G2/M Arrest by 1,25 dihydroxyvitamin D3 in Ovarian Cancer Cells Mediated through the Induction of GADD45 via an Exonic Enhancer

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Running title: GADD45 and Vitamin D Induction of G2/M Arrest
Summary

1,25 dihydroxyvitamin D3 suppresses the growth of multiple human cancer cell lines by inhibiting cell cycle progression and inducing cell death. The present study shows that 1,25 dihydroxyvitamin D3 causes cell cycle arrest at the G2/M transition through p53-independent induction of GADD45 in ovarian cancer cells. Detailed analyses have established GADD45 as a primary target gene for 1,25 dihydroxyvitamin D3. A DR3-type vitamin D response element is identified in the fourth exon of GADD45 that forms complex with the vitamin D receptor/retinoid X receptor heterodimer in electrophoresis mobility shift assays and mediates the dose-dependent induction of luciferase activity by 1,25 dihydroxyvitamin D3 in reporter assays. Chromatin immunoprecipitation assays have shown that the vitamin D receptor is recruited in a ligand-dependent manner to the exonic enhancer but not to the GADD45 promoter regions. In ovarian cancer cells expressing GADD45 anti-sense cDNA or GADD45-null mouse embryo fibroblasts, 1,25 dihydroxyvitamin D3 fails to induce G2/M arrest. Taken together, these results identify GADD45 as an important mediator for the tumor suppressing activity of 1,25 dihydroxyvitamin D3 in human ovarian cancer cells.
Introduction

Vitamin D₃ (VD) is a lipophilic hormone essential for normal bone structure and maintenance of serum calcium (1). VD action is mediated through the vitamin D receptor (VDR) (2), a member of the steroid/thyroid receptor superfamily of ligand-regulated transcription factors (3, 4, 5). This superfamily includes receptors for steroids such as progesterone, androgens, estrogens, glucocorticoids and mineralocorticoids, receptors for non-steroid hormones like vitamin D, thyroid hormones, all-trans retinoic acid and 9-cis retinoic acid as well as orphan receptors for which the ligand is unknown. VDR and other non-steroid receptor members of the superfamily form heterodimers with the retinoid X receptor (RXR), the receptor for 9-cis retinoic acid. In most cases, the VDR/RXR heterodimer binds VD response elements (VDREs) to mediate the biological activities of VD through transcriptional activation or repression of target genes containing VDREs in their regulatory sequence. In response to VD activation, the VDR recruits multiple co-activators, including members of the p160 SRC family (6) which are associated with histone acetyl transferase activity and the DRIP complex (7); the latter serves as a mediator between the VDR and RNA polymerase II complex (Pol II).

In addition to its well defined role in calcium homeostasis and bone cell metabolism, the active metabolite of VD, 1,25-Dihydroxyvitamin D₃ (1,25VD), modulates cellular proliferation and differentiation of both normal and malignant cells (8). 1,25VD and its synthetic analogues can inhibit carcinogenesis in mouse skin (9, 10), decrease the size of transplanted sarcomas, reduce lung metastasis in mice (11), suppress the growth of human colonic cancer cell derived xenografts in immune-suppressed mice (12), increase cell differentiation and decrease proliferation of leukemia (13), breast (14) and prostate cancer (15) cells. Studies in cancer cell lines have shown that 1,25VD causes cancer cells to accumulate in the G1 phase of the cell cycle.
(15), in the G2 phase (16) or undergo apoptosis (17, 18). Genes that mediate each of these specific activities remain largely unidentified.

Similar to breast and prostate cancers, ovarian cancer (OCa) mortality and incident rates are lower in countries within 20 degrees of the equator (19) where there is a high amount of sunlight. In US, women between the ages of 45-54 living in the North have 5 times the OCa mortality rate than women living in Southern states (20, 21). In the epidermis, sunlight controls the first step of 1,25VD synthesis, namely, the photoconversion of 7-dehydrocholesterol to pre-Vitamin D₃. Exposure to sunlight, rather than food consumption, is the primary source of 1,25VD (22). The inverse correlation between sunlight exposure and OCa mortality indicates that decreased synthesis of 1,25VD may contribute to OCa initiation and/or progression. VDR has been found in rat ovaries by immuno-histochemistry (23) and in hen ovaries by ligand-binding assays (24), showing that the ovary is a target organ for VD. VDR expression in gynecologic neoplasms, including OCa (25, 26), has also been described, indicating that VD could be an effective agent in OCa treatment and/or chemoprevention.

GADD45α (GADD45) is a DNA damage-induced and p53-regulated gene that plays an essential role in cell cycle and DNA repair (27, 28, 29). In the current study, GADD45 is identified as a primary target gene for 1,25VD in OCa cells. Regulation of GADD45 expression by 1,25VD is p53-independent and mediated through a VDRE localized to the 3’ un-translated region in the fourth exon. Studies with cells in which GADD45 expression is compromised have shown that GADD45 is required for 1,25VD-induced arrest at G2/M. Overall, our studies identify GADD45 as an important mediator of the tumor-suppressing activity of 1,25VD in OCa cells.

Experimental Procedures
Materials

pHG45-HC (30), pCMV45 and pCMVAS45 (31), pCMVgal (32), p91023B-VDR (2, 33), pCMX-RXRβ (34) and p23 containing rat 24-hydroxylase promoter in pMAMMneoLuc (35) have been described previously. pGL3-promoter, pGL3-basic and pGL3-control vectors were from Promega (Madison, WI). Mouse embryo fibroblasts (MEFs) from wild type and GADD45-null mice have been described (36). 1,25D₃ was from Calbiochem (La Jolla, CA). Baculovirus-expressed human VDR protein, human RXRβ protein and anti-RXRβ antibody were from Affinity BioReagents Inc. (Golden, CO). Anti-VDR antibody was from Chemicon International (Temecula, CA). Anti-Flag M2 and anti-β-actin antibodies were from Sigma (St. Louis, MO). Anti-GADD45 (C-20), anti-cdc2 and anti-cyclin B1 (D11) antibodies were from Santa Cruz Biotech (Santa Cruz, CA). All oligonucleotides were synthesized by Invitrogen (Carlsbad, CA). Their sequence is available from W.B. on request.

Cell growth, flow cytometry and statistical analyses

To measure cell growth, OVCAR3 cells were plated in 96-well plates and treated with 1.25VD or ethanol vehicle (ETOH). Colorimetric methylthiazole tetrazolium (MTT) assays were performed as described (37). OD₅₉₅ was read on a MRX microplate reader (DYNEX Technologies, Chantilly, VA). For each data point, eight samples were analyzed and the experiment reproduced three times.

To determine the cell cycle distribution, cells were fixed with 70% ethanol, stained with 50 μg/ml propidium iodide and analyzed on a FACScan (Becton Dickinson, Mountain View, CA). For each data point, duplicate samples were analyzed and the experiment reproduced three times.
For cell growth and cell cycle analyses, statistical analysis was performed using the independent-samples \( t \) test. \( P<0.05 \) was considered to be statistically significant.

**Northern blot analysis and Gel mobility shift assay (EMSA)**

To determine the level of GADD45 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, Northern blot was performed as described (38). EMSA was performed as described (32, 39) with modifications. Briefly, double-stranded oligonucleotides were end-labeled with \(^{32}\)P using a T4 polynucleotide kinase labeling system (Life Technologies, Rockville, MD). 1 \( \mu l \) of radiolabeled probe (roughly 50,000 cpm) was mixed with 19 \( \mu l \) DNA binding reaction mixture that contains 250 ng VDR and 250 ng RXR in 10 mM Tris-Cl (pH7.9), 100 mM KCl, 0.1 mM EDTA, 15% glycerol, 100 \( \mu g/ml \) poly(dI:dC), 0.1 \( \mu g/\mu l \) bovine serum albumin, 1 mM DTT and \( 10^{-7} \) M 1,25VD. The mixture was incubated at room temperature for 30 minutes. For competition experiments, VDR/RXR was pre-incubated with 2 \( \mu g \) anti-RXR\( \beta \), anti-Flag M2 antibody or excess amount of cold probes on ice for 20 minutes before the EMSA reaction. The reaction mixture was resolved in a 5% non-denaturing polyarylamide gel and protein-oligo complexes were revealed by autoradiography.

**Construction of luciferase reporter plasmid and mutational analyses**

To construct GADDLuc, GADD45 genomic DNA fragment from +366 to +2926 was amplified by PCR using pHG45-HC (30). The forward primer contains a MluI and the reverse primer a BglII site. The amplified PCR fragment was cloned into the MluI and BglII sites of pGL3-promoter vector. Luc1 was generated by digesting the GADDLuc with KpnI and religation. Luc2 was generated by digesting GADDLuc with MluI and EcoRI, filling with Klenow fragments and religation. Luc3 was generated by sub-cloning into BglII site of pGL3-promoter vector a 440 bp DNA fragment released from GADDLuc with BamHI and BglII. Luc4
was generated by digesting GADDLuc with BglII and EcoRI, filling with Klenow fragments and religation. Luc5 was generated by sub-cloning into pGL3-promoter vector at KpnI and BglII sites a 777 bp fragment released from Luc2 with KpnI and BamHI.

Site-directed mutagenesis was performed as described (40) using QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). The sequence of all mutant constructs was verified by DNA sequencing.

**Transcriptional analysis**

For transfection studies, OVCAR3 cells were plated in 15% FBS RPMI 1640 medium at 1 × 10^5 cells/well and HeLa cells in 10% FBS DMEM at 5 × 10^4 cells/well in 12-well plates. On the next day, OVCAR3 cells were transfected by Lipofectamine Plus and HeLa by Lipofectamine. 4 hrs post-transfection, cells were treated with 1,25VD or vehicle in fresh medium for 36 hrs. Cells were harvested and luciferase and β-galactosidase (β-gal) assays were performed as described (40, 41).

**Chromatin Immunoprecipitation (CHIP) assays**

For CHIP assays, OVCAR3 cells were treated with ETOH or 10^{-7} M 1,25VD for 60 min and cross-linked with 1% formaldehyde. Then, the cells were lysed in buffer (pH 8.0) containing 5 mM PIPES, 85 mM KCl, 0.5 % NP 40 and protease inhibitor cocktail. Cell nuclei were pelleted and re-suspended in buffer containing 50 mM Tris-Cl (pH. 8.1), 10 mM EDTA, 1% SDS and protease inhibitor cocktail. Soluble chromatin was prepared by sonication and diluted in buffer containing 16.7 mM Tris-Cl (pH 8.1), 0.01 % SDS, 1.1% Triton X 100, 1.2 mM EDTA, 167 mM NaCl and protease inhibitor cocktail. Immunoprecipitates were prepared with rat anti-VDR antibody or Rat IgG (Sigma). DNA was extracted from the immunocomplexes using a QIAquick Spin Kit (Qiagen, CA). 2 µl (out of 30 µl) DNA extract was used for PCR.
Immunoblotting analysis and *In vitro* immunocomplex kinase assays

Immunoblotting analysis of GADD45 protein was performed as described (38). Immunoblotting analysis of cdc2, cyclinB1, VDR and β-actin was performed similarly as for GADD45 except that the cell extracts were prepared in buffer containing 20 mM Tris-Cl (pH 7.5), 300 mM NaCl, 3 mM EDTA, 3 mM EGTA, 100 µM Na3VO4, 1% NP-40, and protease inhibitor cocktail.

*In vitro* immunocomplex kinase assays were performed as described (42) with minor modifications. In brief, cellular extracts were prepared in buffer containing 20 mM Tris-Cl (pH 7.5), 300 mM NaCl, 3 mM EDTA, 3 mM EGTA, 100 µM Na3VO4, 1% NP-40, protease inhibitor cocktail and immunoprecipitated with anti-cyclin B1 antibody. Kinase assays were performed at 30 °C in 20 µl reaction buffer containing 20 mM HEPES (pH7.5), 5 mM MgCl2, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 20 µM ATP, 3 µg histone H1, and 10 µCi [γ-32P] ATP. Reactions were terminated by adding 2 × SDS-PAGE sample buffer. Samples were analyzed on a SDS-PAGE and the phosphorylated histone H1 visualized by autoradiography.

Establishment of stable clones from OVCAR3 cells

OVCAR3 cells were transfected with 10 µg pCMVAS45 plasmid and 0.5 µg pcDNA3 for the establishment of GADD45 anti-sense stable clones or with pcDNA3 alone for the Vector-OVCAR3 controls. For the establishment of stable clones with GADDLuc reporter, OVCAR3 cells were transfected with 10 µg GADDLuc plasmid and 0.5 µg pcDNA3. All stable clones were obtained through selection with 100 µg/ml G418 for a period of about 4 weeks and clonal isolation with glass cylinders.

Results

1,25VD suppresses OCa cell growth and induces cell cycle arrest at G1/S and G2/M
To better understand the molecular mechanism of VD action in OCa cells, we tested the response of OCa cells to 1,25VD in proliferation assays. The cells were treated with vehicle or 10^{-7} M 1,25VD for 6 or 9 days and cell growth was analyzed by MTT assays. As shown in Figure 1, panel A, OVCAR3 cell growth decreased in the presence of 10^{-7} M 1,25VD in a time-dependent manner, confirming the sensitivity of OVCAR3 to 1,25VD (26). Since the cell growth was not significantly affected by 1,25VD at concentrations of 10^{-8} M or lower (data not shown), it appears that there is a threshold to the action of 1,25VD.

To determine the mechanism underlying the 1,25VD-induced growth suppression, OVCAR3 cells were treated with vehicle or 10^{-7} M 1,25VD for 9 days and analyzed by flow cytometry. Figure 1, panel B, shows that 1,25VD decreased the percentage of cells in the S phase, which was accompanied by an accumulation of cells in G0/G1 and G2/M. This suggests that 1,25VD causes cell cycle arrest at both G1/S and G2/M checkpoints. The increase in G0/G1 was estimated as 13% while the percentage of cells at G2/M increased by 8% (Fig. 1C), suggesting that cell cycle arrest at both checkpoints contributed roughly equally to the growth-suppressing activity of 1,25VD. At variance with drugs used in conventional cancer chemotherapy, inhibition of cancer cell growth by 1,25VD is a chronic process, explaining why the effect on the cell cycle is modest and requires treatment for a longer time.

**GADD45 is a primary and immediate early response gene for 1,25VD in OCa cells**

To identify the genes that mediate the inhibitory effects of 1,25VD on cell cycle progression, a preliminary microarray analysis was performed to screen for genes that are differentially expressed in OCa cells treated with vehicle vs. 10^{-7} M 1,25VD. Among the many differentially expressed genes, GADD45 was one of the genes that were potentially up-regulated by 1,25VD (data not shown). Since GADD45 has a well-established role in cell cycle control
and a suggested role in ovarian tumorigenesis (43), we carried out a Northern blotting to confirm the GADD45 regulation by 1,25VD. Compared to cells treated with vehicle, 10^{-7} M 1,25VD significantly increased GADD45 mRNA levels, whereas 10^{-8} M 1,25VD caused a barely detectable increase (Fig. 2A), showing that the induction of GADD45 mRNA by 1,25VD is dose-dependent. The induction was detectable as early as 2 hrs following 1,25VD treatment with maximum induction detected at 8 hrs (Fig. 2B), suggesting that GADD45 is an immediate early response gene for 1,25VD. Treatment for times longer than 8 hrs maintained the induction but did not further enhance it. The levels of GAPDH mRNA were not affected by 1,25VD, showing the specificity of the effect of 1,25VD on GADD45. The stability of GADD45 mRNA, as measured in the presence of an inhibitor of RNA synthesis, actinomycin D, was not different between cells treated with vehicle and 1,25VD (Fig. 2C), suggesting that the regulation of GADD45 by 1,25VD is transcriptional. Although the induction of GADD45 decreased (Fig. 2D), it persisted in the presence of an inhibitor of protein synthesis, cycloheximide. This shows that GADD45 induction by 1,25VD does not require new protein synthesis, thus identifying GADD45 as a primary target gene for 1,25VD in OCa cells. This is consistent with similar data in squamous cell carcinoma (44, 45).

A novel VDRE in the 3’ un-translated region of GADD45 mRNA mediates the transcriptional up-regulation of GADD45 by 1,25VD

To define the specific DNA elements that mediate the induction of GADD45 by 1,25VD, the genomic sequence of GADD45 was examined for the presence of putative VDREs. Based on similarity to the consensus VDRE sequence (46) and the VDRE sequence of known VD target genes, such as osteopontin (OPN) (47) and osteocalcin (OC) (48), five putative DR3-type
VDREs were identified (Fig. 3A). Four were in introns, one in exon 4 but none in the 5’ promoter region.

To test which of the five putative VDREs bind the receptors, EMSAs were performed with recombinant VDR and RXR proteins. As shown in Figure 3, panel B, our conditions allow the detection of a specific VDR/RXR complex with the OC VDRE. The complex is up-shifted by a RXR antibody and decreased by excess cold OC probe (upper panel). Under the same conditions, all putative VDREs except VDRE-C bound RXR/VDR with an affinity comparable to OC VDRE (Fig. 3B, lower panel). The binding is specific for the VDR/RXR heterodimer since neither VDR nor RXR alone formed a detectable complex with the VDRE probes. The VDREs were displaced from the complexes with an excess amount of cold OC VDRE but not affected by the putative VDRE-C that did not bind VDR/RXR (Fig. 3C). These data show that VDR/RXR binding is specific. The RXR antibody up-shifted the complex while a non-related antibody did not, confirming the presence of RXR in the complex.

To determine whether VDREs binding the receptors in vitro mediate the induction of GADD45 by 1,25(VD in OVCAR3 cells, a reporter gene was constructed with a 2.6 kb genomic DNA fragment of GADD45 containing all the putative VDREs located upstream of SV40 promoter and the cDNA of firefly luciferase gene (Fig. 4A). In OVCAR3 cells transiently transfected with this reporter, 1,25(VD induced the luciferase activity in a dose (Fig. 4B) and time dependent manner (Fig. 4C). This induction was also detected in OVCAR3 cells in which the reporter was stably integrated into the genome (Fig. 4D), showing that it is not an artifact of the transient transfection. Expression of additional VDR, RXR or both did not further increase the reporter activity in OVCAR3 cells (Fig. 4E), suggesting that endogenous VDR and RXR in OVCAR3 cells are sufficient for such induction.
To test whether 1,25VD regulates the GADD45 reporter through VDR, the activation of the reporter was tested in HeLa cells that lack functional VDR. As shown in Figure 5, panel A, 1,25VD did not induce the activity of a known VDR reporter, p23, that was constructed with the promoter of 24-hydroxylase (35). The induction of p23 reporter was restored by the ectopic expression of VDR in HeLa cells. Similar to the p23 reporter, 1,25VD did not cause measurable induction of luciferase activity from the GADD45 reporter in HeLa cells (Fig. 5B). Co-transfection with VDR restored the induction in a manner that depends on the dose of the transfected receptor (Fig. 5B). Transfection with RXR alone did not affect reporter activity but its co-transfection with VDR enhanced the induction compared to cells transfected with VDR alone. Similar to the results with OVCAR3 cells, 1,25VD induced reporter activity in HeLa cells in a dose and time dependent manner after receptor transfection (data not shown). These experiments demonstrate that the regulation of the reporter is VDR-dependent and involves the RXR.

To determine which of the putative VDREs is functional, OVCAR3 cells were transfected with reporter constructs where VDREs, either individually or in combination, were deleted or mutated by altering key nucleotides known to be essential for receptor interaction. As shown in Figure 6, panel A, deletion of the VDRE-A or VDRE-A plus VDRE-B region did not affect the induction. Deletion into the VDRE-D region caused a significant decrease while further deletion into the VDRE-E region eliminated the induction. These analyses suggest that regions around VDRE-D and VDRE-E are essential for regulation of GADD45 by 1,25VD.

Site-directed mutation in VDRE-E eliminated the induction, while single or multiple mutations of key nucleotides in the intronic VDREs had no effect. This suggests that only VDRE-E is essential for the induction. This conclusion is consistent with the lack of the
induction in mutant reporters containing only VDRE-D or both VDRE-A and VDRE-B (Fig. 6A). It is also consistent with the induction of the reporter that contains only VDRE-E (Luc3) and with the loss of this induction by site-directed mutation of VDRE-E (MTELuc3) (Fig. 6A).

Individual mutation of the first two VDREs or combined mutation of all first three VDREs actually increased the induction by 1,25VD, indicating that some VDREs may function in a negative fashion. The deletion of the VDRE-D region, not the mutation of the VDRE-D sequence, caused a decrease in the induction, suggesting that DNA elements in the VDRE-D region for transcription factors other than VDR may cooperate with VDR/RXR binding to VDRE-E to mediate the up-regulation of GADD45. The lack of a VD effect on the activity of pGL3-basic, pGL3-promoter and the pGL3-control vectors indicates that the regulation of the reporters by 1,25VD is specific to the GADD45 sequence.

To demonstrate that the VDRE-E interacts with VDR in vivo, OVCAR3 cells were treated with or without 1,25VD and CHIP assays were performed with anti-VDR antibodies (Fig. 6B). From soluble chromatin prepared from OVCAR3 cells treated with 1,25VD, the anti-VDR antibody precipitated GADD45 DNA fragments containing the VDRE-E but not the promoter regions. The specificity of the CHIP assay was demonstrated by the lack of VDRE-E DNA in the mock as well as in the immunoprecipitates of rat IgG control. The data shows that VDR is recruited to the VDRE-E in vivo. More importantly, the recruitment of VDR to the response element is apparently ligand-dependent since the anti-VDR antibody did not precipitate GADD45 DNA fragments from soluble chromatin prepared from cells treated with vehicle alone.

1,25VD induces the accumulation of GADD45 protein in OCa cells, which is required for the hormone-induced cell cycle arrest at the G2/M but not the G1/S checkpoint
Our studies have established GADD45 as one of the immediate early response genes for 1,25VD, but questions remain whether regulation at the RNA level extends to the protein level and whether GADD45 mediates the growth-suppressing activity of 1,25VD in OCa cells. To address these questions, OVCAR3 cells were treated with 1,25VD for up to 6 days and GADD45 protein expression was examined by immunoblotting. As shown in Figure 7, panel A, 1,25VD increased the level of GADD45 protein in a time dependent manner in OVCAR3 cells whereas the level of β-actin remained constant during the treatment. Comparing to the data on GADD45 mRNA (Fig. 2B), 1,25VD-induced accumulation of GADD45 protein is a much slower process. This suggests the possible presence of additional regulations for GADD45 expression at post-transcriptional steps. The slower accumulation of GADD45 protein in response to 1,25VD may explain why the hormonal effect on the cell cycle requires longer treatment.

To test whether GADD45 mediates the inhibitory effect of 1,25VD on OCa cell cycle progression, OVCAR3 cells were stably transfected with an expression vector containing GADD45 cDNA in the anti-sense orientation (31) or an empty vector. Immunoblot analysis of GADD45 was used to select stable clones with significantly reduced level of GADD45 protein by the anti-sense GADD45 as compared to the control clones (Fig. 7B). Although the anti-sense GADD45 did not completely eliminate GADD45 protein (Fig. 7B), it decreased the expression of GADD45 protein in the presence of 1,25VD to a level lower than basal level of control clones (Fig. 7B). In other words, the anti-sense clones represent a functional “knock out” of GADD45 in terms of the induction by 1,25VD.

Flow cytometry showed that a decrease of GADD45 protein in the anti-sense clones was associated with an increase of the proportion of cells in G2/M phase and a decrease of those in S phase (Figs. 7C and 7D). In the control clones, 1,25VD decreased the percentage of cells in S
phase and increased the cells in G0/G1 and G2/M, showing that the hormone induced a similar cell cycle arrest at both G1/S and G2/M checkpoints similar to that in the parental OVCAR3 cells (Figs. 1B and 1C). In the anti-sense clones, 1,25VD-induced G2/M accumulation was blocked whereas 1,25VD-induced decrease in S phase and increase in G0/G1 still occurred (Figs. 7C and 7D). The data strongly suggest that GADD45 mediates the inhibitory effect of 1,25VD on the G2/M transition in OVCAR3 cells. Similar to the data in colon cancer cells (28), the two untreated anti-sense GADD45 clones had slightly increased G0/G1 and G2/M fractions and slightly lower S fractions, compared with vector-control OVCAR3 cells.

To firmly establish GADD45 as the mediator for the 1,25VD-induced cell cycle arrest at the G2/M transition, we tested the effect of 1,25VD on the cell cycle progression of MEFs established from either wild type or GADD45-null mice (36). Both MEFs expressed similar levels of VDR protein as determined on immunoblots (Fig. 8A). It is known that the MEFs from this strain of mice are a mixture of diploid and tetraploid cells (36). This complicates the flow cytometry analysis of diploid cells, largely due to the inability to distinguish diploid cells at G2/M from tetraploid cells at G0/G1 phases. Therefore, we compared the changes induced by 1,25VD in the cell cycle distribution of tetraploid cells. As shown in Figure 8, panels B and C, 1,25VD induced a consistent increase in the percentage of cells in G2/M and a decrease of that in S phase of wild type MEFs, although the magnitude of the response was less than in OVCAR3 cells. In GADD45-null MEFs, no induction of G2/M arrest by 1,25VD was observed, confirming the conclusion reached in OVCAR3 cells with the anti-sense approach that GADD45 is required for 1,25VD-induced cell cycle arrest at the G2/M transition. In the wild type and GADD45-null MEFs, 1,25VD did not induce G1/S arrest (data not shown). Since G1/S arrest by 1,25VD was
observed in the MEFs derived from another strain of mice (unpublished data), the response to 1,25VD appears to vary among MEFs from different strains.

**GADD45 mediates 1,25VD-induced decrease in Cdc2 kinase activity in OCa cells**

It is well established that the G2/M transition of mammalian cells is controlled by the M phase promoting factor, a heterodimeric complex between cdc2 and cyclin B. Studies in recent years suggest that GADD45 may directly regulate the activation of cdc2 kinase (29). Therefore, the effect of 1,25VD on cdc2 kinase activity in control and GADD45 anti-sense clones was measured by *in vitro* immunocomplex kinase assays. As shown in Figure 9, 1,25VD decreased the kinase activity of cdc2 in the control clone. In the GADD45 anti-sense clones, there is an increase in cdc2 kinase activity (Fig. 9), presumably due to the decrease in the level of GADD45 protein. More importantly, 1,25VD did not decrease the activity to a level below the basal activity of control clones. Immunoblot analysis showed that 1,25VD decreased the level of cyclin B protein and that the decrease was not observed in the anti-sense clones (Fig. 9), suggesting that GADD45 mediates the effect of 1,25VD on cdc2 activity by regulating the cyclin B level in OCa cells. The data clearly suggest that G2/M arrest induced by 1,25VD in OCa cells is mediated through GADD45 and a subsequent decrease in cdc2 kinase activity.

**Discussion**

GADD45 is a nuclear protein that binds to numerous cellular proteins and plays a role in cell cycle progression as well as the maintenance of genomic stability. Our studies have identified GADD45 as one of the primary target genes for 1,25VD and suggest the following model of VD action in OCa cells (Fig. 10). 1,25VD induces binding of the VDR/RXR heterodimer to the VDRE located in the fourth exon at a position downstream from the termination codon for protein translation. Presumably through the recruitment of co-activator
complexes, the activated VDR/RXR interacts with Pol II complex bound to the promoter and increases the rate of GADD45 transcription. This leads to an increase in the amount of GADD45 protein that, through a yet unknown undefined mechanism, decreases the level of cyclin B, the regulatory subunit of cdc2 kinase. The resulting decrease in cdc2 activity is then responsible for disturbed cell cycle progression to M phase.

Induction of GADD45 can be detected in cells within 2 hrs after treatment with 1,25VD (Fig. 2B). With the lack of VD effect on the mRNA stability (Fig. 2C) and the identification of functional VDREs in the genome (Figs. 3-6), our data establish GADD45 as a primary and immediately early response gene for 1,25VD. Furthermore, our data suggest that GADD45 regulation is mediated through a VDR/RXR heterodimer, instead of a VDR/VDR homodimer. This conclusion is reached based on the inability of VDR to bind to all putative VDREs in EMSAs until the addition of RXR and the up-shift of the complexes by RXR antibody (Fig. 3). At variance with the belief that nuclear receptors forming heterodimers with RXRs bind DNA in the absence of ligand, our CHIP assays (Fig. 6B) clearly show that in vivo binding of VDR to the exonic VDRE is ligand-dependent. Recently, Yamamoto et. al. (49) reported a similar observation on VDRE located in the promoter of OPN gene, suggesting that the ligand-dependency is common to VDREs located inside and outside promoter regions.

Since the p53 in OVCAR3 cells is mutated (50), the regulation of GADD45 transcription by 1,25VD obviously occurs independently of p53 activity. This is consistent with the identification of the VDRE in the fourth exon that is distant from the p53 binding site in the third intron (30). This is also consistent with the conclusion reached by a published study in squamous cell carcinoma (44). Furthermore, the regulation is also likely to be independent of BRCA1 since the DNA element for this tumor suppressor is located in the promoter region (51). It is striking
that four of the five putative VDREs bound VDR/RXR equally well in EMSAs, but only VDRE-E mediated the up-regulation of GADD45 reporter by 1,25VD. The other putative VDREs are either nonfunctional or may act in a negative way. Overall, the data suggest that the regulation of GADD45 by 1,25VD is a complex process that may involve the interaction of the receptor bound to the VDRE and other transcription factors. The different VDREs may also function in a cell-specific manner to mediate the regulation of GADD45 expression by 1,25VD.

To the best of our knowledge, known VDREs are located in the promoter region or the upstream regulatory sequence. It is unique that the specific VDRE that is functional in GADD45 induction is located in an exon and falls into the 3’ un-translated region. The VDRE may provide a good model system to study how DNA response elements in the 3’ end of the coding sequence regulate the transcription of a target gene via the Pol II complex bound to the promoter in the 5’ end. Recent studies suggest that site-specific transcription factors (52), including nuclear hormone receptors (53), recruit components of the Pol II complex to the enhancer sequences and, after co-activator mediated chromatin remodeling, the adjacent nucleosome slides downstream for a certain distance to initiate transcription. It remains to be seen whether DNA response elements at the 3’ end of the coding sequence recruit Pol II components and, if so, how the modified nucleosome moves to the right position to permit transcriptional initiation since this nucleosome has to either slide upstream for a significant distance or jump across the coding region to initiate transcription.

Our studies link GADD45 induction specifically to the inhibition by 1,25VD of cell cycle progression through the G2/M checkpoint. This linkage was established using cells in which GADD45 expression was compromised by anti-sense approach or genetic knock out. Obviously, it is important to determine the effect of GADD45 anti-sense on 1,25VD induced growth
inhibition in the stably transfected cells, which would reveal whether the G2/M arrest is a major or minor factor in the overall growth inhibition. However, our analysis in MTT assays revealed little difference in the overall response to 1,25VD between anti-sense GADD45 and control clones (data not shown). Studies in our lab showed that, besides G2/M arrest, 1,25VD also induces G1/S arrest, apoptosis and senescence-like phenotype in OCa cells (data not shown). The contribution of each individual effect may be below the detectable level of MTT assays. The accurate assessment may require the development of new growth assays that are more sensitive and quantitative than MTT.

Previous studies (45) have shown that the induction of GADD45 expression in squamous cell carcinoma by VD is associated with an increased interaction with PCNA. Although we have not examined the association of GADD45 with PCNA, it appears unlikely to be the case in OVCAR3 cells. PCNA is known to be required for DNA replication in S phase but our data show that G1/S arrest still occurs in OVCAR3 cells expressing the anti-sense cDNA of GADD45 (Fig. 7C and 7D). Instead of PCNA, our studies show a 1,25VD-induced decrease in cdc2 activity, which is associated with a decrease in the level of cyclin B1 protein. The decrease was not observed in the stable clones expressing GADD45 anti-sense cDNA (Fig. 9). The data suggest that GADD45 mediates the effect of 1,25VD on cdc2 activity. Our data concur with the proposed role of GADD45 in G2/M arrest induced by certain types of DNA damaging agents (28, 29). Zhan et al. (29) have shown that GADD45 inhibits the interaction between cyclin B and cdc2. Later, it was shown that GADD45-induced cell cycle arrest at G2/M is associated with an altered cellular distribution of cyclin B1 (54), which seems to require a functional p53. Our conclusions, however, differ from the above studies since we detected a decrease in the level of cyclin B1 protein (Fig. 9). As mentioned earlier, OVCAR3 cells contain a mutant p53 (50).
Obviously, GADD45 exerts its effect on cdc2 activity and the G2/M transition in the OCa cells independently of p53. It remains to be determined whether the effect of 1,25VD on G2/M in OCa cells is exerted through GADD45 alone or in combination with another protein that functions similarly to p53.

Besides its role in regulating G2/M transition, GADD45 plays an essential role in DNA repair and in the maintenance of genomic stability. MEFs derived from GADD45-null mice exhibit aneuploidy, chromosomal abnormalities, gene amplification and centrosomal amplification (36). GADD45-null mice display increased sensitivity to dimethylbenzanthracene-induced carcinogenesis (43). It is intriguing that dimethylbenzanthracene increased female ovarian tumors more in GADD45-null mice compared to the wild type (43). GADD45 induction by 1,25VD through VDR suggests that VDR may act in a p53 independent tumor-suppressing pathway to affects ovarian genomic stability. Along this line, VDR has been shown to act as a bile acid sensor for the secondary bile acid lithocholic acid (LCA) (55), a potential enteric carcinogen that induces DNA strand breaks, forms DNA adducts and inhibits DNA repair enzymes. It is interesting to find out whether ovarian carcinogens or DNA-damaging processes (e.g. ovulation) activate VDR in ovarian epithelial cells and, if yes, whether the VD-independent VDR activation functions in DNA repair.

OCa is a fatal disease unless detected at early stages and the overall 5-year survival in patients with this malignancy is less than 40%. The poor response of advanced OCa to current treatments stimulates the search for novel therapeutic strategies. The present study provides a strong rationale for further investigation of 1,25VD along with the less calcemic synthetic VD analogues (56) as a chemo-preventive agent against OCa. Furthermore, half of OCa is estimated to lose p53 function, thus becoming refractory to drugs targeting p53 pathways. These tumors
may however still be sensitive to synthetic VD since 1,25VD induces GADD45 in a p53-independent manner.

Acknowledgements

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References


**Figure Legends**
Fig. 1. 1,25VD inhibits OCa cell growth and induces cell cycle arrest at G1/S and G2/M checkpoints. (A) Suppression of cell growth by 1,25VD. OVCAR3 cells were plated in 96 wells and treated with 10^{-7} M 1,25VD (VD) or ethanol (ETOH) as vehicle. Cell numbers were determined in MTT assays. * P<0.01 (versus ETOH). (B) Induction of cell cycle arrest at G1/S and G2/M checkpoints by 1,25VD. OVCAR3 cells were treated with ETOH or 10^{-7} M 1,25VD for 9 days. Treated cells were subjected to flow cytometry analysis. Three independent experiments were performed and the profile of a representative experiment is shown. The first peak in red shows diploid cells at G0/G1 and the second at G2/M (arrow). Reverse-hatched area shows diploid cells at S phase, green area aggregates and blue area sub-G1. (C) Average percentages of cells at G0/G1, S and G2/M in OVCAR3 cells treated with 1,25VD or ETOH for 9 days, * P<0.05 (versus ETOH).

Fig. 2. 1,25VD increases transcription of GADD45 mRNA in OCa cells. (A) Dose-dependent induction of GADD45 mRNA by 1,25VD. Total RNA was isolated from OVCAR3 cells treated with ETOH or 1,25VD at indicated dosages for 24 hrs. 20 µg RNA was subjected to Northern blot analysis. (B) Time course of the induction of GADD45 mRNA by 1,25VD. OVCAR3 cells were treated with ETOH or 10^{-7} M 1,25VD for indicated times. Total RNA was isolated and Northern blot was performed as in panel A. (C) Lack of VD effect on GADD45 mRNA stability. OVCAR3 cells were treated with ETOH or 10^{-7} M 1,25VD for 24 hrs. The cells were washed and subsequently treated with 5 µg/ml actinomycin D (ActD) for indicated times. Northern blot analysis was performed as in panel A. The signals on Northern blot were quantified using Scion Image Beta 4.02 software. The GADD45 signal was normalized with corresponding GAPDH signal and presented as percentage of the GADD45 mRNA level at time 0. (D) Effect of cycloheximide (CHX) on the induction of GADD45 mRNA by 1,25VD. OVCAR3 cells were
exposed to ETOH or 10^{-7} M 1,25VD for 24 hrs in the absence or presence of 25 \mu M CHX. Northern blot was performed as in panel A. The GADD45 signal was quantified and normalized with corresponding GAPDH signal as in panel C and presented in the bar graph as fold induction.

**Fig. 3.** Multiple putative VDREs are present in GADD45 genome, which interact with VDR/RXR *in vitro*. (A) Schematic representation of the human GADD45 genome and the position of the putative VDREs. The sequences of consensus DR3, mouse OPN (mOPN) VDRE, human OC (hOC) VDRE and the five putative GADD45 VDREs are listed. Hexameric VDRE half sites are shown in bold capital letters. The 3-bp space is shown in small letters. Deviations from the consensus sequence RGKTSA are underlined. (B) *In vitro* interaction of VDR/RXR heterodimer with putative GADD45 VDREs. EMSAs were performed in the presence of 10^{-7} M 1,25VD using hOC (upper panel) or putative GADD45 (lower panel) VDRE probes. Pre-incubation with 2 \mu g anti-RXR\textbeta (RXR-Ab) or 100-fold molar excess of cold hOC VDRE (Cold-hOC) was performed for the super-shift and competition experiments, respectively. (C) Specificity of the interaction between VDR/RXR heterodimer and the putative GADD45 VDREs. EMSAs were performed as in panel B. Specificity of the interaction was demonstrated by competition with 100-fold molar excess of unlabeled hOC VDRE oligoes as a specific competitor and the lack of competition with VDRE-C oligoes as nonspecific competitor. 2 \mu g anti-Flag M2 monoclonal antibody was used as a non-specific antibody control for the super-shifting with anti-RXR antibody.

**Fig. 4.** 1,25VD induces GADD45 reporter activity through endogenous receptors in OCa cells. (A) Schematic representation of GADDLuc construct. (B) Dose-dependent induction of GADD45 VDRE reporter activity by 1,25VD. OVCAR3 cells were transfected with 0.2 \mu g
GADDLuc, 0.05 µg pCMVgal, 0.05 µg p91023B-VDR and 0.05 µg pCMX-RXRβ and treated with ETOH or 1,25VD at the indicated concentrations. Luciferase activity was determined and normalized with cognate β-gal activity. Each data point was analyzed in duplicate and reproduced three times. (C) Time-dependent induction of GADDLuc activity by 1,25VD. OVCAR3 cells were transfected as in panel A and treated with ETOH or 10⁻⁷ M 1,25VD for indicated times. Luciferase activity was determined as in panel B. (D) Induction of reporter activity by 1,25VD in cells stably transfected with the GADDLuc. OVCAR3 cells stably transfected with GADD45 reporter were transfected with 0.05 µg pCMVgal, 0.05 µg p91023B-VDR and 0.05 µg pCMX-RXRβ and treated with ETOH or 10⁻⁷ M 1,25VD for 36 hrs. Luciferase activity from two stable clones was determined and shown as fold induction. (E) Endogenous VDR/RXR in OVCAR3 cells is sufficient for VD induction of GADD45 reporter. OVCAR3 cells were transfected with GADDLuc and pCMVgal either with or without p91023B-VDR (VDR) and pCMX-RXRβ (RXR). The total amount of plasmid DNA was balanced with empty vectors. Luciferase activity was determined as in panel B.

**Fig. 5.** Induction of GADD45 reporter activity by 1,25VD is VDR-dependent. (A) The lack of functional VDR in HeLa cells. Cells were transfected with 0.2 µg p23 and pCMVgal together with or without p91023B-VDR. Transfected cells were treated and luciferase activity was determined as in Fig. 4B. (B) VDR-dependent induction of GADD45 reporter activity by 1,25VD. HeLa cells were transfected with GADDLuc and pCMVgal together with indicated amounts of p91023B-VDR, pCMX-RXRβ or both. The total amount of plasmid DNA was balanced with empty vectors. Luciferase activity was determined as in Fig. 4B.

**Fig. 6.** The VDRE in the fourth exon of GADD45 genome is the functional VDRE that mediates the transcriptional induction of GADD45 by 1,25VD in OCa cells. (A) Mutational analysis of the
GADD45 reporter. OVCAR3 cells were transfected with 0.2 µg of the reporter constructs together with pCMVgal and treated with ETOH or $10^{-7}$ M 1,25VD for 36 hrs. Luciferase activity was determined as in Fig. 4B and shown in the right. Schematic representation of the different reporter constructs is shown in the left. pGL3-basic, pGL3-promoter and pGL3-control vectors were used as controls. (B) CHIP assays. Soluble chromatin was prepared from OVCAR3 cells treated with ETOH or $10^{-7}$ M 1,25VD for 60 min. CHIP assays were performed with control (rat IgG) or anti-VDR antibody. The mock control was performed with immunoprecipitates from buffer that contains no soluble chromatin from OVCAR3 cells. Promoter-1 (-1492/-1241), promoter-2 (-505/-310), VDRE-E region (2565/2767).

**Fig. 7.** Anti-sense GADD45 blocks 1,25VD-induced cell cycle arrest at G2/M, but not G1/S, checkpoint. (A) Induction of GADD45 protein expression by 1,25VD in VD-sensitive human OCa cells. OVCAR3 cells were treated with ETOH or $10^{-7}$ M 1,25VD for indicated times and the level of GADD45 protein was analyzed by immunoblotting. HeLa cells transfected with pCMV45 plasmid was included as a positive control. β-actin was used to show the equal amount of total protein present in each lane. (B) Suppression of GADD45 protein expression by stable expression of GADD45 anti-sense cDNA. OVCAR3 cells stably transfected with control vector (Vector-OVCAR3) or the anti-sense cDNA of GADD45 (AS45-OVCAR3) were treated with ETOH or $10^{-7}$ M 1,25VD for 24 hrs. The level of GADD45 and β-actin protein was determined as (A). (C) Abrogation of 1,25VD-induced G2/M arrest in AS45-OVCAR3 clones. Vector-OVCAR3 and AS45-OVCAR3 clones were treated with ETOH or $10^{-7}$ M 1,25VD for 9 days. Cell cycle distribution was determined and a representative profile presented as in Fig.1B. (D) Bar graphs showing percentage of cells at G2/M, G0/G1, and S phases. * P<0.05; # P>0.05 (versus ETOH).
**Fig. 8.** 1,25VD induces G2/M arrest in wild type (WT) but not in GADD45-null MEFs. (A) Expression of VDR protein in WT and GADD45-null MEFs. The level of VDR protein was determined by immunoblotting with anti-VDR antibody and equal loading was shown by immunoblotting with anti-β-actin antibody. (B) Induction of G2/M arrest in WT but not GADD45-null MEFs by 1,25VD. WT and GADD45-null MEFs were treated with ETOH or 10\(^{-7}\) M 1,25VD for 9 days. Cell cycle distribution was determined and a representative profile presented as in Fig.1B. The peak in red shows diploid cells at G0/G1. The first peak in yellow shows tetraploid cells at G1/G0 and the second at G2/M (arrow). Forward- and reverse-hatched areas show tetraploid and diploid cells, respectively, at S phase. (C) Bar graph showing the percentage of tetraploid cells at G2/M. * P<0.01; # P>0.05 (versus ETOH).

**Fig. 9.** 1,25VD decreases cdc2 kinase activity in OVCAR3 transfected with control vector but not in cells stably transfected with the anti-sense cDNA of GADD45. Vector-OVCAR3 and AS45-OVCAR3 cells were treated with ETOH or 10\(^{-7}\) M 1,25VD for 9 days. Cellular extracts were immunoprecipitated with anti-cyclin B1 antibody. The activity of cdc2 was assayed using Histone H1 as a substrate. The level of cdc2 and cyclin B1 protein was determined by immunoblotting. β-actin blot was included to show equal loading.

**Fig. 10.** A diagram illustrating VD action in regulating G2/M transition in OCa cells with emphasis on the role of GADD45 (see text for details).
A  

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<tr>
<th>ETOH</th>
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10^{-8}M

10^{-7}M

GADD45

GAPDH

B  

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0  

30 min  

2 h  

8 h  

24 h

GADD45

GAPDH

VD (days)

0  

1  

3  

6

GADD45

GAPDH

C  

<table>
<thead>
<tr>
<th>ActD (hours)</th>
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| 0  

2  

4  

8 |

GADD45

GAPDH

VD

[Graph: Relative Level of mRNA (%) vs. Exposure to ActD (hours)]

D  

<table>
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ETOH  

VD  

CHX  

CHX

GADD45

GAPDH

Fold Induction

0.0  

0.5  

1.0  

1.5  

2.0  

2.5

-  

+  

CHX
Consensus DR3-type VDRE
(R: A or G, K: G or T, S: C or G)
\[RGKTSAnnnRGKTS\]

mOPN \[GGTTCA\] cga \[GGTTCA\]
hOC \[GGGTGA\] acg \[GGGG\]

Putative VDREs in GADD45 genome

A 659-\[GGGTCA\] tgg \[GGGGTG\]
B 1482-\[GGGTCA\] gga \[GGGTGG\]
C 2129-\[GTTTCA\] ctc AGGTCA
D 2233-\[GGTTGC\] atg \[GGTTCA\]
E 2694-\[GGCTGA\] gtg \[AGTTCA\]

VDR + - + + + +
RXR + - + + + +
RXR-Ab - - + + - -
Cold-hOC - - - - - -

VDREs

hOC A B C D E

VDRE-A

Free probe

VDRE-B

Free probe

VDRE-D

Free probe

VDRE-E

Free probe
A

B

VDR (ng) | 4 | 40 | 40 | 4 | 4 | 4 | 40
RXR (ng) | - | - | - | 4 | 4 | 40 | 40

ETOH

VD

RLU x 10^-5

0 2 4 6 8 10 12

RLU x 10^-6

0 2 4 6 8 10
A

Wild Type (WT)  Mutant (MT)  SV40 promoter  SV40 Enhancer

Putative VDRE  Putative VDRE

GADDLuc

Luc1

Luc2

Luc3

Luc4

Luc5

MTALuc

MTBLuc

MTDLuc

MTELuc

MTABDLuc

MTELuc1

MTELuc2

MTELuc3

Luc1  Luc2  Luc3  Luc4  Luc5

RLU x 10^-6

0  2  4  6  8  10  12  14  16

B

-  +  -  +  -  +  VD

Promoter - 1

Promoter - 2

Mock  Input  Rat IgG  VDR  VDRE-E

Downloaded from http://www.jbc.org/ by guest on September 1, 2017
A

WT  Null
VDR
β-actin

B

WT  null

ETOH  VD

C

Percentage of Cells in G2/M

WT  Null

ETOH  VD

*  #
G2/M Arrest by 1,25 dihydroxyvitamin D3 in ovarian cancer cells mediated through the induction of GADD45 via an exonic enhancer
Feng Jiang, Pengfei Li, Albert J. Fornace, Jr, Santo V. Nicosia and Wenlong Bai

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