoic acid, the progesterone analog R-5020, and prednisone were found to induce heterologous up-regulation of the VDR. We then determined with ligand binding assays whether 1,25(OH)$_2$-vitamin D$_3$ could influence receptor levels for another hormone in a manner analogous to the heterologous regulation of VDR. Regulation of estrogen receptor (ER) by 1,25(OH)$_2$-vitamin D$_3$ was studied in T-47D and MDA-MB-231 breast cancer cells. Incubation of T-47D cells, which are ER (+), with 10^{-8} M 1,25(OH)$_2$-vitamin D$_3$ did not result in up-regulation of ER. Yet estrogen binding was significantly up-regulated in a cell line that is ER (−), MDA-MB-231. The increased estrogen binding was associated with a shift in binding affinity and ribonuclease protection assay showed absence of ER mRNA in these cells, suggesting an up-regulation of estrogen binding proteins and not of the ER itself.

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INTRODUCTION

1,25(OH)$_2$-Vitamin D$_3$, the biologically active metabolite of vitamin D, regulates epithelial cell growth and differentiation and may be an important factor in progression toward terminal differentiation [1]. 1,25(OH)$_2$-vitamin D$_3$ has been shown to inhibit growth and promote differentiation in a wide array of malignant cell types in which the VDR is expressed [2–6]. This effect is mediated through a specific high affinity vitamin D receptor (VDR) in the cells [7, 8]. The VDR is a member of the steroid hormone receptor superfamily and regulates gene transcription through interaction with hormone response elements (HREs) in the promoter region of target genes [9].

The presence of VDR in breast cancer has been documented both in cell lines and in tumor samples [10–12]. VDR activity is essential for hormonal action [13, 14]. Response to 1,25(OH)$_2$-vitamin D$_3$; correlates with receptor number in several models [13–15]. Thus it is important to understand the determinants of VDR expression.

Several steroid hormones are known to modulate VDR in cells of various origin. Estradiol (E$_2$) has been identified as a regulator of VDR in normal cells from

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intestine [16], uterus [17], liver and kidney [18], and in malignant cells from osteosarcoma [19] and breast [20]. Dihydroxytestosterone (DHT) has also been shown to regulate VDR in breast cancer cells, possibly through an estrogen receptor (ER)-mediated mechanism [20]. Similarly, 1,25$(OH)_2$-vitamin D_3 and several vitamin D analogs have been shown to regulate VDR in a variety of mammalian cells [21–26].

In this study, we evaluated the potential for 1,25$(OH)_2$-vitamin D_3 to modulate VDR and ER in two human breast cancer cell lines. Regulation of ER expression represents a possible mechanism of 1,25$(OH)_2$-vitamin D_3 growth regulation. Expression of ER and VDR has been well studied in T47D human breast cancer cells [10, 27]. It is ER (+) and VDR (+), is growth stimulated by E_2 and is growth inhibited by 1,25$(OH)_2$-vitamin D_3. MDA-MB-231 is a poorly-differentiated breast cancer cell line which expresses VDR in insignificant quantities and does not express functional ER [3, 28]. Together, these cell lines represent an effective model in which to study modulation of VDR and ER. We employed radiolabeled hormone binding assays and a ribonuclease protection assay to determine the effect of 1,25$(OH)_2$-vitamin D_3 on VDR and ER.

MATERIALS AND METHODS

Cell culture conditions

T-47D and MDA-MB-231 human breast cancer cells were obtained from the American *Type Culture* Collection, Rockville, MD. Cells were cultured in 75 cm^2 flasks in RPMI 1640 media (Biofluids, Rockville, MD) supplemented with L-glutamine (1 ×), pen-strep (10 U), and 5% fetal calf serum (FCS), and maintained at 37°C in a humidified atmosphere of 5% CO_2-air.

Chemicals

1,25$(OH)_2$-vitamin D_3 was kindly provided by Dr Milan Uskokovic, Hoffmann-LaRoche (Nutley, NJ). The progesterone analog, R-5020, was obtained from DuPont (Wilmington, DE). All-*trans*-retinoic acid (*t*-RA), diethylstilbestrol (DES) and prednisone were purchased from Sigma Chemicals (St Louis, MO).

Cytosol binding assay

VDR. T-47D cell cultures were trypsinized (0.05% trypsin and 0.02% versene) when they approached 80% confluence. Cells were then cultured overnight in Iscove's modified essential media (IMEM) without phenol red, with 5% charcoal-stripped FCS. 0.5 mg of cytosolic protein extract in a total volume of 200 μl was incubated for 18 h with 1,25$(OH)_2$-vitamin D3 in an ice water bath. This non-radioactive ligand was added at a 200-fold molar excess to determine non-specific binding. $[^3H]$1,25$(OH)_2$-vitamin D_3 (SA 165 Ci/mmol) (DuPont NEN, Boston, MA) was added to achieve final concentrations from 0.01 to 4 nM. After 18 h incubation on ice, 300 μl of 2.5% dextran-coated charcoal was added to remove excess radiolabeled ligand. Radioactivity in the supernatant was determined using a scintillation counter. Specific uptake of $[^3H]$1,25$(OH)_2$-vitamin D_3 was calculated by Scatchard analysis as the difference between total and non-specific binding [29].

ER. The protocol was identical to that for the VDR except that MDA-MB-231 cells were incubated for 18 h with DES, which is an effective competitor for ER. $[^3H]$estradiol (SA 126 Ci/mmol) (Amersham, Arlington Heights, IL) was added to achieve final concentrations ranging from 1 to 50 nM.

Whole cell binding assay

VDR. In order to study VDR modulation by 1,25$(OH)_2$-vitamin D_3, T-47D cells were incubated with 1,25$(OH)_2$-vitamin D_3 at concentrations ranging from 10^{-6} 10^{-11} M for 72 h. The cells were gently washed with phosphate buffered saline (PBS) and incubated at 4°C for 5 h with $[^3H]$. 1,25$(OH)_2$-vitamin D_3 at a final concentration of 1 nM. Cells were incubated with a 200-fold molar excess of non-radioactive 1,25$(OH)_2$-vitamin D_3 in order to determine non-specific binding. Radioactivity of the cell lysate was measured with a scintillation counter. This experiment was repeated with an incubation with a fixed 10^{-8} M concentration of 1,25$(OH)_2$-vitamin D_3 for treatment lengths ranging in duration from 30 min to 72 h.

The ability of other steroid hormones to modulate VDR was assessed by whole cell binding assay. T-47D cells were incubated for 72 h with *t*-RA, R-5020, or prednisone at 10^{-6}–10^{-11} M concentrations prior to the binding assay. Medium was changed every 24 h during the incubation.

ER. Modulation of ER by 1,25$(OH)_2$-vitamin D_3 in both an ER (+) and an ER (−) breast cancer cell line was assessed with the whole cell binding assay described above. T-47D and MDA-MB-231 cells were incubated with 10^{-8} M 1,25$(OH)_2$-vitamin D_3 for 72 h. The binding assay for ER was performed with DES and $[^3H]$estradiol.

Statistical analysis

A two-tailed Student's *t*-test was used to assess differences between experimental groups. P values < 0.05 were considered statistically significant.

Ribonuclease protection assay

VDR and ER modulation by 1,25$(OH)_2$-vitamin D_3 was assessed by RNase protection assay. The complementary DNA coding for the human VDR was obtained from Dr J. Wesley Pike. The VDR probe corresponds to 319 bp protected fragment at the 3′-end of the hVDR coding sequence. The ER probe, which corresponds to a 270 bp fragment in exon 2, was obtained from Dr Mary Beth Martin. This assay was performed as previously described [15]. A radiolabeled probe for the constituitively expressed human acidic

ribosomal phosphoprotein, 36B4, served as the loading control. The experiment was repeated twice with consistent results.

RESULTS

Scatchard analysis of 1,25(OH)$_2$-vitamin D_3 binding to VDR in T-47D human breast cancer cells yielded a linear plot (Fig. 1). A K_d of 0.054 ± 0.003 nM was calculated for VDR. This result was similar to that reported by Escaleira and colleagues, $K_d = 0.079$ nM [20]. These two results differ from that reported by Findlay and colleagues, $K_d = 0.0076$ nM [30]. The discrepancy in measured receptor affinity may be accounted for by variability in culture conditions, incubation time and temperature, and assay technique—whole cell versus cytosolic method.

To determine 1,25(OH)$_2$-vitamin D_3-mediated regulation of VDR in breast cancer, T-47D cells were harvested after 72 h treatment with 1,25(OH)$_2$-vitamin D_3 concentrations ranging from 10^{-6} M to 10^{-11} M. The receptor level was determined by radiolabeled ligand binding assay (Fig. 2). 1,25(OH)$_2$-vitamin D_3 significantly up-regulated the amount of VDR at concentrations from 10^{-9} to 10^{-6} M ($P < 0.05$). T-47D cells were treated with a concentration of 10^{-8} M 1,25(OH)$_2$-vitamin D_3 for varying lengths of time (Fig. 3). The receptor level peaked with a 7-fold increase at 4 h treatment, decreased at 24 h, and returned to near-baseline levels at 72 h. The receptor levels at 4, 24 and 48 h were significantly greater than control ($P < 0.05$). When VDR message levels were analyzed by RNase protection assay, no changes in the amount of VDR mRNA was found at time periods up to 24 h (Fig. 4). Densitometric readings of VDR message levels for 30 min, and 3, 5, 8, 12, and 24 h, expressed as % control, were as follows: 110, 108, 100, 116, 96, and 87%, respectively.

Next, we sought to determine whether different steroid hormones regulated the amount of VDR in T-47D cells. The cells were treated with t-RA, R-5020, and prednisone at concentrations from 10^{-6} to

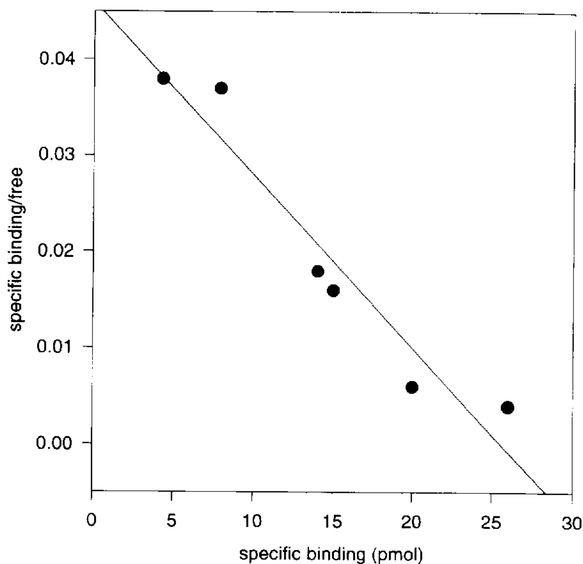

Fig. 1. Scatchard analysis of specific [^{3}H]1,25(OH)$_2$-vitamin D_3 binding in T-47D cells. Cells were cultured overnight in IMEM medium with 5% charcoal-stripped FCS. Cells were then incubated with increasing concentrations (0.01–4 nM) of [^{3}H]1,25(OH)$_2$-vitamin D_3 in the presence or absence of a 200-fold excess of 1,25(OH)$_2$-vitamin D_3 at 4° C for 5 h. Specific [^{3}H]-1,25(OH)$_2$-vitamin D_3 uptake was calculated by subtracting the radioactivity measured in the presence of unlabeled 1,25(OH)$_2$-vitamin D_3; from that measured in its absence. Each point was derived from the mean of duplicate determinations. The assay was performed twice with equivalent results. The vitamin D receptor displayed a K_d of 0.054 ± 0.003 nM.

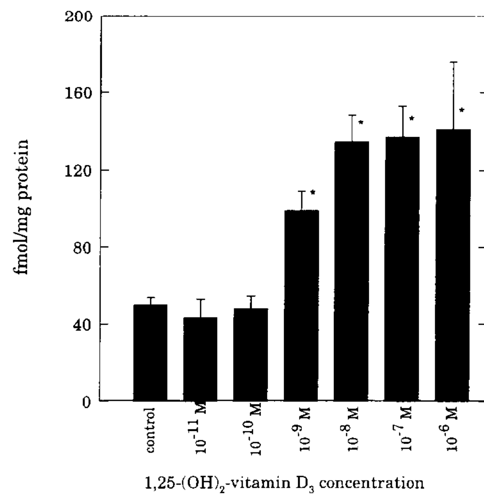

Fig. 2. Influence of increasing concentrations of 1,25(OH)$_2$-vitamin D_3 on specific binding of VDR in T-47D cells. Monolayers of T-47D cells were pre-incubated with 1,25(OH)$_2$-vitamin D_3 for 72 h. Data are the mean ± SD of triplicate determinations. (*) Denotes a statistically significant increase in specific binding vs control ($P < 0.05$).

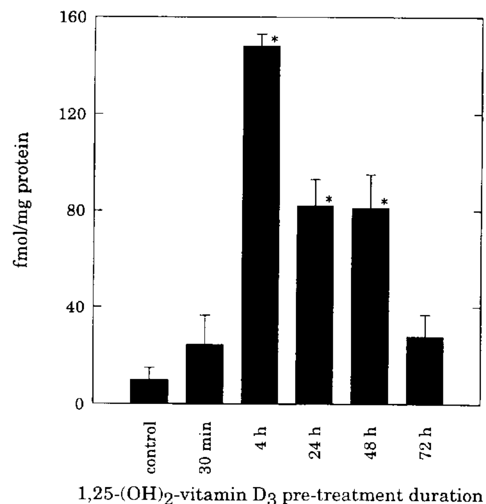

Fig. 3. Effect of increasing lengths of treatment with 10^{-8} M 1,25(OH)$_2$-vitamin D_3 on VDR in T-47D cells. Ligand binding assays were performed in duplicate; data points are the mean ± SD. (*) Denotes a statistically significant increase in specific binding vs control ($P < 0.05$).

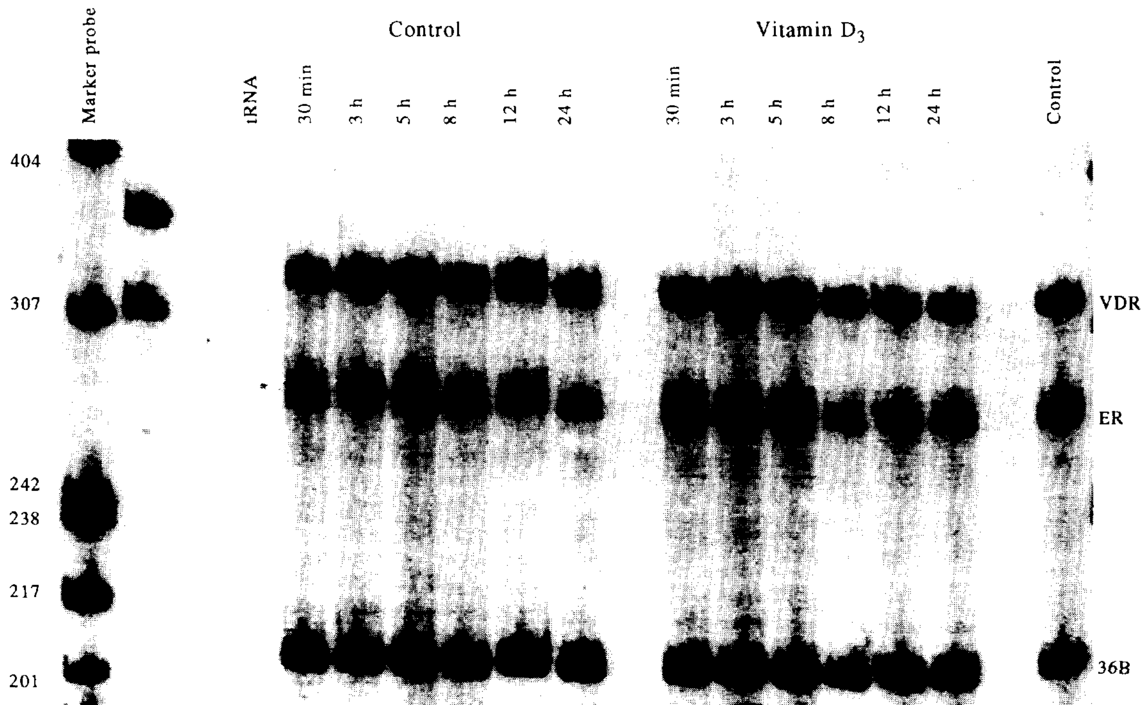

Fig. 4. Ribonuclease protection assay for VDR and ER in T-47D cells treated with 10^{-8} M 1,25(OH)$_2$-vitamin D$_3$ for varying lengths of time to 24 h.

10^{-11} M for 72 h. Ligand binding assay demonstrated that *t*-RA concentrations from 10^{-8} M through 10^{-11} M down-regulated VDR specific binding while higher concentrations, 10^{-7} and 10^{-6} M, effected an up-regulation (Fig. 5). A similar effect was observed with R-5020 treatment, except that 10^{-8} M was associated with an up-regulation of VDR. However, the results for prednisone treatment demonstrated an increase in the amount of active VDR at concentrations from 10^{-6} M through 10^{-10} M.

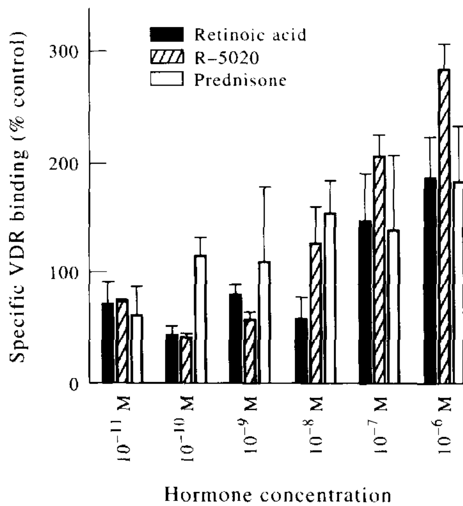

Fig. 5. Effect of steroid hormones on specifically bound [^{3}H]1,25(OH)$_2$-vitamin D$_3$ in T-47D cells. Monolayers of T-47D cells were pre-incubated with 10^{-6}–10^{-11} M concentrations of *t*-RA, R-5020, or prednisone for 72 h. Binding assays were performed in duplicate; data points are the mean ± SD.

The effect of 1,25(OH)$_2$-vitamin D$_3$ on expression of ER in breast cancer cells was analyzed. T-47D cells were treated with 10^{-8} M 1,25(OH)$_2$-vitamin D$_3$ for varying lengths of time. This experiment demonstrated no significant change in the amount of ER as determined by radiolabeled ligand binding assay ($P > 0.05$) (Fig. 6). RNase protection assay results for ER corresponded with the binding assay findings. ER message levels, which were performed concomitantly with VDR, were also essentially unchanged (Fig. 4). No pattern was evident with % control values for 30 min,

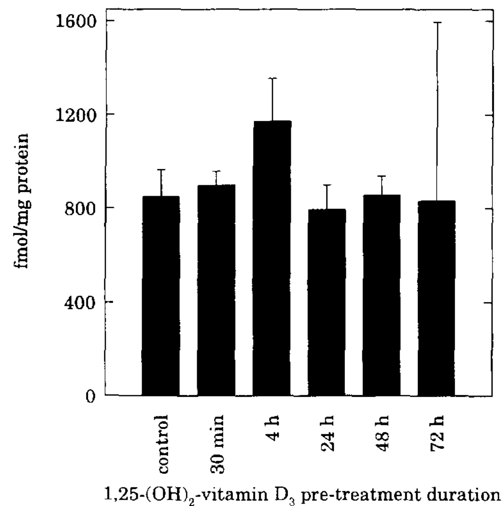

Fig. 6. Effect of 10^{-8} M 1,25(OH)$_2$-vitamin D$_3$ on ER in T-47D cells. Ligand binding assays were performed in duplicate for time points ranging to 72 h; data points are the mean ± SD.

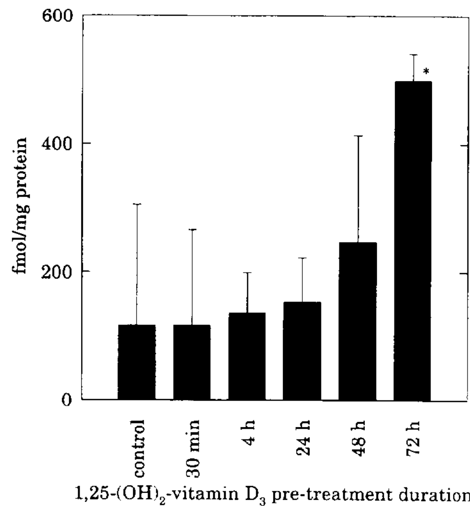

Fig. 7. Time course of 10^{-8} M 1,25(OH)$_2$-vitamin D$_3$-induced up-regulation of ER in MDA-MB-231 cells. The binding assay was performed in triplicate; data points are the mean ± SD. (*) Denotes a statistically significant increase in specific binding vs control ($P < 0.05$).

and 3, 5, 8, 12, and 24 h as follows: 122%, 113, 109, 100, 112, and 141%, respectively.

MDA-MB-231 cells which were treated with 10^{-8} M 1,25(OH)$_2$-vitamin D$_3$ for varying lengths of time up to 72 h, demonstrated an increase in E$_2$ binding at 48 h and a statistically significant increase at 72 h ($P < 0.05$) (Fig. 7). This assay was repeated three times with whole cells.

A competitive binding assay for ER was performed with MDA-MB-231 cells which were either treated for 72 h with 10^{-8} M 1,25(OH)$_2$-vitamin D$_3$ or without 1,25(OH)$_2$-vitamin D$_3$ (Fig. 8). Scatchard analysis indicated that 1,25(OH)$_2$-vitamin D$_3$ increases the expression of a cellular protein which bound E$_2$ with an affinity that differed from the untreated ($K_d = 0.11$ nM vs 0.067 nM).

DISCUSSION

VDR and its functional ligand, 1,25(OH)-vitamin D$_3$, interact with other members of the steroid hormone receptor superfamily [16–20]. This has attracted considerable interest, especially in light of the antiproliferative effect of 1,25(OH)$_2$-vitamin D$_3$ on breast cancer cells. T-47D breast cancer cells are growth inhibited by 10^{-8} M 1,25(OH)$_2$-vitamin D$_3$ and are high expressors of both VDR mRNA and receptor protein that is capable of binding ligand [11, 14]. In this study, we assessed the potential of 1,25(OH)$_2$-vitamin D$_3$ to modulate its own receptor. Additionally, we sought to determine the effect of 1,25(OH)$_2$-vitamin D$_3$ on ER expression. Regulation of ER expression represents a possible mechanism of 1,25(OH)$_2$-vitamin D$_3$ growth regulation.

Specific 1,25(OH)$_2$-vitamin D$_3$ binding activity was up-regulated by 1,25(OH)$_2$-vitamin D$_3$ concentrations ranging from 10^{-9} through 10^{-6} M (Fig. 2). A time course evaluation with 10^{-8} M 1,25(OH)$_2$-vitamin D$_3$ determined that hormone binding activity peaked at 4 h, maintained a plateau for up to 48 h treatment, and returned to near baseline levels by 72 h (Fig. 3). Homologous up-regulation of VDR has been demonstrated in several models, both *in vitro* [21–24] and *in vivo* [25, 26]. We found that the 7-fold increase in functional receptor was not associated with any increase VDR mRNA through 24 h of treatment with 1,25(OH)$_2$-vitamin D$_3$ (Fig. 4). This finding is consistent with the work of Weise and DeLuca, who have shown that treatment with 1,25(OH)$_2$-vitamin D$_3$ resulted in increased levels of VDR without an antecedent increase in VDR mRNA in mouse fibroblasts and rat intestinal epithelial cells [31]. The up-regulation of VDR is probably the result of ligand-induced stabilization of the receptor. Evidence supporting this hypothesis has also been demonstrated in rat kidney cells [32]. 1,25(OH)$_2$-Vitamin D$_3$ bound to VDR may prevent receptor degradation, possibly by protecting it from endogenous proteases [31].

Alternatively, there have been several studies which suggest that VDR is up-regulated at a transcriptional level. Mouse 3T6 cells have elevated levels of VDR after 24–72 h treatment with 1,25(OH)$_2$-vitamin D$_3$ [33]. VDR mRNA levels were elevated at 6 h of 1,25(OH)$_2$-vitamin D$_3$ treatment, but returned to control level by 24 h in vitamin D deficient rats given a single dose of 1,25(OH)$_2$-vitamin D$_3$ [26]. Other cell lines, including human osteosarcoma [19], HL-60 cells [34], and rat parathyroid gland [35] have all demonstrated up-regulation of VDR mRNA in response to 1,25(OH)$_2$-vitamin D$_3$ treatment. It remains possible that VDR mRNA in T-47D cells may rise in response

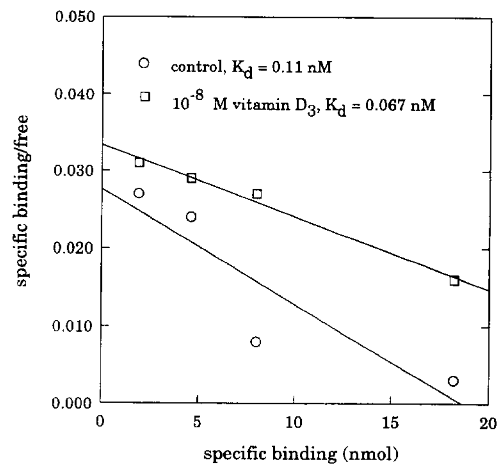

Fig. 8. The effect of 1,25(OH)$_2$-vitamin D$_3$ on the affinity and concentration of ER in MDA-MB-231 cells. [^{3}H]estradiol binding data for MDA-MB-231 cell monolayers pre-incubated for 72 h in the absence (○) or presence of (□) 10^{-8} M 1,25(OH)$_2$-vitamin D$_3$ are presented as Scatchard plots. Each point was derived from the mean of duplicate determinations. The assay was performed three times with equivalent results.

to 1,25$(OH)_2$-vitamin D_3 at a time point beyond 24 h, it would not, however, account for the increased quantity of receptor present from 4–48 h (Fig. 3).

The ability of other steroid hormones, including *t*-RA, E_2, and DHT, to modulate VDR expression in cancer cells has been evaluated [19, 36–39]. DHT and E_2 have been shown to increase levels of VDR via an ER-mediated mechanism in T-47D cells [19]. *t*-RA modulated the number but not the affinity of VDR binding sites in osteosarcoma cells [36]. In T-47D cells, *t*-RA enhanced the antiproliferative response to 1,25$(OH)_2$-vitamin D_3 without increasing the number of receptors [38]. We have shown up-regulation of VDR binding with *t*-RA, R-5020, and prednisone (Fig. 5). While *t*-RA and R-5020 increased 1,25$(OH)_2$-vitamin D_3 binding only at higher concentrations (10^{-7}, $^{-10}$ M and 10^{-8}, ^{10}, $^{-7}$, $^{-10}$, $^{-6}$ M, respectively), prednisone induced up-regulation of VDR from 10^{-10} to 10^{-6} M. The up-regulation of VDR in T-47 D cells with *t*-RA treatment for 72 h differs from 24 h results obtained by Koga and Sutherland, who found no change in VDR quantity at low *t*-RA concentrations and down-regulation of VDR at 10^{-6} M *t*-RA [38]. The treatment duration difference may account for the difference in receptor levels. A late (72 h) up-regulation of receptor may be consistent with transcriptional up-regulation.

The cross-reactivity between other steroid hormones and VDR may be due to heterologous stabilization of VDR. Alternatively, heterologous up-regulation of VDR, which has been demonstrated with a variety of different agents, including the above-mentioned *t*-RA [38], E_2 and DHT [20], as well as dibutyryl cAMP, phorbol esters, sodium butyrate, and forskolin may occur at the transcriptional level [24, 34, 40]. This explanation can be accounted for by the well-conserved DNA binding domain of the different steroid hormone receptors. Interaction between the various steroid hormones with HREs for other steroids possessing similar consensus sequences suggests that complex regulatory mechanisms may control the amount of receptors for a given steroid hormone [41].

In turn, we then questioned whether 1,25$(OH)_2$ vitamin D_3 was capable of influencing other receptor levels in a manner analogous to the heterologous regulation of VDR. ER and progesterone receptor (PR) in T-47D cells have been well described [27, 42]. The down-regulation of the highly-expressed PR in T-47D by *t*-RA is associated with a decreased level of PR mRNA [42]. T-47D cells, low expressors of ER, are nonetheless growth-stimulated by exogenous E_2 [38]. We found that 1,25$(OH)_2$-vitamin D_3 treatment for up to 72 h did not alter the amount of ER (Fig. 6). This suggests that 1,25$(OH)_2$-vitamin D_3 neither stabilized nor transcriptionally up-regulated ER in T-47D.

Yet 1,25$(OH)_2$-vitamin D_3 seems to have a significant impact on ER in other cell lines. MCF-7 human breast cancer cells, strongly positive for ER, have been shown to have less ER message and fewer functional receptors when treated with 1,25$(OH)_2$-vitamin D_3 [43]. *t*-RA and 1,25$(OH)_2$-vitamin D_3 inhibit E_2-induced growth and transcription in MCF-7 cells, an effect which is potentiated by tamoxifen [44]. E_2-induced growth of T-47D cells, however, is not influenced by *t*-RA [38]. In MCF-7 cells, a mechanism by which 1,25$(OH)_2$-vitamin D_3 can block ER function (including E_2-induced transcription) has been partially elucidated. Activated VDR may impair ER binding to estrogen response elements through formation of ER/VDR heterodimers [44]. *t*-RA, however, does not effect ER dimerization. Its effect is mediated though inhibition of ER binding to estrogen response elements in the DNA promoter region [44].

The difference between the antiproliferative responses of T-47D and MCF-7 may be attributable to ER expression in each cell line. Thus we evaluated the ER response in the MDA-MB-231 human breast cancer cell line. MDA-MB-231 cells are not growth inhibited by 1,25$(OH)_2$-vitamin D_3 and have virtually no ER, as determined by RNase protection assay using a probe specific for a portion of the ER hormone binding domain (data not shown). When treated with 10^{-8} M 1,25$(OH)_2$-vitamin D_3 for 48 h these cells demonstrated a 4-fold increase in E_2 binding activity (Fig. 7). The protein responsible for this binding differed in binding affinity from that of the low levels of E_2 binding activity that existed in untreated cells (Fig. 8.). The altered binding affinity after 1,25$(OH)_2$-vitamin D_3 treatment suggests induction of binding proteins that do not bind E_2 with the same affinity as the classic ER ($K_d = 0.35$–0.45 nM) [45, 46]. Increased expression of estrogen binding proteins in response to 1,25$(OH)_2$-vitamin D_3 represents another mechanism through which 1,25$(OH)_2$-vitamin D_3 may interfere with estrogenic stimulation of breast cancer cells.

In conclusion, these studies suggest that 1,25$(OH)_2$-vitamin D_3 is capable of modulating VDR expression in T-47D through a mechanism that is not transcriptionally regulated. Other steroid hormones also up-regulate VDR in a dose-dependent manner. While different steroid hormones regulate VDR, 1,25$(OH)_2$-vitamin D_3 itself does not regulate heterologous steroid hormone receptors such as ER in breast cancer cells.

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