Overexpression of ER and VDR is not sufficient to make ER-negative MDA-MB231 breast cancer cells responsive to 1α-hydroxyvitamin D₅

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1α-hydroxyvitamin D₅ [1α(OH)]D₅ is an active vitamin D analog showing promising chemopreventive effect in breast carcinogenesis. We previously reported that estrogen receptor (ER)-positive breast cancer cells were sensitive, whereas ER-negative breast cancer cells were relatively resistant to their antiproliferative effects. In the present study, we used ER-negative MDA-MB231, ER-transfected MDA-MB231 (S30) and ER-positive BT474 cell lines to elucidate the possible association between ER status and cellular sensitivity to 1α(OH)D₅ treatment. Our results demonstrate that ER expression in ER-negative breast cancer cells (S30) did not increase the sensitivity to 1α(OH)D₅ whereas in ER-positive BT474 cells, the significant antiproliferative effect of 1α(OH)D₅ was correlated with the downregulation of ER and progesterone receptor expression. Further analysis indicated that both MDA-MB231 and S30 cells express low vitamin D receptor (VDR) at transcriptional level and protein level. However, transfection of VDR failed to restore the sensitivity to 1α(OH)D₅ in MDA-MB231 and S30 cells, although VDR direct target gene CYP24 was more responsive to 1α(OH)D₅ treatment in MDA-MB231 and S30 cells overexpressing VDR. In addition, nuclear receptor cofactors NCoR1 and SRC1 that could potentially affect VDR action were also low in both MDA-MB231 and S30 cells in comparison with ER-positive, vitamin D-sensitive BT474 cells. These results suggest that in addition to the increased ER and VDR expression, the intact VDR signaling machinery as present in ER-positive, vitamin D-sensitive cells is essential for the antiproliferative action of vitamin D, whereas the direct VDR target genes such as CYP24 can remain responsive to augmented VDR expression.

Introduction

The role of estrogen receptor (ER) ζ in breast cancer has been extensively studied and is a well-established major target for breast cancer treatment (1–3). ER-positive breast cancer cells are generally more responsive to antiestrogen therapy and chemopreventive agents, including retinoids, vitamin D analogs, tamoxifen, in comparison with ER-negative breast cancer cells (4,5). The purpose of the present study was to evaluate whether ER or VDR overexpression in ER-negative, vitamin D-resistant MDA-MB231 cells increases the sensitivity to 1α(OH)D₅ treatment. Whereas Narvaez et al. (10) demonstrated that a vitamin D3-resistant MCF-7 variant (MCF-7ΔVDR) expressed comparable level of functional VDR protein in comparison with the sensitive parental MCF-7 cells, the VDR was uncoupled from a functional apoptotic pathway. Hansen et al. (11) developed another vitamin D-resistant MCF-7 cell line with increased sensitivity to the pure antiestrogen IC182780. Overall, in ER-positive, vitamin D-resistant MCF-7 cells, there seems to be significant dissociation between ER and VDR signaling, since the ER-positive cells remained sensitive to antiestrogens (10,11). In ER-negative MDA-MB231 breast cancer cells that are relatively resistant to the antiproliferative effect of vitamin D, the gene expression pattern induced by 1,25(OH)₂D₃ was also very different from that in MCF-7 cells (12). This provides an ideal model where one can understand the interactions between ER- and VDR-mediated functions by stably transfecting ER and/or VDR in ER-negative breast cancer cells. In this report, we designed experiments to test whether ER-negative cells become responsive to 1α-hydroxyvitamin D₅ [1α(OH)D₅], an active vitamin D analog, after stably transfected with ERζ or VDR. The results would in turn help to further elucidate the role of ER and VDR signalling in response to vitamin D in breast epithelial cells.

The active metabolite of vitamin D, 1,25-dihydroxyvitamin D₅, has been shown to be an efficacious inhibitor of ER-positive breast cancer cell proliferation. The possible clinical use for 1,25(OH)₂D₃ is precluded due to its toxicity. To circumvent this problem >1000 analogs of vitamin D₃ have been synthesized and many of them have been evaluated to identify effective but non-toxic analogs of vitamin D. Very few of these have been considered for further clinical trials. In our laboratory, we synthesized one such 1α-hydroxy, 24 ethyl analog of vitamin D₃ [1α(OH)D₅] and showed that this is an efficacious inhibitor of breast and colon carcinogenesis and it is currently being developed for clinical trials. 1α(OH)D₅ has an extra ethyl group at C 24 and does not possess hydroxyl group at C 25 in comparison with 1,25(OH)₂D₃ (13). 1α(OH)D₅ is less toxic in comparison with other vitamin D analogs and at 1 μM, 1α(OH)D₅ showed significant inhibition of the development of carcinogen-induced mammary alveolar and ductal lesions in mouse mammary gland organ culture without any signs of cytotoxicity (13). The main mode of action reported for vitamin D analogs is via binding to the VDR resulting in the regulation of key genes involved in antiproliferation, differentiation and/or apoptosis (14). 1α(OH)D₅ has been shown to be most effective in cell lines that express high levels of VDR, such as the ER-positive BT-474 breast carcinoma cells, whereas cell lines with low levels of VDR, such as the MDA-MB-231 have been shown to be less responsive to 1α(OH)D₅ (5). The purpose of the present study was to evaluate whether ER or VDR overexpression in ER-negative, vitamin D-resistant MDA-MB231 cells increases the sensitivity to 1α(OH)D₅ treatment.

Materials and methods

Cell culture

BT474 and MDA-MB231 cell lines were purchased from American Type Tissue Collection (Manassas, VA) and cultured as described previously (5). ER-transfected MDA-MB231 (S30) cells were kindly provided by Dr V.C.Jordan (Northwestern University, Chicago, IL) and cultured as described (15). The vitamin D analog, 1α(OH)D₅ was synthesized and dissolved as described previously (16). 1,25(OH)₂D₃ was purchased from Sigma-Aldrich Corp. (St Louis, MO) and served as a control in some of the experiments.

Cell proliferation assay and cell cycle analysis

Cell proliferation was evaluated using MTT assay (4). For cell cycle analysis, cells were grown in six-well plates. Following treatments with 1 μM of 1α(OH)D₅, the cells were washed twice with phosphate-buffered saline, trypsinized, washed in phosphate-buffered saline, fixed in 70% ethanol, collected in 12 × 75 mm tubes and stored at –20°C until evaluation. For FACS analysis, the cells were washed with phosphate-buffered saline, resuspended in 0.2 ml
propidium iodide staining solution with RNAase A for 30 min and then analyzed by a Coulter EPICS Elite ESP Flow Cytometer (Coulter Corp., Miami, FL). At least 10,000 cells from both control and 1α(OH)D$_3$-treated cells were analyzed for cell cycle distribution (17). BrdU incorporation assay was performed using a kit from Calbiochem (La Jolla, CA) according to the manufacturer’s instructions. Cells were seeded in 96-well plates at 2000 cells per well, treated with vitamin D for 48 h and BrdU was added to the medium 6 h before termination of the experiment. BrdU incorporation assay is a more sensitive assay for cell proliferation that was used to evaluate cell proliferation potential in S30 cells transiently transfected with VDR.

Western blot
When cells grew to 50–70% confluence, cell lysates were prepared and subjected to western blot analysis as described previously (4). Rat anti-VDR monoclonal antibody and ER mouse monoclonal antibody were purchased from NeoMarkers (Fremont, CA). CYP24 mouse polyclonal antibody was from Abnova Corp. (Taipei, Taiwan). All secondary antibodies were from Santa Cruz Biotech (Santa Cruz, CA).

Transfection of VDR in MDA-MB231 and S30 cells
The VDR expression vector pcDNA3.1VDR was generated by PCR cloning using pcDNA3.1/V5-His TOPO TA Expression Kit (Invitrogen, Carlsbad, CA). The PCR primers for cDNA from T47D cells using primers containing start and stop codons and using pcDNA3.1/V5-His TOPO TA Expression Kit (Invitrogen, Carlsbad, CA) by using iQ™ SYBR Green PCR Supermix (Bio-Rad) according to manufacturer’s guidelines. The PCR cycling conditions used were 40 cycles 15 s at 95°C, 15 s at 60°C, and 1 s at 72°C. Fold inductions were calculated using the formula 2$^{-ΔΔCt}$, where ΔCt is ΔCt(treatment) − ΔCt(control), ΔCt is Ct(treatment) − Ct(control), Ct is the cycle at which the threshold is crossed. The gene primer pairs (and product size) for the gene analyzed here were as follows: VDR gene forward 5'-TGAAGCAG-GCCACTATTCCACTC-3' and reverse 5'-ACTCTTCTTCTACATGCCG-TAGTTCA-3' (123 bp), Prohibitin gene forward 5'-ACCACTGGGGAATGCTGACATCGTCA-3' and reverse 5'-TAGTCTCC-TCTC-GATGTGTGCTG-3' (126 bp), CYP24 gene forward 5'-CTCAGACGCGT-TAGTGCGATT-3' and reverse 5'-AGCAGTCTTATATGTCGTTTCCAC-3' (122 bp), β-actin gene forward 5'-CTCAGCACCAGG-GCTGCATG-3' and reverse 5'-GGCGAGGGTCAAGGCAGTA-3' (101 bp), progesterone receptor (PgR) gene forward 5'-GATTCAAAGCGAGCGCAGAG-3' and reverse 5'-CCACTTGGGATTCCTTCAGAG-3' (119 bp), NCoR1 gene forward 5'-GGAGGGC-GGGTTGGAAC-3' and reverse 5'-GGCCAGCTGTCCCTTCCAG-3' (147 bp), SRC1 gene forward 5'-AAATGATCCCGCCACCTTGA-3' and reverse 5'-CGGATTCGAGTCCTGCTA-3' (147 bp). PCR product quality was monitored using post-PCR melt curve analysis.

RNA extraction and real-time reverse transcription-polymerase chain reaction (RT-PCR)
Total RNA extraction and RT reaction were performed as described previously (4). RNA was further subjected to DNase I (Ambion, Austin, TX) digestion and purification using RNase Mini Kit (Quagen, Valencia, CA) before RT. Two RT reactions for each sample were pooled and diluted with equal amount of DNase/Rnase-free water. Real-time PCR was performed with 2 μl diluted RT product in a MyiQ Real-time PCR Detection System (Bio-Rad, Hercules, CA) by using iQ™ SYBR Green PCR Supermix (Bio-Rad) according to manufacturer’s guidelines. The PCR cycling conditions used were 40 cycles of 15 s at 95°C, 15 s at 60°C, and 20 s at 72°C. Fold inductions were calculated using the formula 2$^{-ΔΔCt}$, where ΔCt is ΔCt(treatment) − ΔCt(control), ΔCt is Ct(treatment) − Ct(control), Ct is the cycle at which the threshold is crossed. The gene primer pairs (and product size) for the gene analyzed here were as follows: VDR gene forward 5'-TGAAGCAG-GCCACTATTCCACTC-3' and reverse 5'-ACTCTTCTTCTACATGCCG-TAGTTCA-3' (123 bp), Prohibitin gene forward 5'-ACCACTGGGGAATGCTGACATCGTCA-3' and reverse 5'-TAGTCTCC-TCTC-GATGTGTGCTG-3' (126 bp), CYP24 gene forward 5'-CTCAGACGCGT-TAGTGCGATT-3' and reverse 5'-AGCAGTCTTATATGTCGTTTCCAC-3' (122 bp), β-actin gene forward 5'-CTCAGCACCAGG-GCTGCATG-3' and reverse 5'-GGCGAGGGTCAAGGCAGTA-3' (101 bp), progesterone receptor (PgR) gene forward 5'-GATTCAAAGCGAGCGCAGAG-3' and reverse 5'-CCACTTGGGATTCCTTCAGAG-3' (119 bp), NCoR1 gene forward 5'-GGAGGGC-GGGTTGGAAC-3' and reverse 5'-GGCCAGCTGTCCCTTCCAG-3' (147 bp), SRC1 gene forward 5'-AAATGATCCCGCCACCTTGA-3' and reverse 5'-CGGATTCGAGTCCTGCTA-3' (147 bp). PCR product quality was monitored using post-PCR melt curve analysis.

Results
ER expression in MDA-MB231 cells does not increase cellular sensitivity to 1α(OH)D$_3$
We first examined cellular sensitivity to 1α(OH)D$_3$ using MTT assay in both the parental ER-negative MDA-MB231 and ER-transfected MDA-MB231 (S30) cells and compared them with ER-positive BT474 breast cancer cells. As shown in Figure 1A, 2, 5, and 7 days treatment with 0.5 μM 1α(OH)D$_3$ caused differential response in both MDA-MB231 and S30 cells. There was a consistent minor inhibition in cell proliferation after 7 day treatment with vitamin D in both cell lines. In ER-positive BT474 cells, 1α(OH)D$_3$ treatment caused consistent and significant inhibition in cell proliferation in a time-dependent manner.

Further analysis of vitamin D direct target gene CYP24 by real-time RT–PCR (Figure 1B) demonstrated dramatic induction of CYP24 transcription (~1.2 × 10$^9$-fold higher compared with its basal level) in MDA-MB231 (S30) cells and compared them with ER-positive BT474 breast cancer cells. As shown in Figure 1A, 2, 5, and 7 day treatment with 0.5 μM 1α(OH)D$_3$ caused differential response in both MDA-MB231 and S30 cells. There was a consistent minor inhibition in cell proliferation after 7 day treatment with vitamin D in both cell lines. In ER-positive BT474 cells, 1α(OH)D$_3$ treatment caused consistent and significant inhibition in cell proliferation in a time-dependent manner.

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BT474 cells and only moderate induction of CYP24 in both MDA-MB231 and S30 cells (7.5- and 26-fold higher compared with their basal levels). S30 cells expressed slightly lower basal CYP24 than that of MDA-MB231 cells. 1α(OH)D₃ treatment induced CYP24 by ~2-fold in S30 cells in comparison with that of parental MDA-MB231 cells, suggesting that ER expression in ER-negative breast cancer cells sensitized the cells to VDR direct target gene response. BT474 cells expressed extremely low basal level of CYP24 transcripts; whereas MDA-MB231 and S30 cells expressed relative high basal level of CYP24 (25- to 32-fold of BT474 basal level). Western blot analysis demonstrated that 1α(OH)D₃ treatment increases VDR mRNA expression at 8–20 h (data not shown), therefore the increased VDR protein expression after 1α(OH)D₃ treatment could be due to protein stabilization after ligand-VDR binding as reported previously (18) and increased VDR transcriptional level at early time points. In comparison with BT474 cells, both MDA-MB231 and S30 cells expressed low basal level of VDR at mRNA and protein levels.

Table I. Cell cycle analysis of MDA-MB231, S30 and BT474 cells following 1α(OH)D₃ treatment

<table>
<thead>
<tr>
<th>Cell line and treatment</th>
<th>Duration (days)</th>
<th>Cell cycle distribution</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>% G₁</td>
<td>% S</td>
</tr>
<tr>
<td>MDA-MB231</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
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</tr>
<tr>
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</tr>
<tr>
<td>S30</td>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>1α(OH)D₃</td>
<td>3</td>
<td>85.2</td>
</tr>
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Cells during exponential phase of growth were treated for 1, 3 and 6 days with 1 μM of 1α(OH)D₃. Data presented are mean of two independent experiments with each sample in duplicate wells per experiment.

Fig. 2. VDR expression in breast cancer cells after treatment with 1α(OH)D₃. (A) Real-time RT-PCR analysis of VDR mRNA level. Cells were treated for 24 h. VDR mRNA basal level in the control sample of BT474 cells was set as 100 after normalized to β-actin. VDR mRNA level in each sample was normalized to β-actin and the basal level of BT474 cells. Data are expressed as a mean ± SEM of three independent experiments with duplicate analyses of each treatment. (B) VDR protein expression in breast cancer cells after 48 h treatment with 1α(OH)D₃. Same membrane was reprobed with β-actin that served as a loading control.
ERα. 1α(OH)D₃ treatment decreased PgR mRNA expression by 69%. Immunohistochemistry analysis demonstrated the same pattern of PgR protein expression after 1α(OH)D₃ treatment (data not shown). These data confirm that MDA-MB231 cells are ERα- and PgR-negative, S30 cells are PgR negative even with high expression of ERα, whereas BT474 cells are ERα- and PgR-positive and their expressions were downregulated by 1α(OH)D₃ treatment.

Expression of nuclear receptor cofactors in MDA-MB231, S30 and BT474 cells
Nuclear receptor co-repressor (CoR) NCoR1 and co-activator (CoA) SRC1 play an important role in VDR function, we therefore assessed their transcriptional level using real-time RT–PCR. Both MDA-MB231 and S30 cells expressed NCoR1 mRNA at similar level, which is lower (by 40–50%) than that of BT474 cells (Figure 4A). SRC1 mRNA basal level in S30 cells was about half of that in MDA-MB231 cells, but both cell lines expressed reduced SRC1 mRNA by 40–75% in comparison with that of BT474 cells (Figure 4B). 1α(OH)D₃ treatment did not have significant effect on the transcriptional expression of these nuclear receptor cofactors in all three cell lines regardless of their differential sensitivity to 1α(OH)D₃ (Figure 4).

Transfection of VDR in MDA-MB231 cells fails to restore sensitivity to 1α(OH)D₃
Since VDR is the key molecule involved in the antiproliferative effect of 1α(OH)D₃ and VDR expressed at low level in both MDA-MB231 and S30 cells that are resistant to 1α(OH)D₃ treatment, we transfected MDA-MB231 cells with VDR expression vector to restore the VDR level to test if VDR overexpression sensitize the cells for the antiproliferative effect of 1α(OH)D₃. We established two cell lines: MB231-vector as a control cell line that was stably transfected with an empty vector and MB231-VDR that stably overexpressed VDR (Figure 5A and B). The VDR transcriptional level in MB231-VDR cells is comparable with that of BT474 cells. The basal transcriptional level of VDR in MB231-VDR cells is higher than that of parental MDA-MB231 cells, probably due to G418 selection. The basal transcriptional level of VDR in MB231-VDR cells is ~10-fold higher than that of parental MDA-MB231 cells, and 1α(OH)D₃ treatment increased the transcriptional VDR expression by ~20% in both parental MDA-MB231 and MB231-VDR cells (Figure 5A). At protein level, MB231-VDR cells expressed significant higher level of VDR than the control cell line MB231-vector, but the VDR protein level was still lower than that of BT474 cells (Figure 5B) regardless of the comparable transcriptional VDR level in MB231-VDR and BT474 cells.
Further characterization demonstrated that VDR direct target gene CYP24 was more responsive to 1\(\alpha\)(OH)\(_D\)\(_3\) treatment in MB231\(_{VDR}\) cells than that in parental MDA-MB231 cells and control MB231\(_{vector}\) cells (Figure 5C), which is correlated to its increased VDR expression level. Cell sensitivity for the antiproliferative effect of 1\(\alpha\)(OH)\(_D\)\(_3\) was determined by using MTT assay and the results demonstrated that MB231\(_{VDR}\) cells were still resistant to 1\(\alpha\)(OH)\(_D\)\(_3\) (Figure 5D), which is similar to that of parental MDA-MB231 cells, suggesting that the functional VDR was uncoupled with its functional antiproliferative pathway in this cell line.

**Transient transfection of VDR in S30 cells failed to restore sensitivity to 1\(\alpha\)(OH)\(_D\)\(_3\)**

Since VDR transfection did not restore the sensitivity to 1\(\alpha\)(OH)\(_D\)\(_3\) in MDA-MB231 cells, it was considered possible that both VDR and ER may be required to respond to vitamin D. We therefore transiently transfected S30 cells with VDR expression vector to restore the VDR level to test if VDR overexpression sensitizes the cells for the antiproliferative effect of 1\(\alpha\)(OH)\(_D\)\(_3\). As shown in Figure 6A, transient transfection of VDR (S30\(_{VDR}\)) caused extremely high level of VDR transcription as examined by quantitative RT–PCR, but the VDR protein expression was still not as high as in BT474 cells, although it is much higher than that of control vector-transfected S30 cells (Figure 6B). Similar to that of MB231\(_{VDR}\) cells, further characterization demonstrated that VDR direct target gene CYP24 was more responsive to 1\(\alpha\)(OH)\(_D\)\(_3\) treatment in S30\(_{VDR}\) cells than that in control S30\(_{vector}\) cells (Figure 6C) that is correlated to its increased VDR expression level. BrdU incorporation assay did not show increased sensitivity of VDR-transfected S30 cells to both 1\(\alpha\)(OH)\(_D\)\(_3\) and 1,25(OH)\(_2\)D\(_3\) for their antiproliferative effects after 48 h treatment (Figure 6D). This result was confirmed using MTT and crystal violet assays after 4 and 6 day treatment with 1\(\alpha\)(OH)\(_D\)\(_3\) and 1,25(OH)\(_2\)D\(_3\) (data not shown).

**Discussion**

Breast cancer is generally characterized by estrogen-dependent growth, and the proliferative actions of estrogen are mediated via ER. Hence, presence or absence of ER is critical to the cell and forms the basis for its classification. The mechanism of action of ER is similar to other nuclear receptors. The binding of ligand induces an activating conformational change in the ER, leading to homodimerization and binding to specific DNA response elements (ERE) within the regulatory regions of the target gene (19). ER-positive cells are usually well differentiated and represent a phenotype that is more amenable to treatment. Some studies have suggested a cross talk between 1,25(OH)\(_2\)D\(_3\) and estrogen-signaling pathway (6,7). In ER-positive breast cancer cells such as MCF-7 cells, the antitumor effects of 1,25(OH)\(_2\)D\(_3\) may be secondary to disruption of estrogen-mediated survival signals (20). One way to understand the role of ER and vitamin D function is to introduce ER into VDR-negative breast cancer cells and evaluate the role of vitamin D on cell proliferation. Thus, the current study is an attempt to address the role of ER in VDR signaling by using ER-negative cells overexpressing exogenous functional ER and VDR and determining the antiproliferative effects of 1\(\alpha\)(OH)\(_D\)\(_3\).

In our previous studies, we have consistently observed similar effects of both 1\(\alpha\)(OH)\(_D\)\(_3\) and 1,25(OH)\(_2\)D\(_3\) except that 1\(\alpha\)(OH)\(_D\)\(_3\) needs higher dose to reach similar effects of 1,25(OH)\(_2\)D\(_3\) (5,13,21); this study mainly focused on cellular response to 1\(\alpha\)(OH)\(_D\)\(_3\).

We first compared the differential sensitivity of three breast cancer cell lines in response to 1\(\alpha\)(OH)\(_D\)\(_3\): ER-negative MDA-MB231, MDA-MB231-expressing functional ER (S30) and ER-positive BT474 cells. Both MTT assay and cell cycle analysis consistently demonstrated that MDA-MB231 and S30 cells were resistant to the antiproliferative effect of 1\(\alpha\)(OH)\(_D\)\(_3\) although the primary VDR target gene CYP24 was more responsive in S30 cells, while BT474 cells were most sensitive to 1\(\alpha\)(OH)\(_D\)\(_3\) treatment among the three cell lines. S30 cell line was initially established by V.C.Jordan’s group to examine the mechanism...
overexpression of ER and VDR in MDA-MB231 and vitamin D response

Fig. 6. Characterization of S30 cells after transiently transfected with VDR. (A) Real-time RT–PCR analysis of VDR mRNA in control vector (pcDNA3.1)-transfected S30 (S30vector) and VDR expression vector-transfected S30 (S30VDR) cells. Twenty-four hours after transfection, cells were trypsinized and resuspended in culture dishes. After overnight incubation, cells were treated for 24 h with 1α(OH)D3. VDR mRNA level in each sample was normalized to β-actin and the basal VDR level of S30vector cells (VDR basal level in S30vector cells was set as 1). Data are expressed as the mean value of duplicate analyses of two independent experiments with two RT reactions from each sample. (B) VDR protein expression in S30vector and S30VDR cells after 48 h treatment with 1α(OH)D3. BT474 cells were included as a control, β-actin served as a loading control. (C) Real-time RT–PCR analysis of CYP24 mRNA expression in the same samples as described in (A). The magnitude of induction of CYP24 transactivation by 1α(OH)D3 in S30vector cells was significantly higher than that in S30vector cells. CYP24 mRNA level in each sample was normalized to β-actin and its own basal level. (D) Effect of 1α(OH)D3 on cell proliferation in VDR-transfected S30 cells. Transfected S30 cells were treated with 500 nM 1α(OH)D3 and 10 nM 1,25(OH)2D3 for 2 days and subjected to BrdU incorporation assay. Data are expressed as the mean of absorbance ± SD of 12 wells per treatment. Data shown are representative of two independent experiments.

of action of estrogen and antioestrogen (15). Even though S30 cells regained hormonal responsiveness, estrogen inhibited rather than stimulated cell growth in S30 cells (15). Similarly, a short 2 day treatment of S30 cells with 1α(OH)D3 stimulated cell proliferation rather than inhibited as showed by MTT assay (Figure 1A). Further analysis demonstrated that both MDA-MB231 and S30 cells expressed low level of VDR in comparison with ER-positive BT474 cells. On the other hand, in BT474 cells, 1α(OH)D3 treatment significantly reduced the ER and PgR expression. Consistent with our results, Swami et al. (7) also reported that 1α(OH)D3 treatment downregulated ER at transcriptional level in breast cancer cells, resulting in suppression of estrogen-induced cell proliferation. One of the estrogen-inducible genes is PgR; because 1α(OH)D3 has been observed to have some action as PgR modulator (22), 1α(OH)D3 might either decrease PgR via downregulating ER expression or directly act as a PgR modulator. These results support the concept that in ER-positive and vitamin D-sensitive breast cancer cells, the antioestrogenic effects of vitamin D may be in part due to vitamin D-mediated downregulation of ER and PgR. In ER-negative cells, there must have been significant alteration for ER signalling, as a result, when functional exogenous ER was introduced, estrogen inhibits cell proliferation in S30 cells instead of enhancing it (15). This may be also a reason that the antioestrogenic effect of 1α(OH)D3 in S30 cells is not observed. It appears that the presence of intact ER and estrogen-signal cascade contribute to the vitamin D sensitivity in ER-positive breast cancer cells such as BT474. One may argue that the low level of VDR expression could partially account for the resistance to vitamin D, but overexpressing VDR in both MDA-MB231 and S30 cells also failed to restore the sensitivity to 1α(OH)D3, although CYP24 was more responsive in the VDR-transfected cells, suggesting that a significant alteration of VDR signaling must have occurred in these ER-negative, low VDR-expressing MDA-MB231 cells. Although VDR transfection greatly increased VDR mRNA expression (Figures 5A and 6A) that is comparable with or higher (such as in S30vector) than the level in BT474 cells, VDR protein expression was not as high as in BT474 cells, indicating that the translation machinery might be ‘inert’ in MDA-MB231 and S30 cells, which could also contribute to the insensitivity of these cells. It should be pointed out that BT474 cells express higher level of VDR in comparison with other ER-positive cell lines such as MCF-7 and T47D (data not shown). In addition, treatment with vitamin D caused significant increase of functional VDR protein (Figure 2B), although MDA-MB231 and S30 cells were still resistant. This suggests that VDR protein level might not be the key factor for vitamin D resistance in these cell lines. This has been previously reported in vitamin D-resistant MCF-7 variant, where VDR protein level was comparable with sensitive parental MCF-7 cells, but VDR was uncoupled from a functional apoptotic pathway (10).

The VDR introduction to MDA-MB231 (MB231VDR) cells is similar to ER introduction to MDA-MB231 cells (S30). In both cell types (S30 and MB231VDR), the respective target genes TGFα (for ER) and CYP24 (for VDR) were more responsive to estradiol (23) and 1α(OH)D3, respectively, (Figure 5C). However, the signaling pathways related to their cell proliferation in response to these ligands were not restored. Similar results have also been reported for ERβ introduction to MDA-MB231 cells; the ERβ transfection did not cause significant cell growth regulation when treated with estrogen (23). These results may suggest that there is a profound signaling alteration of nuclear receptor-mediated proliferation in ER-negative MDA-MB231 cells in comparison with ER-positive breast cancer cells such as MCF-7 and BT474 cells. Restoration of certain nuclear receptor in MDA-MB231 cells may only partially restore the responsiveness of its primary target genes and be unable to fully restore the cell proliferation regulatory function of the steroid receptor in response to the respective
lignan. Regulation of cell proliferation is much more complex as compared with single target gene regulation; treatment of ER-negative breast cancer might need strategy other than restoring single nuclear receptor expression. It has been recently reported that cotreatment of MDA-MB231 cells with histone deacetylrylation inhibitor trichostatin A dramatically increased the sensitivity to the antiproliferative effect of 1,25(OH)2D3 (24). Consistently, our recent data also showed that cotreatment of MDA-MB231 cells with trichostatin A significantly enhanced the antiproliferative effect induced by 1α(OH)D3 (data not shown). These data suggest that silencing of multiple genes related to proliferation regulation through histone deacetylation-mediated chromatin condensation could compose at least part of the mechanism underlying the resistance to vitamin D in MDA-MB231 cells.

In exploring the insensitivity of MDA-MB231 and S30 cells, we further compared nuclear cofactors (CoRs and CoAs) that potentially affect VDR function in these three cell lines. CoR proteins, such as NCoR, SMRT and Alien, link non-liganded, DNA-bound VDR–RXR heterodimers to enzymes with histone deacetylase activity that cause chromatin condensation (25). This gives VDR intrinsic repression properties comparable with retinoic acid and thyroid hormone receptors. CoA proteins such as SRC1, TIF and RAC3 (26), link the ligand-activated VDR to enzymes displaying histone acetyltransferase activity that cause chromatin opening. The conformational change within VDR’s ligand-binding domain after binding of 1,25(OH)2D3 or its analog results in replacing a CoR by a CoA protein. Ligand-activated VDR–RXR heterodimers fulfill two tasks, opening chromatin and activating transcription (27). We initially hypothesized that BT474 cells would express low level of CoR and high level of CoA at transcriptional level. Elevated NCoR1 has been reported to attenuate VDR signaling in breast cancer cells (28), whereas SRC1 increases VDR–DNA binding and enhances function (29,30). To our surprise, MDA-MB231 and S30 cells expressed lower transcriptional levels of both NCoR1 and SRC1 in comparison with that of BT474 cells, whereas S30 cells expressed lowest SRC1 among the three cell lines. Whether the low level of NCoR1 in the two cell lines is correlated to its higher basal level of CYP24 still requires further investigation; it is possible that high level of CoR and CoA in BT474 can help to tightly and precisely control the CYP24 transcription. The magnitude of CYP24 transcription induced by 1α(OH)D3 in BT474 cells is much greater than that in MDA-MB231, S30, MB231VDR, and S30VDR cells. The high level of NCoR1 in BT474 and the cellular responsiveness to 1α(OH)D3 are in contrast to a proposed model whereby elevated CoR levels lead to epigenetic silencing of the transcriptional responsiveness of key antiproliferative target genes and insensitivity to vitamin D (24,28). This suggests another possible biological significance of CoRs—right control of transactivation mediated by nuclear receptor ligands; further investigation is needed to address this issue.

In summary, loss of ER in breast cancer cells appear to be irreversible, there is profound alteration in proliferation signaling mediated by ER and VDR, since introduction of exogenous ER and VDR in ER-negative breast cancer cells failed to restore the sensitivity to the antiproliferative effect of 1α(OH)D3. Endogenous VDR, although expressed at lower basal level, is functional in both MDA-MB231 cells and S30 cells, since VDR direct target gene CYP24 was responsive to 1α(OH)D3 treatment. Low levels of NCoR1 and SRC1 were observed in MDA-MB231 and S30 cells in comparison with BT474 cells; these proteins could also contribute to the cellular sensitivity to vitamin D treatment in these cells. These studies further our understanding on vitamin D resistance in ER-negative breast cancer cells and the biological significance of nuclear receptor CoRs in ER-positive breast cancer cells in response to 1α(OH)D3.

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References


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