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Vitamin D Arrests Thyroid Carcinoma Cell Growth and Induces p27 Dephosphorylation and Accumulation through PTEN/Akt-Dependent and -Independent Pathways

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We investigated the effects of 1,25-dihydroxycholecalciferol vitamin D_3 (VD) and its noncalciomimetic analog EB1089 on thyroid carcinoma cell growth. VD and EB1089 exhibited anti-proliferative effects in a dose-dependent manner as determined by [^{3}H]thymidine incorporation and MIB-1 immunolabeling. VD or EB1089 resulted in similar G_1-phase arrest. Neither apoptosis nor differentiation was affected. VD and EB1089 induced increased nuclear protein expression of the cyclin-dependent kinase inhibitor, $p27^{kip1}$ (p27). VD/EB1089 effects paralleled but were not additive to those of the proteasome inhibitor LLnL, consistent with reduced p27 degradation. As p27 phosphorylation and association with Skp2 is a key step in its degradation, we examined the effects of VD/EB1089 on this reaction. Despite increased total p27, the pThr content of p27 remained unaffected, an effect confirmed by diminished association with Skp2 as well as *in situ* phosphorylation. Moreover, phosphatase inhibition abrogated the effect of VD/EB1089 on p27 accumulation consistent with a role for phosphatase action in mediating this VD effect. Although VD/EB1089 resulted in comparable increases in p27 in WRO and NPA cells, only WRO but not NPA cells demonstrated a change in the phosphatase PTEN and its downstream target pAkt/PKB in response to VD/EB1089. Transfection of PTEN resulted in p27 accumulation and was partially additive to the effect of VD/EB1089. Moreover, treatment with PI-3 kinase inhibitors decreased pAkt/PKB and increased p27 in both WRO and NPA cells highlighting the potential role of this downstream pathway in regulating p27 in the thyroid. These findings point to a novel mechanism of action for VD/EB1089 inhibition of thyroid carcinoma cell growth by p27 hypophosphorylation, diminished association with Skp2, and consequent accumulation. This effect can be mediated but is not essentially dependent on the phosphatase PTEN/Akt/PKB pathway. These properties support the potential utility of VD analogs in the treatment of thyroid carcinomas irrespective of their PTEN/pAkt status. *(Am J Pathol 2002, 160:511–519)*

Thyroid carcinoma has a very wide spectrum of differentiation from some of the most indolent carcinomas (papillary microcarcinoma) to the most rapidly lethal of human malignancies (anaplastic carcinoma).[1] These carcinomas have several markers of differentiation status that are extremely sensitive, such as thyroglobulin. Moreover, it has been shown that they follow a pattern of cumulative genetic defects that correlate with tumor differentiation and proliferation.[2] These cells, therefore, provide an ideal model in which to examine the effects of targeted modulation of cell growth and differentiation in tumors of various stages of dedifferentiation and with specific genetic defects.

In addition to its role in calcium homeostasis, the hormonal form of vitamin D, 1α,25-dihydroxy vitamin D_3 (VD), has been recognized to play a role in the modulation of the proliferation and differentiation of several cell types.[3–6] VD has been reported to induce apoptosis in human breast cancer[7] and leukemic cell lines.[8] Several VD analogs that lack unwanted side-effects of hypercalcemia, hypercalciuria, and soft tissue calcification have been shown to have anti-proliferative or apoptotic effects, and their promise as an important therapeutic tool has been recognized.[6] However, the regulatory mechanism(s) by which these agents exert their influence on the cell cycle remains to be elucidated. It has been suggested that vitamin D compounds act by inducing apoptosis, but the mechanism(s) of an anti-proliferative action through cyclin-dependent kinases (CDKs) and/or their inhibitors (CDKIs) remains to be elucidated.

In this study we examined whether VD and its noncalciomimetic analog EB1089 can inhibit growth of several

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thyroid cancer cell lines either by inhibition of proliferation, induction of differentiation, or induction of apoptosis. Our data indicate that the CDK inhibitor $p27^{kip1}$ (p27) is an important cellular target for the action of VD and its analog on thyroid cancer.

Materials and Methods

Compounds

The expression level of p27 was investigated during a 72-hour incubation of the cells with VD or EB1089. VD and its analog EB1089 (22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3) were provided by Dr. L. Binderup of LEO Pharmaceutical Products (Ballerup, Denmark). To demonstrate the mechanism of increased p27 expression by VD and EB1089, NPA and WRO cells were incubated with VD and EB1089 for 72 hours and in the presence or absence of 50 μmol/L of the proteasome inhibitor LLnL (Sigma, St. Louis, MO) for 5 hours. To demonstrate the role of PI-3 kinase-mediated pathways in VD/EB1089-mediated reduction of p27 degradation, we incubated cells with the PI-3 kinase inhibitors wortmannin 0.2 μmol/L (Sigma) or LY900402 20 μmol/L (New England Biolabs, Beverly, MA) for 12 hours. To evaluate the requirement for phosphatase action in mediating VD or EB 1089 action on p27, we incubated cells with the phosphatase inhibitor pervanadate (Sigma) at 0.1 μmol/L for 15 to 60 minutes. PTEN expression was performed using a full-length wild-type pGZ21δ xZ vector kindly provided by Dr. K. Yamada (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD).

Cell Culture

To determine VD effects on cells with variable degrees of differentiation, several human thyroid carcinoma cell lines were studied. TPC-1 cells (a gift from Dr. S. M. Jhiang, Ohio State University, Columbus, OH), a well-differentiated papillary thyroid carcinoma line with a ret/PTC-1 gene rearrangement and intact p53, were cultured in Dulbecco's modified Eagle's medium supplemented with 5% FCS, 2 mmol/L L-glutamine. The NPA cell line is a poorly differentiated papillary carcinoma cell line with mutant p53, WRO cells are a well-differentiated follicular carcinoma cell line with mutant p53, and FRO is an anaplastic carcinoma cell line with wild-type p53 status. These cells were obtained from Dr. J. Fagin (University of Cincinnati, Cincinnati, OH) and cultured in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 1 $\times$ nonessential amino acid (Sigma-Aldrich Co. Ltd., Irvine, UK). Cell number and viability was assessed at the beginning and end of each experiment using trypan blue.

Proliferation Assay

[^{3}H]Thymidine incorporation was used to assess effects of VD and EB1089 on proliferation of the above cell lines. Briefly, the cells were cultured in 6-well plates. After overnight recovery, the cells were incubated with VD or EB1089 at graded concentrations ranging from 10^{-6} to 10^{-10} mol/L for 72 hours. After refeeding the cells with the same medium and compound as above, the cells were exposed to 1 μCi [^{3}H]thymidine/well for 5 hours. The medium was discarded and the cells were washed three times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). Incorporated [^{3}H]thymidine was measured with a β-counter. Inhibition of cell proliferation was expressed as the percentage of mean [^{3}H]thymidine incorporation relative to that incorporated by vehicle-treated control cells.

Cell-Cycle Analysis

Cells were incubated with VD or EB1089 for 72 hours. After trypsinization, 1 to 3 $\times$ 10^6 cells were washed with PBS and fixed with cold 80% ethanol for 1 hour on ice. The fixed cells were washed with staining buffer (0.2% Triton X-100 and 1 mmol/L ethylenediaminetetraacetic acid, pH 8.0, in PBS) and resuspended in the staining buffer containing 50 μg/ml RNase A (Sigma) and 50 μg/ml propidium iodide for 1 hour. Cell-cycle analysis was done by fluorescence-activated cell sorting (FACScan; Becton Dickinson, San Jose, CA) using Cellquest Analysis and specific S phase was analyzed using Modfit DNA Analysis (Verity Software House Inc., Topsham, ME) programs.

Preparation of Cell Pellets

The cells were cultured in 10-cm plates. After overnight recovery, the medium was changed and the cells were exposed to VD or EB1089 at graded concentrations for 72 hours. The cells were trypsinized, washed, and centrifuged into pellets that were coated in 2% bactoagar until solidified, fixed in 10% formalin, and embedded in paraffin.

Immunocytochemistry

Sections of cell pellets were cut at 4 μm. Endogenous peroxidase activity was blocked with 3% aqueous hydrogen peroxidase and nonspecific binding was blocked with 20% protein blocker (Signet Laboratories, Inc., Dedham, MA). For cell proliferation analysis, the monoclonal anti-MIB-1 antibody (Immunotech, Marseilles, France) was used at 1:600 dilution for 60 minutes. For cell differentiation, thyroglobulin content was determined with a polyclonal antibody (DAKO Corporation, Carpinteria, CA) applied at 1:8000 dilution for 30 minutes after pepsin pretreatment. For localization of p27, a monoclonal antibody (Transduction Laboratories, Lexington, KY) was used at 1:1000 for 60 minutes after microwave antigen retrieval. The immunological reactions were visualized with the UltraStreptavidin detection system level 2 (Sig-

net) and 3,3,-diaminobenzidine tetrahydrochloride as the chromogen. As negative controls, the primary antibody was replaced with normal mouse ascites and omission of primary antibody alone was performed.

Apoptosis Analysis

To determine whether DNA fragmentation characteristic of apoptosis occurred, we stained 4-μm sections of cell pellets using the terminal dUTP nick-end labeling (TUNEL) technique (ApopTag kit; Oncor). Briefly, sections were treated with 2% hydrogen peroxide to quench endogenous peroxide for 30 minutes and exposed to 5 μg/ml of proteinase K for 15 minutes at room temperature. The sections were washed extensively and exposed to equilibration buffer for 10 minutes. Each slide was then incubated with terminal deoxytransferase and digoxigenin-labeled TdT at 4°C overnight followed by horseradish peroxidase-conjugated anti-digoxigenin antiserum for 1 hour. The peroxidase reaction was visualized using 3,3,-diaminobenzidine tetrahydrochloride. Control sections were similarly stained but in the absence of terminal deoxytransferase, digoxigenin-conjugated TdT, or anti-digoxigenin antiserum.

Western Blotting

Cells were lysed in lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 8.0, 5 mmol/L ethylenediaminetetraacetic acid, 1% Nonidet P-40) containing 1 mmol/L phenylmethyl sulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Total cell lysates were quantified by the Lowry method, equal amounts (50 μg) were separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel by electrophoresis, and transferred onto nitrocellulose membranes. Nonspecific binding was blocked with 5% nonfat milk and 0.1% Tween-20 in Tris-buffered saline. Primary antibodies were directed against total p27 (1:1000, Transduction Laboratories), phosphatase, and tensin homologue deleted on chromosome 10 (PTEN) (1:100; Chemicon International, Inc., Temecula, CA), Akt/PKB or phosphorylated Akt/PKB-Ser 473 (pAkt/PKB)(1:1000, New England Biolabs), or actin (1:500, Sigma). Protein bands were visualized using chemiluminescence (Amersham, Oakville, Ontario, Canada) and band intensities were quantified by densitometry.

Immunoprecipitation and Immunoblotting

After incubation of VD and EB1089 for 72 hours, cells were lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethyl sulfonyl fluoride, aprotinin, and sodium orthovanadate in PBS). Total cell lysates were quantified and equal amounts of protein were immunoprecipitated with monoclonal p27 antibody (Transduction Laboratories), divided into equal parts and analyzed by Western blotting with antisera to phosphothreonine (Santa Cruz Biotechnology Inc., Santa Cruz, CA), pThr187-p27 (1:100; Zymed, San Francisco, CA), Skp2 (1:200, Santa Cruz), or total p27.

p27 Phosphorylation Assay

After 72 hours of VD or EB1089 treatment, cells were trypsinized and 5×10^6 cells were incubated with ortho-32 P (0.25 mCi/ml) in RPMI 1640 without sodium phosphate (Life Technologies, Inc., Grand Island, NY) for 1.5 hours at 37°C. This procedure was also performed with simultaneous labeling and treatment with VD or EB1089 for 8 hours. After washing with PBS four times, cells were lysed with RIPA buffer and equal amounts of lysate were immunoprecipitated with either p27 antibody (Santa Cruz) or actin and separated on a 12% SDS-polyacrylamide gel, followed by drying and autoradiography.

Statistical Analysis

Data are presented as mean ± SE. Differences were assessed by Student's paired *t*-test. Significance level was assigned at $P < 0.05$.

Results

VD and EB1089 Inhibit Growth of Thyroid Cancer Cells

Effects of VD and EB1089 on cell proliferation were analyzed by measurement of [^{3}H]thymidine incorporation. Both compounds exerted a parallel growth inhibition of ~50% in all cell lines. Representative experiments are shown in Figure 1 in WRO and NPA cells. These cell lines showed a dose-dependent response to both compounds at concentrations from 10^{-10} to 10^{-6}mol/L The combined use of both compounds did not result in an additive effect (data not shown). Trypan blue exclusion confirmed that there was no toxicity attributable to these agents (>95%, data not shown).

Cell growth inhibition index can also be determined by analysis of the Ki-67 antigen, a DNA-binding nuclear protein that is localized with the MIB-1 antibody. The proliferation index of cells treated with VD or EB1089 show similar inhibitory patterns as the [^{3}H]thymidine incorporation (Figure 2).

VD and EB1089 Arrest G_1/S Phase Transition in Cell Cycle

Cell-cycle analysis from three independent experiments revealed that treatment with VD or EB1089 (10^{-7} mol/L) caused a decrease in the percentage of NPA cells in S phase (7.9 ± 0.3, or 7.7 ± 0.3 *versus* control 19.4 ± 0.1, $P < 0.001$), respectively. Similar results were found with the other cell lines (data not shown). The reductions in S-phase fractions were associated with corresponding ac-

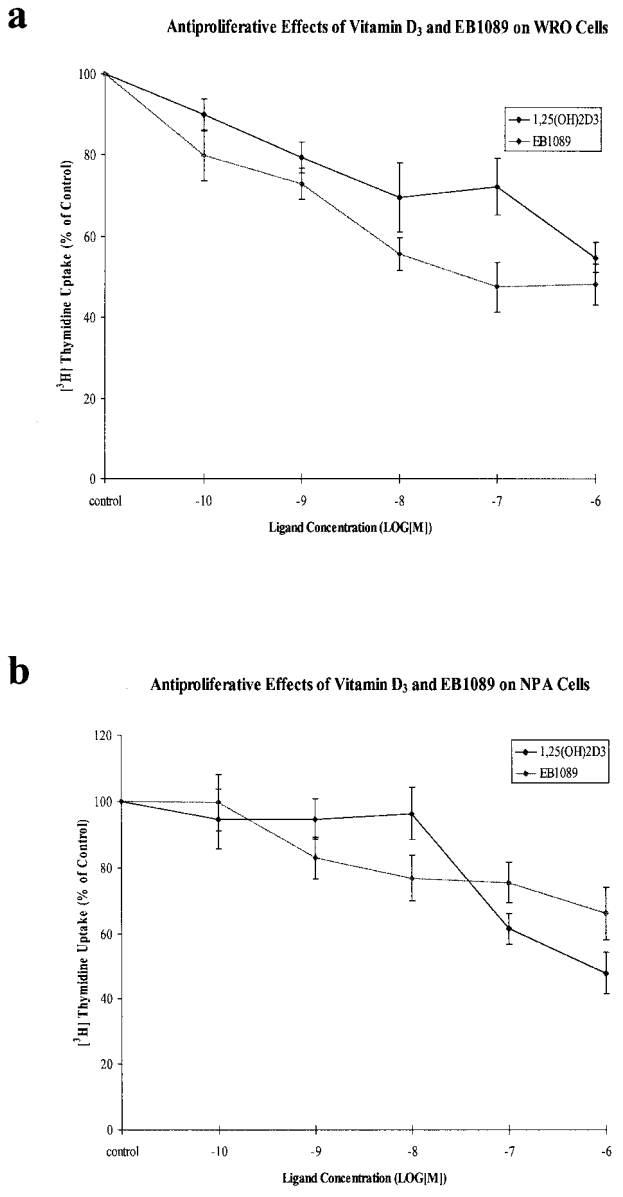

Figure 1. Dose response of VD and EB1089 on thyroid cancer cell proliferation as assessed by thymidine incorporation. WRO cells (**a**) and NPA cells (**b**) were treated with VD or EB1089 at the concentrations indicated for 72 hours. Both cell lines shows a comparable ~50% reduction in response to VD at 10^{-6} mol/L. TPC-1 and FRO cells showed similar responses to both compounds (not shown).

cumulations of cells in G_1 phase and no significant change was observed in the percentage of cells in G_2 + M.

VD and EB1089 Do Not Induce Differentiation of Thyroid Carcinoma Cell Lines

To examine whether VD and EB1089 induced differentiation of human thyroid cancer cells, thyroglobulin immunostaining was used as a differentiation marker. Neither compound, however, resulted in thyroglobulin induction in any of the cell lines examined at concentrations ranging from 10^{-10} to 10^{-6} mol/L (data not shown).

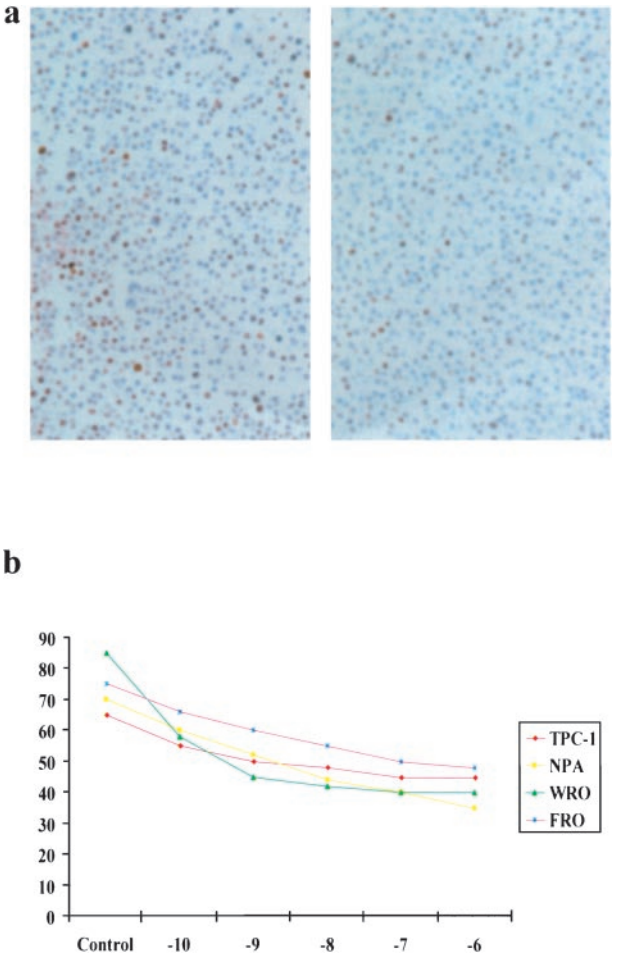

Figure 2. Effect of VD on MIB-1 labeling of thyroid carcinoma cell lines. **a:** A representative photomicrograph of NPA cells showing control cells (**left**) and cells treated with VD 10^{-6} mol/L (**right**). **b:** All cell lines show a similar dose-dependent reduction in MIB-1 labeling with maximum inhibition of 40 to 55%.

VD and EB1089 Do Not Induce Apoptosis in Thyroid Carcinoma Cell Lines

It has been reported that VD and its analogs can induce apoptosis in several tumor cell lines. We examined the effects of EB1089 or VD on the various thyroid carcinoma cell lines using the TUNEL technique. Neither compound showed any sign of enhanced apoptosis at concentrations ranging from 10^{-10} to 10^{-6} mol/L (data not shown).

VD and EB1089 Increase p27 Protein Accumulation

p27, a cyclin-cdk inhibitor, plays a key role in G_1-S phase transition via binding cyclin/CDK complexes, decreasing kinase activity, and arresting G_1/S phase transition in the cell cycle. To assess the possible effects of VD and EB1089, p27 expression was evaluated by immunoblotting. Both compounds increased p27 protein levels in thyroid cancer cell lines in a dose-dependent manner in concentrations ranging from 10^{-10} to 10^{-6} mol/L(Figure 3, a and b).

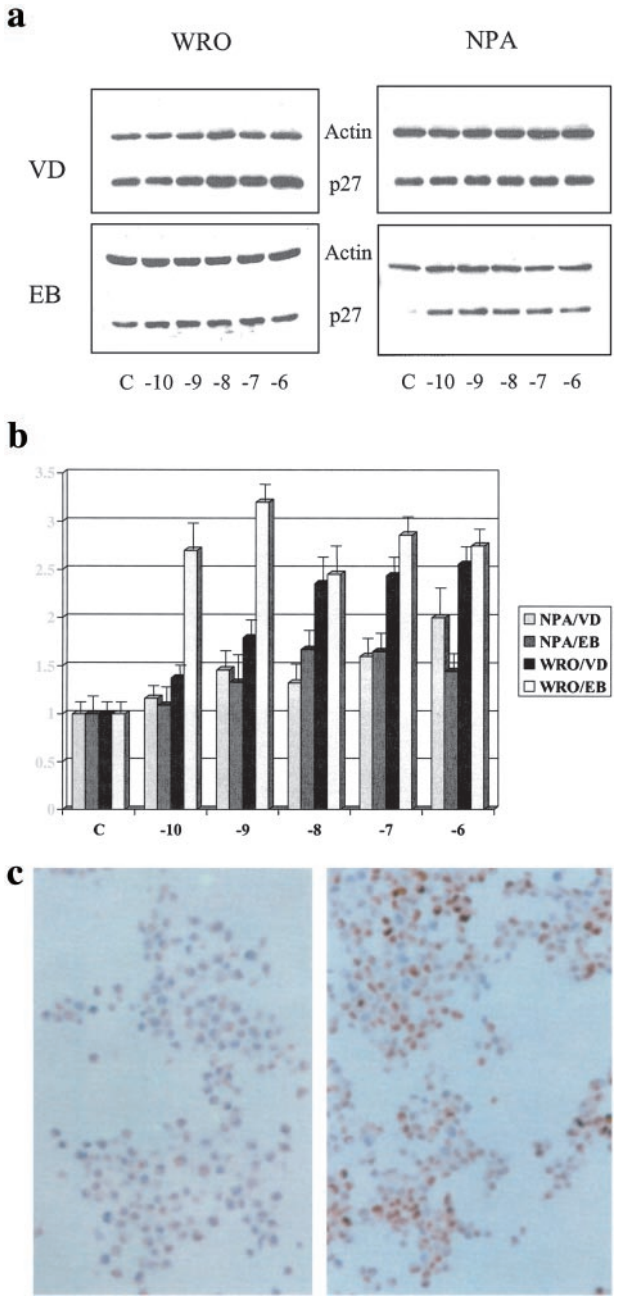

Figure 3. Effect of VD or EB1089 on p27 expression. **a:** WRO (**left**) and NPA cells (**right**) were treated with VD (**top**) or its analog EB1089 (**bottom**) at the concentrations indicated. After 72 hours of incubation, equal amounts (50 μg) of cell lysates were subjected to immunoblotting with antibodies to p27 and actin as shown. **b:** The graph depicts a quantitative densitometric analysis of the p27/actin ratio representing the mean + SEM of three separate experiments, each in triplicate for each treatment. **c:** Immunohistochemical localization of p27 was performed on pellets of control and VD-treated cells as described above. Control cells (**left**) show weak nuclear staining of p27. After treatment with VD (**right**) nuclear staining is increased and no cytoplasmic staining is seen.

VD and EB1089 Induce Nuclear p27 Accumulation

It has been reported that thyroid tumor cell lines exhibit cytoplasmic p27 staining and that expression of cyclin D3 induces cytoplasmic retention of p27.[9] In our system, immunolocalization of p27 was primarily nuclear with the exception of NPA cells that had both cytoplasmic and nuclear staining. Treatment with VD or EB1089 increased nuclear staining in all cell lines (Figure 3c); no translocation of p27 into the cytoplasm was seen.

VD and EB1089 Interfere with Proteasome-Mediated p27 Degradation

p27 expression is regulated at the posttranscriptional level via ubiquitin-dependent and nonubiquitin-dependent degradation pathways.[10] To determine the mechanism of increased p27 expression by VD and EB1089, NPA and WRO cells were treated with these VD compounds (10^{-7} mol/L) alone or in combination with the proteasome inhibitor LLnL (50 μmol/L) for up to 8 hours. Both VD and EB1089 increased p27 expression, an effect that was similar to but not significantly additive to that achieved by LLnL alone (Figure 4). As p27 degradation is dependent on the activity of the SCFSkp2 (Skp2) protein complex that targets phosphorylated proteins for ubiquitin-dependent proteolysis,[11] we demonstrated that VD treatment results in diminished Skp2 expression (Figure 4b) as well as reduced p27 association with Skp2 (Figure 4c). Taken together, these data suggest that increased p27 expression by VD and EB1089 is, at least partly, mediated by diminished targeting by Skp2 of p27 to the ubiquitin-dependent degradation pathway.

The Inhibitory Effect of VD/EB1089 on p27 Degradation Is Mediated through Reduction of p27 Phosphorylation

p27 is phosphorylated at threonine 187 and this phosphorylation contributes significantly to enhanced ubiquitination and degradation by the proteasome.[12] To further elucidate the mechanism of p27 accumulation induced by VD and EB1089, we examined the effect of these compounds on p27 phosphorylation. Lysates from VD/EB1089-treated cells were immunoprecipitated with anti-p27 antibody, followed by immunoblotting with anti-phosphothreonine or anti-p27-Thr187 antibodies. Despite an increase in total p27, pThr content of p27 was not increased in response to VD/EB1089 treatment (Figure 5a). To further demonstrate the effect of VD/EB1089 on p27 phosphorylation, cells were metabolically labeled in the presence or absence of VD or EB1089 for 8 hours (Figure 5b). Diminished p27 phosphorylation was noted under such conditions (Figure 5b) and also when cells were pretreated with VD or EB1089 for 72 hours before metabolic labeling (not shown).

PTEN Is Not Essential for VD/EB1089 Action on p27 Accumulation

PTEN is a tumor suppressor that has been shown to mediate G_1 cell-cycle arrest via the PI3-kinase/Akt/PKB pathway.[13–15] Moreover, multiple lines of evidence support the significance of PTEN in thyroid carcinogenesis.

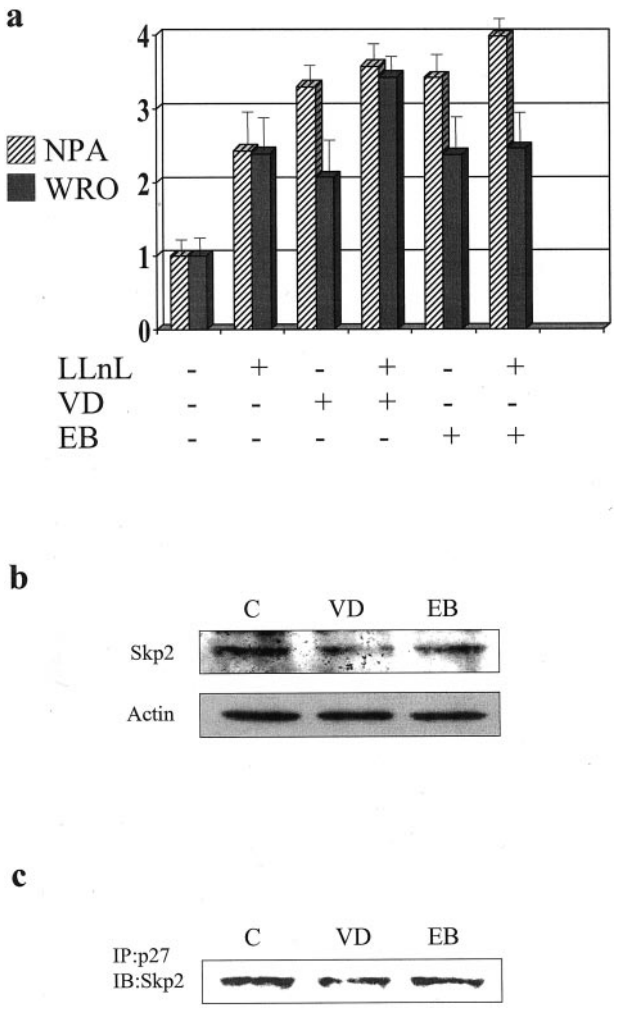

Figure 4. Effect of VD, EB1089, and/or the proteasome inhibitor LLnL on p27 accumulation. **a:** WRO and NPA cells were treated with either VD, EB1089, or LLnL alone or in combination as detailed under Materials and Methods. Each **bar** represents the quantitative densitometric analysis of the p27/actin ratio as a mean + SEM of at least three separate wells from each treatment group from three independent experiments. **b:** Effect of VD or EB1089 treatment on expression of the Skp2 component of the ubiquitin ligase complex. **c:** Immunoprecipitated p27 from VD- or EB1089-treated WRO cells was immunoblotted with anti-Skp2. The blot reveals reduced association between p27 and Skp2 in the presence of VD or EB1089 consistent with diminished p27 targeting to the proteasome for degradation.

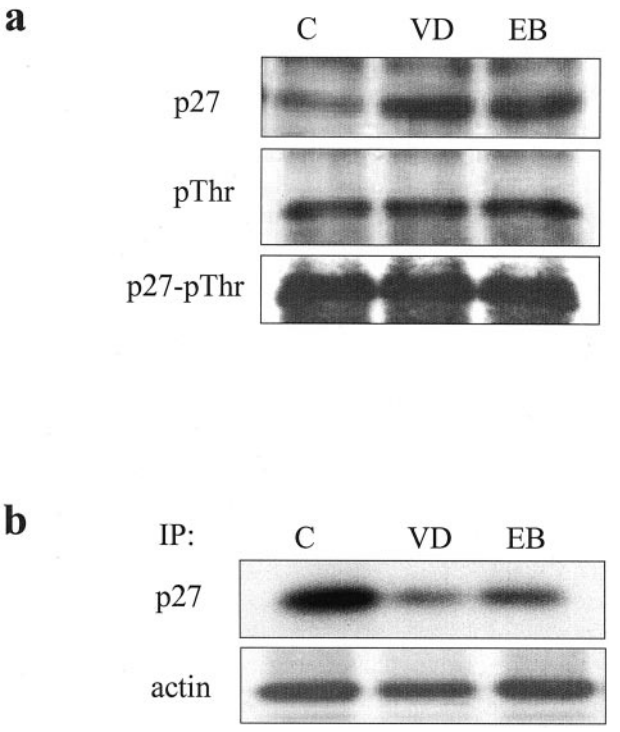

Figure 5. Effect of VD or EB1089 on p27 phosphorylation. **a:** In this representative experiment, WRO cells were treated with VD or EB1089 and lysates immunoprecipitated with a p27 antibody and immunoblotted with p27 antibody (**top**), total phosphothreonine antibody (**middle**), or pThr-p27 (**bottom**). Total p27 levels are significantly greater after treatment with VD or EB1089 but in contrast p27 pThr content remained unchanged by these treatments. **b:** WRO cells were incubated with ortho-32 P with simultaneous labeling and treatment with VD/EB1089 for 8 hours. Equal amounts of lysates were immunoprecipitated with antibodies to p27 or actin (as indicated), separated on SDS-polyacrylamide gel electrophoresis, and autoradiographed.

Loss of heterozygosity of PTEN has been documented in thyroid follicular carcinoma[16–18] and some thyroid carcinoma cell lines.[19,20] Moreover, germline mutations of PTEN result in follicular thyroid adenoma and carcinoma.[21] Thus, to begin to test the potential of the PTEN/PI3-kinase/Akt/PKB pathway, cells were treated with the phosphatase inhibitor pervanadate that we and others have previously shown to activate this pathway.[22] Treatment with this inhibitor diminished basal p27 levels and abrogated VD/EB1089 induction of p27, consistent with a phosphatase role in mediating this VD/EB1089 effect (Figure 6a). To specifically investigate whether VD or EB1089 can regulate p27 through PTEN in thyroid carcinoma cells, WRO and NPA cells were treated with these compounds and cell lysates were analyzed by immunoblotting for PTEN and its downstream target pAkt/PKB.[15] Interestingly, despite the similar effects of VD/EB1089 on p27 accumulation in both cell lines, only WRO but not NPA cells showed an increase in PTEN expression (Figure 6b) or associated reduction in pAKT/PKB responses (Figure 6d). We also examined the effect of VD/EB1089 treatment in the presence or absence of PTEN transfection. Transfection of PTEN resulted in increased p27 accumulation an effect that was modestly additive in the presence of VD/EB1089 (Figure 6c). Taken together these findings suggest that PTEN is at least partially involved in mediating the effect of VD/EB1089 on p27.

To further elucidate the signaling cascade involved in p27 induction in thyroid cancer cells, WRO and NPA cells were incubated with the PI-3 kinase inhibitors wortmannin or LY294002 in the presence or absence of VD/EB1089. Treatment with wortmannin (0.2 μmol/L) or LY294002 (20 μmol/L) resulted in decreased pAkt/PKB (Figure 6d) with p27 accumulation in WRO and NPA cells, further highlighting the potential importance of the Akt pathway in the regulation of p27 in both thyroid cancer cell lines.

Discussion

We have shown that VD and its noncalciomimetic analog EB1089 inhibit thyroid carcinoma cell growth. Inhibition

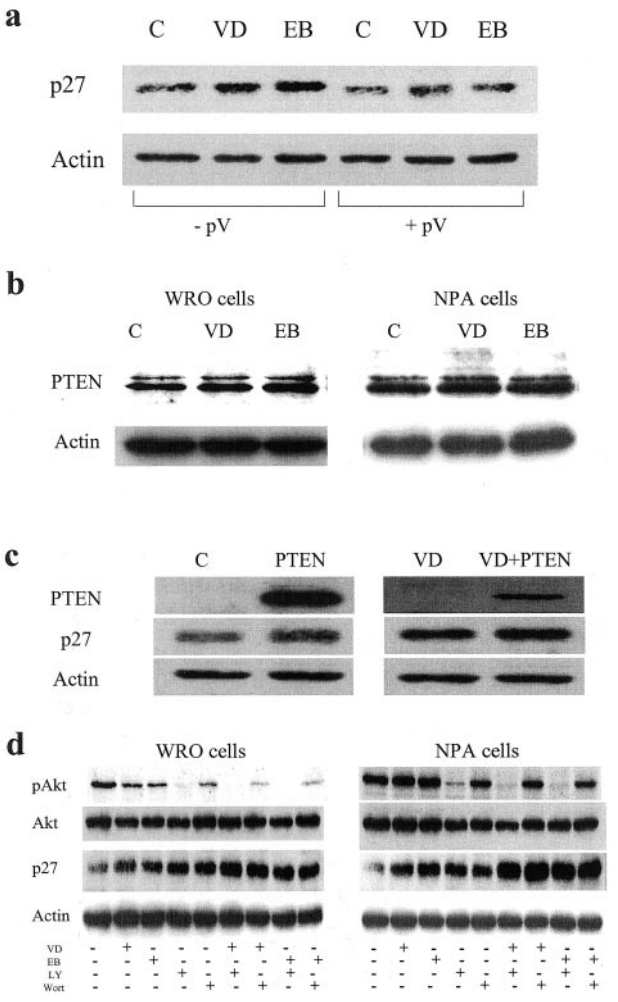

Figure 6. The role of PTEN/Akt/PKB pathway in p27 regulation by VD. **a:** WRO cells were treated with VD or EB1089 in the presence or absence of the phosphatase inhibitor pervanadate (pV) as indicated. Cell lysates were immunoblotted with antibodies to p27 or actin as indicated. Note the effect of phosphatase inhibition on abrogation of VD/EB1089-mediated p27 accumulation consistent with a role for phosphatase action in mediating VD/EB1089 effect on p27. **b:** WRO and NPA cells were treated with either VD or EB1089 as detailed under Materials and Methods. Note the induction of PTEN expression by VD/EB1089 in WRO but not NPA cells. Blots were stripped and reprobed with an anti-actin antibody. **c:** Transfection of PTEN results in p27 accumulation 24 hours after PTEN transfection (**left**) an effect not significantly enhanced when cells were subsequently treated for 24 hours with VD (**right**). Note the effect of PTEN introduction on p27 accumulation that was minimally enhanced by the subsequent treatment with VD. **d:** To investigate the effect of the downstream PI3 kinase pathway on p27 accumulation in thyroid carcinomas, WRO and NPA cells were treated with VD, EB1089, or the PI3-kinase inhibitors wortmannin (wort), or LY900402 (LY) alone or in combination as indicated. Lysates were resolved on SDS-polyacrylamide gel electrophoresis and probed with specific antisera to pAkt/PKB, total Akt/PKB, or p27. Blots were stripped and reprobed with anti-actin antibody. Note the effect of VD/EB1089 on reduction of pAkt/PKB in WRO but not NPA cells consistent with the induction of PTEN activity by VD/EB in WRO but not NPA cells. Note, however, the comparable effects of PI-3 kinase inhibitors on pAkt/PKB and p27 in WRO and NPA cells highlighting the importance of this pathway in p27 regulation in thyroid cancer cells. The results are representative of three separate experiments each of which included at least three separate wells pooled for each treatment group.

by VD and its analog 22-oxacalcitriol (OCT) in the thyroid anaplastic (TTA-1,TAC-1) and papillary (TPC-1,2,3,4) carcinoma cell lines has also been shown by others.[23] Both VD and OCT exert dose-dependent inhibition of cell growth as assessed by an MTT assay. Interestingly, these effects were more pronounced in the anaplastic than in differentiated papillary carcinoma cell lines.[23] Our data reveal that VD's inhibitory effects can be demonstrated in well-differentiated as well as poorly differentiated thyroid carcinoma cell lines. Consistent with our findings, OCT was shown to bind to its receptor at the same rate as $1,25(OH)_2D_3$ and inhibited the proliferation of NPA cells *in vitro* in a dose-dependent manner.[24]

The mechanisms by which VD and its analogs exert their anti-proliferative effects are not well established. VD is known to induce the mRNA and protein expression of Bcl-2 but has no effect on Bcl-x or Bax expression. The increase in Bcl-2 expression correlates with protection of thyrocytes against the induction of apoptosis by either staurosporine or UV irradiation.[25] Moreover, VD-induced increases in the expression of Bcl-2 could be mimicked by analogs with high nuclear VDR affinity, but not by analogs with only nongenomic actions.[26] Our data indicate that VD and it's analog with nongenomic action EB1089 display anti-proliferative actions in thyroid carcinoma cell lines. These effects were associated with induced p27 protein expression without a direct increase in cell apoptosis.

Progression of dividing mammalian cells is governed by a series of cyclin proteins that positively regulate a family of CDKs. The sequential activation/inactivation of cyclin/CDK complexes in turn is modulated by the interaction between constitutively expressed CDKs and oscillating cyclin levels. CDK activity is positively regulated by phosphorylation at a conserved threonine by a CDK-activating kinase (CAK). Conversely, CDK inhibitors (CDI) are classified into two major families. The INK4 (p15, p18, p19) proteins bind and inhibit CDK4 and CDK6 preventing cyclin D association.[27] The Cip/Kip family includes p21 and p27 that inhibit a broader group of CDKs by binding to several cyclin/CDK complexes that are essential for G_1 progression and S-phase entry. The cyclin E/CDK2 complex represents a major target of p27 inhibitory action. Whereas p21 expression is diminished in quiescent cells, p27 is induced on serum starvation and is required for G_1 arrest.[28] Moreover, increased p27 expression has been shown to mediate G_1 arrest resulting from contact inhibition, interferon, or interleukin treatment.[27] Increased p27 expression has also been reported to be associated with increased susceptibility to apoptosis in some systems[29] but not others.[30] Indeed, cleavage and inactivation of p27 have been suggested to be important steps in the induction of apoptosis in some cell types.[27] Here we demonstrate, however, that VD and its analog EB1089 increase p27 expression, an effect that was associated with G_1 arrest in thyroid carcinoma cells without concomitant induction of apoptosis. Our findings are consistent, however, with the effects of VD analogs on growth arrest of MCF-7 breast cancer cells without increased apoptosis.[30]

p27 is considered to be a tumor-suppressor protein playing a critical role in the pathogenesis and possibly prognosis of several human malignancies.[31,32] In particular, thyroid neoplasms exhibit significantly lower p27 protein levels than normal thyroid tissue with poorly differentiated carcinomas having the lowest p27 immuno-

histochemical staining.[31,33,34] Others have suggested that the localization of p27 may be of importance in the thyroid where cyclin D3 expression has been reported to be associated with cytoplasmic localization of p27. Expression of cyclin D3 in thyroid carcinoma cell lines induced cytoplasmic retention of co-transfected p27, an effect considered to remove p27 from intranuclear complexes that contain cyclins A to E and Cdk2.[9] In our study, we noted that the accumulation of p27 remained within the nuclear compartment with no evidence of cytoplasmic translocation.

Recent evidence suggests that VD may stimulate the transcription of the p27 gene although the latter lacks a VD response element.[35] Using gel shift assays, VD was shown to stimulate the transcription factors Sp1 and NF-Y DNA binding activities in inducing the p27 gene,[35] indicating that VD may indirectly modulate p27 at the transcriptional level. Nevertheless, p27 expression is primarily regulated at the posttranscriptional level. On mitogen stimulation, p27 undergoes rapid degradation by the ubiquitin-proteasome pathway.[36] Multiple lines of evidence support the notion that VD-induced p27 accumulation is the result of diminished degradation. We show here that under the same treatment conditions, the proteasome inhibitor LLnL mimics the effects of VD or EB1089 on p27 accumulation. Moreover, phosphorylation of p27 by cyclin E/CDK2 has been shown to trigger ubiquitin-mediated degradation of p27.[37] Degradation of p27 is also dependent on the activity of the SCFSkp2 a protein complex that targets phosphorylated proteins for ubiquitin-dependent proteolysis.[11] SCFSkp2 specifically associates with phosphorylated Thr-187 in targeting p27 degradation.[11] We show here that VD and its analog EB1089 abrogate the rise of phosphothreonine content of p27 inasmuch as these compounds' effects were uncoupled from their parallel effects on total p27. These findings were corroborated by direct p27 phosphorylation assays. We also show diminished Skp2 expression as well as association with p27 in response to VD treatment. These data suggest that VD-induced p27 accumulation and reduced degradation are mediated, at least partly, through diminished p27 phosphorylation and association with Skp2.

Mutations and/or deletions of PTEN have been implicated in various malignancies, including thyroid carcinoma.[38,39] Moreover, mice heterozygous for PTEN deletion (+/−) develop papillary thyroid cancer[40–42] further highlighting the relevance of intact PTEN function in normal thyroid cell growth. Indeed down-regulation of PTEN and p27 have been noted in a subset of human sporadic thyroid tumors[16–18] as well as thyroid carcinoma cell lines.[19,20] WRO cells express low levels of PTEN mRNA and protein compared to NPA cells that express low levels of mRNA but normal to high levels of PTEN protein. We show here that despite the comparable effects of VD/EB1089 on p27 in WRO and NPA cells, PTEN/pAkt/PKB protein levels were regulated by VD/EB1089 in WRO but not NPA cells. Transfection of PTEN also resulted in p27 accumulation an effect that was only modestly enhanced by subsequent treatment with VD. Taken together, these findings suggest that although PTEN may participate in mediating VD action in some thyroid cells, it is not an essential component for the effect of VD on p27 in thyroid carcinoma cells.

Loss of PTEN function leads to the constitutive activation of Akt/PKB via enhanced phosphorylation of threonine 308 and serine 473.[43,44] Consistent with these findings, we demonstrate that VD/EB1089-induced PTEN expression in WRO cells is associated with downstream reduction in pAkt/PKB. NPA cells that failed to demonstrate an increase in PTEN immunoreactivity in response to VD/EB1089 treatment also demonstrated no change in pAkt/PKB. In both cell lines, however, treatment with PI-3 kinase inhibitors, wortmannin, and LY294002 regulated p27 and pAkt/PKB consistent with a potential PTEN/Akt/PKB-dependent but not essential role for this pathway in regulating p27 in thyroid cancer cells. Our findings of the lack of VD/EB1089-induced apoptosis in our model further supports the Akt-independent pathway in VD/EB1089 action, because Akt is recognized to play an important role in triggering apoptotic pathways.[19]

The active form of VD has long been known to have anti-proliferative actions. The hypercalcemic potential of VD limits its clinical use, however, noncalciomimetic analogs such as EB1089 may be clinically useful. In our studies, VD and EB1089 resulted in similar effects on p27 accumulation and cell-cycle arrest. The common inhibitory effects on cell-cycle progression and p27 protein accumulation lend themselves to the potential use of noncalciomimetic VD analogs in the treatment of thyroid cancer irrespective of PTEN/Akt status.

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