Resistance of HBL100 human breast epithelial cells to vitamin D action

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Vitamin D analogs are effective inhibitors of breast cancer cell growth, but many breast cancer cell lines show various degrees of resistance to the growth inhibitory effect of vitamin D. In this study, we investigated the mechanism of vitamin D3 receptor (VDR) in HBL100 and a vitamin D-sensitive ZR75-1 breast cancer cell line. We determined the expression, DNA binding and transcriptional activity of vitamin D3 receptor in HBL100 cells. At 120 ng VDR concentration, the inhibition was completely reversed. Thus the loss of the growth inhibitory effect of vitamin D3 in HBL100 cells may be caused by the expression of the large T antigen in the cells, and provide further evidence that VDR is required for efficient growth inhibition by vitamin D3.

Introduction

Vitamin D and analogs are known to inhibit the proliferation of cultured breast cancer cells and to cause regression of experimental mammary tumors in vivo, due to their ability to induce differentiation and/or apoptosis of breast epithelial cells (1–6). The molecular mechanism by which vitamin D and its analogs inhibit the growth of cancer cells is currently the subject of intensive research. There is evidence that the hormonally active form of vitamin D, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], can generate biological responses through its transcriptional regulation of genes involved in cell growth and differentiation. They include transforming growth factor β (7), epidermal growth factor receptor (8), c-myc (9), insulin-like growth factor-binding protein (10), cell cycle regulators p21 and p27 (11) and cell survival factor Bcl-2 (12). Transcriptional activity of 1,25(OH)2D3 is mainly mediated by vitamin D receptors (VDR) expressed in target cells (6). VDR belong to a large superfamily of steroid/thyroid receptors that function as ligand-dependent transcriptional factors by binding to a set of specific DNA sequences on target genes (13–15). Vitamin D response elements (VDRE) have been identified in several genes and consist of a direct repeat of the hexanucleotide sequences (GGGTGA) separated by a 3 nucleotide spacer (16,17). VDR bind to VDRE either as homodimers or as heterodimers with retinoid X receptor (RXR), i.e. VDR/RXR (18). Transcriptional activation by nuclear hormone receptors requires their interaction with co-activators that appear to provide a direct link of nuclear receptors to the core transcriptional machinery and to modulate chromatin structure (19–27). Several receptor co-activators have been identified so far. One of them, the CREB-binding protein (CBP), functions not only as nuclear receptor co-activator but also as a co-activator of many other transcriptional factors (19–27). Results from previous studies have demonstrated a general correlation between the levels of VDR present and the degree of differentiation of breast cancer cells (6,28,29). In addition, loss of VDR may be responsible for decreased anti-proliferative effect of vitamin D3 in cancer cells (6,28,29). Moreover, VDR polymorphism has been shown to be associated with increased incidence of a variety of diseases (30). Thus, VDR can mediate the biological action of vitamin D3 and may be involved in cancer development.

Although a functional VDR is necessary for the growth regulatory effect of vitamin D3, 1,25(OH)2D3 does not always show growth inhibitory effect on human breast cancer cell lines, despite their expression of VDR (6,31). Typically, both ER+ (MCF-7 and ZR75–1) and ER− breast cancer cell lines (MCF-10neo, BCA-4, SK-BR-3) show growth inhibitory response to vitamin D analogs. Yet some breast cancer cell lines have shown resistance to vitamin D analogs regardless of their steroid receptor status. These include MDA-MB-231, MDA-MB-436, MCF-7-D-resistant and HBL100 cells (6,32,33). The mechanisms by which resistance to vitamin D3 occurs are largely unknown. One possibility may be a non-functional state of VDR in certain cells.

In the present study we have focused on evaluating the mechanism of resistance to the active metabolite of vitamin D, 1,25(OH)2D3, in HBL100 cells. The human breast epithelial
cell line HBL100, originally derived from the milk secretion of a nursing mother without detectable breast lesion, has been extensively characterized as immortal but not tumorigenic in nude mice due to the presence of Simian virus 40 (SV40), large T antigen (34). The SV40 large T antigen is known to contribute to virus-induced tumorigenesis by interacting with and altering the function of key cellular regulatory proteins, such as members of the retinoblastoma gene family (35), members of the CBP family of transcriptional co-activator proteins (36,37) and p53 tumor suppressor (38,39). Unlike many other breast cancer cell lines, HBL100 cells do not show a clear response to the growth inhibitory effect of 1,25(OH)2D3. In addition to HBL100 cells, we selected ER+, ZR75-1 breast cancer cells as a positive control to establish clear resistance to effects of 1,25-(OH)2D3 in HBL100 cells. Our results demonstrate that 1,25(OH)2D3 resistance in HBL100 cells may be attributed to the expression of SV40 large T antigen in these cells and provide evidence for the role of functional VDR in mediating growth inhibition by 1,25(OH)2D3.

Materials and methods

Cell culture

The breast epithelial cell lines HBL100 and ZR75-1 were obtained from the American Type Culture Collection (Rockville, MD). HBL100 cells were maintained in minimal essential medium with Earl’s salts (MEME) medium supplemented with 10% fetal bovine serum (FBS). The ZR75-1 cells were maintained in RPMI 1640 supplemented with 10% FBS.

Growth inhibition assay

For growth inhibition studies, cells were seeded in 96-well plates at a density of 1000 cells/well, in 100 µl/well in a medium containing 10% steroid-stripped serum. After 24 h, cells were incubated with 1 and 10 nM concentrations of 1,25(OH)2D3. An aliquot of 50 µl of medium with fresh 1,25(OH)2D3 was added to each well after 3 days of culture. The viable cell number was determined after 7 days of cultures using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation/cytotoxicity assay kit (Promega, Madison, WI). The assay measures the capacity of cells to convert a tetrazolium salt to a blue formazan (40). Results obtained were confirmed by cell counting with a hemocytometer.

Western blot analysis

Cells were lysed in a buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 1 µg/ml each PMSF, aproteinin, leupeptin and pepstatin. Equal amounts of lysates were boiled in sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using NOVEX precast 4–20% gels. After transfer, the nitrocellulose membrane was blocked with 1% milk TBST [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20], and subsequently incubated for 2 h at room temperature in TBST containing anti-VDR polyclonal antibody (Biogexen, San Ramons, CA). The membrane was then washed with TBST and incubated for 1 h at room temperature in TBST containing horseradish peroxidase-linked anti-immunoglobulin. After three washes with TBST the protein was detected with an enhanced chemiluminescence.

Preparation of nuclear protein extracts and gel retardation assay

Nuclear extracts were prepared essentially according to the method described previously (41). Briefly, following the vitamin D treatment HBL100 and ZR75-1 cells were washed with ice-cold phosphate-buffered saline and collected using a rubber policeman. The cells were pelleted by low-speed centrifugation and washed with buffer B containing 10 mM HEPES–KOH (pH 7.9), 1.5 mM MgCl2, 0.2 mM EDTA and 0.5 mM DTT. Nuclear proteins were extracted with 200 µl of high salt buffer containing 10 mM HEPES–KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA and 0.5 mM DTT. All buffers contained protease inhibitors PMSF (100 µg/ml), leupeptin (1 µg/ml) and aproteinin (1 µg/ml). This method was used to construct VDR expression vector, cDNA for the VDR gene was cloned into the pRc/CMV expression vector (Invitrogen, San Diego, CA) as described previously (42). The resulting recombinant construct was then stably transfected into HBL100 cells by the calcium phosphate precipitation method and screened with G418 (0.5 mg/ml) (Gibco BRL, Grand Island, NY).

Results

We investigated the growth inhibitory effect of 1,25(OH)2D3 on HBL100 cells by the MTT assay. The reporter VDRE-tk-CA T was obtained by inserting one copy of VDRE oligonucleotide into the BamHI site of the pBlCAT2 as described previously (43). For transient transfection assay in HBL100 and ZR75-1 cells, 5×103 cells were seeded in six-well culture plates. A modified calcium phosphate precipitation procedure was used for transfection as described previously (43). Briefly, 250 ng of VDRE-tk-CAT reporter plasmid, 250 ng of β-galactosidase (β-gal) expression vector (pHL110; Pharmacia, Piscataway, NJ) and various amounts of VDR and/or RXRα expression vector were mixed with carrier DNA (pBluescript) to 2.5 µg of total DNA per well. For CV-1 cells, 1×105 cells/well were seeded in 24-well plates. For transfection, 100 ng of reporter plasmid, 100 ng of β-gal expression vector and expression vectors for VDR, RXRα and large T antigen were mixed with carrier DNA to 1 µg of total DNA/well. CAT activity was normalized for transfection efficiency by the corresponding β-gal activity. Large T antigen expression vector was constructed by cloning the large T antigen cDNA into a eukaryotic expression vector pCE4 (45).

Stable transfection

To construct VDR expression vector, cDNA for the VDR gene was cloned into the pRc/CMV expression vector (Invitrogen, San Diego, CA) as described previously (42). The resulting recombinant construct was then stably transfected into HBL100 cells by the calcium phosphate precipitation method and screened with G418 (0.5 mg/ml) (Gibco BRL, Grand Island, NY).
Analysis of vitamin D resistance in HBL100 cells

Fig. 2. Expression of VDR in HBL100 and ZR75-1 cells. Cell extracts were prepared from HBL100 and ZR75-1 cells treated with or without 10 nM 1,25(OH)2D3 for 24 h and analyzed for the expression of VDR by western blot analysis using a polyclonal anti-VDR antibody. The results shown are from one of the two independent experiments.

Fig. 3. VDRE binding of nuclear proteins prepared from HBL100 and ZR75-1 cells. Nuclear proteins were prepared from HBL100 and ZR75-1 cells untreated or treated with 10 nM 1,25(OH)2D3 for 24 h and analyzed for their DNA binding activity using VDRE as a probe. For comparison, the binding of VDR/RXRα heterodimers using in vitro synthesized receptor proteins is shown. Arrow indicates the specific VDRE binding complex. The results of the gel shift shown here are from duplicate reactions of the two independent experiments. Relative binding activity was determined after densitometric analysis. Binding of nuclear proteins prepared from HBL100 cells in the absence of 1,25(OH)2D3 was defined as 1. HBL100 cells treated with dihydroxyvitamin D3 showed relative intensities of 1 and 3; whereas ZR75-1 cells treated with dihydroxyvitamin D3 showed the relative binding values of 9 and 12.

functional, we investigated VDRE-binding activity of nuclear proteins prepared from untreated or vitamin D3-treated HBL100 and ZR75-1 cells. For comparison, in vitro synthesized VDR and RXRα proteins were used. Nuclear proteins from ZR75-1 cells formed strong complexes with the VDRE; the binding was further enhanced by 9-fold when ZR75-1 cells were treated with 1,25(OH)2D3. In contrast, nuclear proteins of untreated HBL100 cells did not show any detectable binding complex on VDRE and only a weak complex was formed when HBL100 cells were treated with 1,25(OH)2D3 (Figure 3). The VDRE transactivation activity in both HBL100 and ZR75-1 cell lines was determined by the use of CAT reporter gene containing VDRE (VDRE-tk-CAT). When the reporter gene was transfected into HBL100 cells, transcription of the reporter gene was barely induced when cells were treated with either 1 nM or 10 nM of 1,25(OH)2D3. However, ~10-fold increase of reporter activity was observed in ZR75-1 cells when the cells were treated with 1 or 10 nM of 1,25(OH)2D3 (Figure 4). The results indicating that VDR could not bind and transactivate VDRE reporter in HBL100 cells (Figures 3 and 4) suggest that the loss of these activities may be due to low expression levels of VDR or RXRα. To confirm this hypothesis, we co-transfected VDR and/or RXRα expression vectors together with the reporter gene into HBL100 and ZR75-1 cells. As shown in Figure 5, co-transfection of 100 ng of RXRα expression vector did not show any effect on the reporter activity in HBL100 cells either in the absence or presence of 1,25(OH)2D3. In contrast, co-transfection of 100 ng of VDR expression vector strongly induced the reporter activity by 7- to 8-fold in response to 1,25(OH)2D3. The addition of RXRα did not show any enhancing effect on co-transfected VDR activity. These data suggest that loss of VDRE transactivation activity in HBL100 cells is probably due to a low level of functional VDR. When the effect of transfected VDR or RXRα was evaluated in ZR75-1 cells, we did not observe any effect of RXRα and only a slight induction...
Fig. 6. Stable expression of VDR restores 1,25(OH)₂D₃ sensitivity in HBL100 cells. VDR was stably transfected into HBL100 cells. A stable clone (HBL100/VDR) that expressed exogenous VDR was analyzed for the growth inhibitory effect of 1,25(OH)₂D₃. Growth inhibition assay and cell viability were performed as discussed in the legends for Figure 1. Data shown are representative of at least three independent experiments (± SD).

Fig. 7. Effect of the expression of SV40 large T antigen and VDR on VDRE transcriptional activity in CV-1 cells. (A) Inhibition of 1,25(OH)₂D₃-induced VDRE activity by large T antigen. The VDRE-tk-CAT was transiently transfected into CV-1 cells with or without VDR (20 ng) and RXRα (10 ng) in the presence of the indicated amount of large T antigen. (B) Reversal of the inhibitory effect of large T antigen by co-transfection of VDR. The VDRE-tk-CAT was transiently transfected into CV-1 cells together with the indicated amounts of VDR (20 ng), RXRα (10 ng) and large T antigen (50 ng) expression vectors. The cells were then treated with 1,25(OH)₂D₃ and assayed for CAT as described in the legends for Figures 4 and 5. Results represent means ± SD. The experiment was repeated twice.

Discussion
In this study, we investigated the possible mechanism of 1,25(OH)₂D₃ resistance of HBL100 cells to the growth inhibitory effect of 1,25(OH)₂D₃. Our results demonstrated that VDR, although well expressed in HBL100 cells, could not effectively bind and transactivate the VDRE. Furthermore, our data suggest that the loss of VDR function in HBL100 cells may be due to the presence of SV40 large T antigen. Western blot analysis indicates that the expression level of the VDR gene in HBL100 cells was comparable to that in 1,25(OH)₂D₃-sensitive ZR75-1 cells. In addition, the level of overexpression of VDR in HBL100 cells could restore growth inhibition by 1,25(OH)₂D₃.
VDR could be up-regulated by 1,25(OH)₂D₃ similar to that observed in ZR75-1 cells (Figure 2). However, VDR expressed in ZR75-1 cells effectively bound to and transactivated a VDRE, whereas the VDRE-binding and transactivation activities were impaired in HBL100 cells (Figures 3 and 4). Our observation that 1,25(OH)₂D₃ could induce VDR expression in HBL100 cells (Figure 2) suggests that certain aspects of VDR function are normal. Thus, it is likely that the growth inhibitory effect of VDR is specifically diminished in HBL100 cells due to the non-functional state of VDR in these cells. Previous studies have also demonstrated a partial vitamin D₃ resistance in MCF-7 breast cancer (31) and leukemia cell lines (45). In both cases, cells selected for resistance to growth inhibition by 1,25(OH)₂D₃ express VDR. In the case of the leukemia cells, induction of 24-hydroxylase by 1,25(OH)₂D₃ is retained (45), whereas up-regulation of VDR by vitamin D₃ occurs in vitamin D₃-resistant MCF-7 cells (31). Thus, different mechanisms may be utilized by VDR to regulate genes involved in growth control and genes involved in other functions, and they appear to be dissociated. Such dissociated biological functions and the underlying regulatory mechanisms are supported by our recent identification of a novel vitamin D₃ analog, 1α(OH)D₃, that exerts prominent anti-proliferative effect against cancer cells, but has reduced effect on serum calcium levels (46). Such an analog may induce VDR conformation, which only allows regulation of genes involved in growth inhibition. Thus, our study provides another piece of evidence supporting the concept that the anti-proliferative effect of VDR can be separated from other VDR-mediated functions, such as the hypercalcemic effect. Similar dissociation of the anti-proliferative effect from other hormone-dependent functions also occurs for vitamin A receptor in which its anti-proliferative activity can be separated from its receptor transactivation function (44).

Our observation that VDR expressed in HBL100 cells could not bind (Figure 3) and transactivate (Figure 4) the VDRE suggests that VDR function is altered in HBL100 cells. Efficient DNA binding and transactivation of VDR requires heterodimerization of VDR with RXRα (13–15). Based on our observation that co-transfection of VDR, but not RXR, in HBL100 cells could restore 1,25(OH)₂D₃ transactivation activity (Figure 5), the loss of VDRE transactivation and growth inhibition by 1,25(OH)₂D₃ is unlikely to be due to a low level of RXR, but likely to be due to a low level of functional VDR. However, the western blot analysis (Figure 2) shows a comparable VDR expression level in both HBL100 and 1,25(OH)₂D₃-sensitive ZR75-1 cell lines. This observation suggests that loss of VDR function may be attributed to either a mutation in VDR gene or expression of a VDR inhibitor in HBL100 cells. Such a mutation in VDR, however, may only affect its binding and transactivation of the VDRE as well as its regulation of genes involved in growth control, whereas the mutation does not alter its ability to up-regulate VDR expression.

Whether HBL100 cells express a mutated VDR remains to be elucidated. We have investigated the possibility that a VDR inhibitor is present in the cells. HBL100 cells are well known as immortal due to the presence of functional SV40 large T antigen (36). It is, therefore, likely that the large T antigen may also play a role in the development of 1,25(OH)₂D₃ resistance. A previous study has shown that stable expression of SV40 large T antigen in the brown adipocyte cell line is responsible for insulin resistance, without affecting insulin receptor expression (47). When we investigated the effect of large T antigen on VDR function, we observed that VDR-mediated VDRE transactivation was strongly inhibited by large T antigen in a concentration dependent manner (Figure 7A). Recent studies have demonstrated that the large T antigen may contribute to virus-induced tumorigenesis by targeting key cellular regulatory proteins, such as three members of the retinoblastoma family, pRb, p107 and p130, the tumor suppressor p53 and members of the CBP family of transcriptional adapter proteins (37–41). While interaction with pRb and p53 may render cells resistant to the anti-proliferative effect of 1,25(OH)₂D₃, interaction with CBP could directly affect VDR DNA binding and transactivation. CBP is known to act as a co-activator of a number of nuclear hormone receptors and is required for their efficient DNA binding and transactivation (36,37). Thus, it is conceivable that inhibition of VDR transactivation activity by large T antigen (Figure 7A) may be due to its interaction with CBP, resulting in decreased availability of CBP for VDR to bind and activate the VDRE. Alternatively, large T antigen may directly interact with VDR and inhibit its DNA-binding and transactivation activity. The fact that co-transfection of additional VDR could abolish the inhibitory effect of large T antigen (Figure 7B) suggests that the inhibitory effect of the large T antigen may be reversible or that the level of the large T antigen was limiting. This is also consistent with the stable transfection results, which showed that over-expression of VDR could restore growth inhibition by 1,25(OH)₂D₃ (Figure 6). Interestingly, the inhibitory effect of the large T antigen could be also observed with retinoic acid receptor (data not shown), indicating that the large T antigen could be a general inhibitor of a number of nuclear receptors.

Our observation that stable expression of VDR restored 1,25(OH)₂D₃ sensitivity in HBL100 cells provides a possibility for overcoming the eventual viral block or possible VDR mutation by transfecting a functional VDR into HBL100 cells. The finding also provides a piece of direct evidence to support the concept that the growth inhibitory effect of 1,25(OH)₂D₃ is in part mediated by the VDR. It remains to be determined if the transfected VDR activates alternative pathways to induce growth inhibition through bypassing the cell cycle checkpoints blocked by the virus. Alternatively, it could be hypothesized that stable expression of VDR may be able to overcome the blocking capacity of the virus by simply increasing the expression levels of the virus-controlled cellular regulatory protein. Our observation that VDR and the large T antigen inhibit each other’s activity suggests that VDR may exert its anti-proliferative effect through its interaction with the large T antigen or similar proteins, thereby enhancing the biological function of p53 and pRb.

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