p27Kip1 (p27) is a tumor suppressor whose stability is controlled by proteasome-mediated degradation, a process directed in part by cyclin-dependent kinase 2 (CDK2)-mediated phosphorylation of p27 at Thr187 and its subsequent interaction with the Skp1-Cullin-F-box protein/Skp2 (Skp2) ubiquitin ligase. The present study shows that 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) arrests ovarian cancer cells in G1 by stabilizing the p27 protein. 1,25(OH)2D3 initiates a chain of events by decreasing the amounts of cyclin E and cyclin E-associated CDK2 activity. As a result, p27 phosphorylation at Thr187 and consequently the interaction with Skp2 are decreased. 1,25(OH)2D3 also increases p27 stability by decreasing the abundance of Skp2. It is the combined effect of 1,25(OH)2D3 on both the CDK2-dependent phosphorylation of p27, and thus its affinity for Skp2, and Skp2 expression that dramatically increases the stability of the p27 protein. Similar to its effects in ovarian cancer cells, 1,25(OH)2D3 induces p27 accumulation in wild type mouse embryo fibroblasts and arrests wild type but not p27-null mouse embryo fibroblasts in G1. Stable expression of Skp2 in OVCAR3 cells diminishes the G1 arrest and decreases the growth response to 1,25(OH)2D3. Taken together, the results of this study identify p27 as the key mediator of 1,25(OH)2D3-induced growth suppression in G1 and show that the hormone achieves this by decreasing the activity of CDK2 and reducing the abundance of Skp2, which act together to degrade p27.

The active metabolite of vitamin D, 1,25(OH)2D3, is a lipophilic hormone essential for bone homeostasis and the maintenance of serum calcium. The classical target tissues for 1,25(OH)2D3 include bone, intestine, and kidney. In addition to the above well defined role, 1,25(OH)2D3 regulates the proliferation and differentiation of both normal and malignant cells of many tissue types. 1,25(OH)2D3 and its synthetic analogs inhibit carcinogenesis in mouse skin (3, 4), decrease the size of transplanted sarcomas, reduce lung metastasis in mice (5), suppress the growth of human colon cancer cell-derived xenografts in immune-suppressed mice (6), and increase the differentiation and decrease the proliferation of leukemia (7), breast cancer (8), prostate cancer (9), colon cancer (10), and squamous carcinoma (11) cells. Growth analyses have demonstrated that 1,25(OH)2D3 causes cancer cells to accumulate in G1 (9) or G2 (12, 13) or undergo apoptosis (14, 15), suggesting that 1,25(OH)2D3 or its synthetic analogs are potential agents for cancer treatment or chemo-prevention.

Together with breast and prostate cancers, ovarian epithelial cancer (OCA) mortality and incident rates are lower in countries within 20 degrees of the equator (16) where the amount of sunlight exposure is high. For women between the ages of 45–54, the OCA mortality rate is five times greater for those living in the northern United States than in the southern United States (17). In the epidermis, sunlight controls the first step of 1,25(OH)2D3 synthesis, namely, the photoconversion of 7-dehydrocholesterol to pre-vitamin D3. Exposure to sunlight, rather than food consumption, has been shown to be the primary source of 1,25(OH)2D3 (18). Therefore, the inverse correlation between sunlight exposure and OCA mortality suggests that decreased synthesis of 1,25(OH)2D3 may contribute to OCA initiation and/or progression. Immunohistochemical analyses and ligand binding assays have found vitamin D receptor in rat (19) and hen (20) ovaries, indicating that the ovary is a target organ for 1,25(OH)2D3. Studies have also described vitamin D receptor expression in gynecologic neoplasms including OCA (21, 22), raising the possibility that 1,25(OH)2D3 or its synthetic analogs may be effective against OCA.

Previous studies have indeed shown that the growth of OVCAR3 cells is suppressed by 1,25(OH)2D3 (22) and that 1,25(OH)2D3 arrests OVCAR3 cells in G1/M by a mechanism involving GADD45 (13). The current study shows that 1,25(OH)2D3 arrests OCA cells in G1 by increasing the abundance of p27, an inhibitor of cyclin-dependent kinase (CDK) activity. 1,25(OH)2D3 stabilized the p27 protein by decreasing the activity of cyclin E-associated CDK2 and the abundance of Skp2. 1,25(OH)2D3 also increased p27 levels in mouse embryo fibroblasts (MEFs) and arrested wild type MEFs but not p27-null MEFs in G1. Overall, our study identifies p27 as an important mediator of the growth-suppressing activity of 1,25(OH)2D3 in OCA cells.

The abbreviations used are: 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; OCA, ovarian epithelial cancer; CDK, cyclin-dependent kinase; MEF, mouse embryo fibroblast; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.
**EXPERIMENTAL PROCEDURES**

**Materials**—1,25(OH)2D3 was purchased from Calbiochem (La Jolla, CA). Plasmid pcDNA3-Skp2 has already been described (23). Anti-cyclin E, anti-c-CDK2, and anti-p-p27(Thr180) antibodies were from Santa Cruz Biotechnology Inc. (California), anti-p27 antibody was from Transduction Laboratories (Lexington, KY), anti-cyclin A Ab-1 antibody was from NeoMarkers, Inc. (Fremont, CA), anti-Skp2 antibody was from Zymed Laboratories, Inc. (South San Francisco, CA), and anti-p21(145-151)(p21) antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Gene-specific primer/probe sets for real time PCR, including p27, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Skp2, and cyclin E were purchased from Applied Biosystems (Foster City, CA).

**Cells and Cell Culture**—The human ovarian cancer cell line OVCAR3 (obtained from American Type Culture Collection, HTB-161) was cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum, 2 mM L-glutamine, penicillin (50 units/ml), streptomycin (50 μg/ml), 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/liter glucose, 1.5 g/liter sodium bicarbonate, and 10 μg/ml bovine insulin. Other human ovarian cancer cell lines including 2008, CAOV3, OVCAR5, OVCAR10, SKOV3 cells, Chinese hamster ovary cells, MEFs derived from p27-null (24), p21-null (25), and the corresponding wild type mice were all maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum at 37 °C in a humidified incubator with 5% CO2. For 1,25(OH)2D3 treatment, the cells were replaced with fresh medium containing vehicle or 1,25(OH)2D3 every third day.

**Colorimetric MTT Assay and Cell Cycle Analysis**—MTT assays were performed as described (26). For each data point, eight samples were analyzed in parallel. Absorption at 595 nm (A595) was measured on a MRX microplate reader (DYNEX Technologies, Chantilly, VA). Cell cycle distribution of sample was determined by flow cytometry analysis of cells stained with propidium iodide as described previously (27). 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.

**Real Time PCR**—RNA was prepared using the RNeasy RNA isolation kit (Qiagen) and reverse transcribed. An RNA pool was generated by mixing aliquots of RNA from cells treated with vehicle or 1,25(OH)2D3 for various times. Concentrations of the pooled RNA ranging from 0 (buffer alone) to 10 ng/μl were used in the PCR analysis to generate the standard curve for each gene. The C value was generated using the ABI PRISM 7700 SDS software version 1.7 and then exported to an Excel spreadsheet where equations from the standard curve were generated. Using the C values, the concentrations of the p27, Skp2, cyclin E, and GAPDH mRNAs were calculated from the equations. Each sample was analyzed at two different concentrations (50 ng/μl and 0.5 ng/μl) with one resulting in the most sensitive region of the standard curve present. Samples at each concentration were analyzed in triplicate. The value of each gene of interest was divided by the average value of the cognate GAPDH value to provide normalized values.

**Pulse-Chase Experiment**—OVCAR3 cells were treated for 6 days with 1,25(OH)2D3 or ethanol (EtOH) and replicated in 60-mm dishes to ensure that the starting number of cells used for the pulse-chase was the same for all samples treated with 1,25(OH)2D3 or vehicle. The cells were rinsed with Met/Cys-deficient RPMI 1640 medium (Invitrogen) and incubated in the same medium for 1 h. [35S]Methionine (metabolic labeling grade, Promix; Amersham Biosciences) was added to a final activity of 0.1 mCi/ml, and the cells were pulsed for 2 h. The cells were then washed and chased for different times in medium containing 10 mM methionine. p27 protein was immunoprecipitated and resolved on a 12% SDS gel. The gels were then dried and exposed to an x-ray film. The bands were cut out and counted by liquid scintillation counting.

**Immunological Analysis**—For immunoprecipitations, cell extracts were prepared in ice-cold buffer containing 20 mM Tris, pH 7.6, 250 mM NaCl, 3 mM EDTA, 5 mM β-glycerophosphate, 100 μM Na3VO4, 0.5% Noutet P-40, and protease inhibitor mixture (Roche Applied Science). The protein concentrations were determined by the Bio-Rad protein assays (Richmond, CA). The extracts (200 μg of protein) were incubated with 2 μg of antibody for 2 h at 4 °C and subsequently with 25 μl of a 50% slurry of protein A-agrose for an additional 2 h at 4 °C. The beads were washed three times and boiled for 5 min in 2× SDS-PAGE sample buffer.

**Antibodies**—Immunoprecipitates or cell extracts (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was performed as described previously (27).

For immunocomplex kinase assays, cell extracts containing equal amounts of protein (200 μg) were incubated with anti-cyclin E or anti-cyclin A antibodies. Immunoprecipitates were incubated for 30 min at 30 °C in 30 μl of a buffer containing 20 mM HEPES, pH 7.4, 10 mM p-nitrophenyl phenylphosphonate, 20 mM MgCl2, 2 mM EDTA, 2 mM EGTA, 100 μM Na3VO4, 20 mM ATP, 10 μCi of γ-32P]ATP, and 3 μg of histone H1 as substrate. The reactions were terminated by adding 2× SDS-PAGE sample buffer. After boiling, the supernatants were resolved by SDS-PAGE, and phosphorylated substrate was visualized by autoradiography.

**Establishment of OVCAR3 Stable Clones**—OVCAR3 cells were transfected with 10 μg of pcDNA3-Skp2 plasmid for the establishment of Skp2 stable clones or with pcDNA3 for the Vector-OVCAR3 controls. All of the stable clones were obtained through selection in medium containing 100 μg/ml G418 for a period of about 4 weeks followed by clonal isolation with glass cylinders as described previously (13).

**RESULTS**

**1,25(OH)2D3 Suppresses OCA Cell Growth and Induces Cell Cycle Arrest in G1**—Because our laboratory is interested in identifying the specific proteins that mediate the growth-suppressing activity of 1,25(OH)2D3 in OCA cells, we started our investigation by characterizing the response of OCA cells to 1,25(OH)2D3 in proliferation assays. OVCAR3 cells were treated with different concentrations of 1,25(OH)2D3 for 3, 6 and 9 days, and cell proliferation was determined by MTT assays. As shown in Fig. 1A, 10−7 M 1,25(OH)2D3 inhibited the growth of OVCAR3 cells. 38 and 53% reductions in cell proliferation were seen on days 6 and 9, respectively. 1,25(OH)2D3 did not affect the proliferation of OVCAR3 cells at concentrations of 10−4 M or less.

To determine the mechanism underlying 1,25(OH)2D3-induced growth suppression, OVCAR3 cells were treated with vehicle or 10−7 M 1,25(OH)2D3 for 6 days, and the positions of cells in the cell cycle were determined by flow cytometry (Fig. 1B). Treatment with 1,25(OH)2D3 caused a 15% decrease in the percentage of cells in S phase and a concomitant increase in the percentage of cells in G0/G1 (Fig. 1C), showing that 1,25(OH)2D3 arrests OVCAR3 cells at G0/S checkpoint.
1,25(OH)2D3 Increases p27 but Not p21 Abundance in OCa Cells—It has been reported that 1,25(OH)2D3 regulates the expression of p21, p27, or both depending on the cell type (28, 29). p21 is a primary target gene of 1,25(OH)2D3, and its expression is regulated at the transcriptional level by the hormone through vitamin D response elements in its promoter (30). Because p21 and p27 are potential mediators of the negative effects of 1,25(OH)2D3 on the G1 progression of OCa cells, their abundance in OVCAR3 cells treated with vehicle or 1,25(OH)2D3 was compared in immunoblots. As shown in Fig. 2A, treatment of cells with 1,25(OH)2D3 for 3, 6, or 9 days had little effect on p21 levels. In contrast, 1,25(OH)2D3 increased the amounts of p27 in a time-dependent manner (more than 5-fold after 9 days). The level of β-actin was not altered by 1,25(OH)2D3, showing that the increased level of p27 in the treated cells is not due to loading variations.

To determine whether the p27 accumulation correlates with the sensitivity of OCa cells to 1,25(OH)2D3, multiple OCa cell lines were treated with 10−7 M 1,25(OH)2D3, and then growth suppression and p27 induction was determined by MTT assays and immunoblotting analysis. As shown in Fig. 2B, 1,25(OH)2D3 inhibited the growth of OVCAR5, OVCAR10, and Chinese hamster ovary cell lines by 16–40% (upper graph), but had little effect on the growth of OVCAR3. The levels of p27 protein in sensitive cells, not resistant cells, were induced in a time-dependent manner by 1,25(OH)2D3 as in OVCAR3 cells (Fig. 2C). These data suggest that the p27 induction is not limited to OVCAR3 cells. Instead, the induction correlates with the growth response of OCa cells to 1,25(OH)2D3.

1,25(OH)2D3-induced p27 Accumulation is Due to Increased p27 Protein Stability—To determine whether the increased levels of p27 protein in 1,25(OH)2D3-treated cells is due to up-regulation at the transcriptional level, amounts of p27 mRNA were determined by real time PCR. As shown in Fig. 3A, the level of p27 mRNA after normalization with GAPDH was decreased by 50% instead of increased by 1,25(OH)2D3. The data suggest that the increase in the level of p27 protein in cells exposed to 1,25(OH)2D3 is not due to increased transcription rate or increased stability of the p27 mRNA.

Because protein stability is a critical step for p27 regulation (31) in mammalian cells, we next examined whether 1,25(OH)2D3 affected p27 stability in a pulse-chase experiment. In cells treated with vehicle, p27 protein was almost undetectable after chasing for 1 h (Fig. 3B). In contrast, amounts of p27 did not appreciably decrease in 1,25(OH)2D3-treated cells during a 4-h chase. Based on the estimation of the S35 signal (Fig. 3C), the half-life of p27 is about 45 min in the control cells but is longer than 4 h in cells treated with 1,25(OH)2D3. These data clearly show that accumulation of p27 protein in treated cells is due to the suppression of p27 degradation by the hormone. The fact that 1,25(OH)2D3 decreased the level of p27 mRNA but...
increased the level of p27 protein indicates that the p27 protein stability, not the transcription rate or mRNA stability, is the limiting step that determines the level of p27 protein expression in OVCAR3 cells.

1,25(OH)2D3 Decreases p27 Phosphorylation at Thr187 and CDK2/Cyclin E Kinase Activity—It is known that p27 degradation is controlled by Thr187 phosphorylation (32). Therefore, the level of p27 phosphorylation at Thr187 was examined by immunoblotting OVCAR3 cell extracts with Thr187 phospho-specific anti-p27 antibody. As shown in Fig. 4A, 1,25(OH)2D3 decreased the level of p27 phosphorylation at Thr187 and, as expected, increased the level of p27 protein in a parallel analysis. Normalization showed that 1,25(OH)2D3 decreased the Thr187 phosphorylation per p27 molecule and that the decrease is greater at 6 (by 67%) and 9 (by 74%) days than at 3 (by 43%) days (Fig. 4B). This suggests that Thr187 is the major phosphorylation site targeted by 1,25(OH)2D3.

Because CDK2 kinase activity is responsible for Thr187 phosphorylation, it was measured in cyclin E immunoprecipitates prepared from cells treated with vehicle or 1,25(OH)2D3. As shown in Fig. 5A, 1,25(OH)2D3 decreased CDK2 activity in a time-dependent manner. 1,25(OH)2D3 did not affect the abundance of CDK2 but reduced the abundance of cyclin E. Unlike the decrease in CDK2 activity, which began 3 days post treatment, the decrease in cyclin E levels was not obvious until the cells were treated for 6 days. This suggests that 1,25(OH)2D3 decreases CDK2 activity through two mechanisms: one that directly inhibits CDK2 activity at early times and one that indirectly reduces CDK2 activity at later times by reducing amounts of cyclin E.

To determine whether the decreased level of cyclin E protein is due to transcriptional repression of the cyclin E gene, amounts of cyclin E mRNA were determined by real time PCR. As shown in Fig. 5B, treatment with 1,25(OH)2D3 for 24 and 48 h decreased the level of cyclin E mRNA by 32 and 40%, respectively, suggesting that the decrease in the level of cyclin E protein is due to 1,25(OH)2D3-induced reduction at mRNA level.
1,25(OH)2D3 Decreases the Affinity of p27 for Skp2 and Amounts of Skp2 Protein in OCa Cells—After site-specific phosphorylation at Thr187, p27 interacts with Skp2 ubiquitin ligase, which results in the ubiquitination of p27 and its degradation in the proteasome. Because 1,25(OH)2D3 decreased the level of p27 phosphorylation at Thr187, it is expected that the hormone will also reduce the interaction between p27 and Skp2. To measure this interaction, Skp2 was precipitated from cells treated with vehicle or 1,25(OH)2D3, and the amount of p27 in the precipitates was detected by immunoblotting. As shown in Fig. 6A, the amount of both p27 and Skp2 in the immunoprecipitates was decreased by 1,25(OH)2D3. After normalization of the signals, it is clear that 1,25(OH)2D3 decreases the interaction of p27 with Skp2 (Fig. 6A). The decrease caused by 1,25(OH)2D3 at 9 days post treatment is about 40%.

In addition to the decreased affinity for p27, the data in Fig. 6A indicated that the amount of Skp2 in OCa cells might also be decreased by 1,25(OH)2D3. Thus, the level of Skp2 protein was measured by immunoblotting without prior immunoprecipitations. As shown in Fig. 6B, 1,25(OH)2D3 decreased the level of Skp2 protein at 6 and 9 days. The parallel analysis showed that 1,25(OH)2D3 increased the level of p27 protein as expected. These data clearly indicated a tight inverse correlation between the level of p27 and Skp2. The decrease in the level of Skp2 protein in the direct immunoblotting analysis (Fig. 6B) was obviously more dramatic than that observed using immunoprecipitates (Fig. 6A), suggesting that the amount of Skp2 antibody used for the immunoprecipitation in Fig. 6A was limited with respect to the amount of Skp2 protein present in the extracts.

To determine whether 1,25(OH)2D3 decreases the level of Skp2 mRNA, real time PCR was performed. As shown in Fig. 6C, 1,25(OH)2D3 decreased Skp2 mRNA by 46% at 48 h post treatment.

p27 Is Required for 1,25(OH)2D3-induced Cell Cycle Arrest in G1 and Contributes to the Overall Growth Response to the Hormone—Because 1,25(OH)2D3 affected many of the cell cycle regulators working at the G1/S checkpoint, an important question that remained to be addressed was whether p27 accumulation is required for 1,25(OH)2D3-induced cell cycle arrest in G1.

As shown in Fig. 7, 1,25(OH)2D3 induced accumulation of p27 in wild type MEFs, and for the obvious reasons, no p27 expression was detected in p27-null MEFs. The induction of p27 in wild type MEFs occurred more slowly than has been reported previously (33). Immunoblotting analysis showed that vitamin D receptor expresses similarly in both wild type and...
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Fig. 8. Effect of 1,25(OH)₂D₃ on the growth and cell cycle distribution of OVCAR3 cells stably transfected with Skp2 (Skp2-OVCAR3). A, OVCAR3 stable clones were treated with EtOH or 10⁻⁷M 1,25(OH)₂D₃ for indicated times. Skp2 and p27 proteins were determined by immunoblotting. The Skp2 membrane was stripped and reprobed with anti-β-actin antibody. B, OVCAR3 stable clones were treated with EtOH or 10⁻⁷M 1,25(OH)₂D₃ and subjected to flow cytometry analysis. Each data point represents duplicate samples. **, p < 0.01 versus control. C, OVCAR3 stable clones were plated in 96-well plates and treated with EtOH or 10⁻⁷M 1,25(OH)₂D₃ for 6 days. Cell growth was determined by MTT assays as for Fig. 1A.

p27-null MEFs (data not shown). In MTT assays, 1,25(OH)₂D₃ decreased the proliferation of wild type MEFs (by 25%) but not p27-null MEFs (Fig. 7B), suggesting that p27 is required for the growth-suppressing activity of 1,25(OH)₂D₃ in MEFs. As a control, both wild type and p27-null MEFs responded similarly to 1,25(OH)₂D₃ (Fig. 7B). Consistent with the result from MTT assays, flow cytometry analysis (Fig. 7C) showed that 1,25(OH)₂D₃ increased the percentage of wild type MEFs in G₀/G₁ from 62 to 71% and correspondingly reduced the percentage of cells in the S phase. The G₁ arrest was not observed in p27-null MEFs. These data show that the G₁ arrest induced by 1,25(OH)₂D₃ depends on p27.

At variance with OVCAR3 cells, no G₀/M arrest was observed in the 1,25(OH)₂D₃-treated MEFs of this particular mouse strain. In a separate study (13), G₀/M arrest was observed in MEFs from another mouse strain. It is thus clear that MEFs from different mouse strains vary in their response to the treatment by 1,25(OH)₂D₃. The lack of G₀/M arrest in MEFs from p27 mice explains why the response of the MEFs to 1,25(OH)₂D₃ is lower than that of OVCAR3 cells.

To determine whether p27 induction is responsible for G₁ arrest and contributes to the overall growth response in OCa cells, we established Skp2-OVCAR3 clones that stably express Skp2 at higher levels (Fig. 8A). Different from control clones containing the empty vector in which 1,25(OH)₂D₃ decreased the level of Skp2 protein, no apparent Skp2 reduction by 1,25(OH)₂D₃ was detected in the stable clones. As expected, the level of p27 protein in the Skp2 clones was much lower than that in the control clones. Although 1,25(OH)₂D₃ increased the level of the p27 in the Skp2 clones, the amount was not increased above the basal level in control clones. Thus, the Skp2 expression in the stable clones “functionally” eliminated the effect of 1,25(OH)₂D₃ on p27.

Flow cytometry analysis (Fig. 8D) showed that 1,25(OH)₂D₃ did not increase the percentage of Skp2 clones in G₀/G₁ nor decrease the percentage in S phase. These data show that the arrest at the G₁/S checkpoint induced by 1,25(OH)₂D₃ on OVCAR3 cells depends on the alteration in the level of the Skp2 and p27. In MTT assays, 1,25(OH)₂D₃ decreased the growth of both the Skp2 and the control clones (Fig. 8C). However, the degree of inhibition after 6 days of treatment is about 45% in control clones and 28% in both of the Skp2 clones, showing that the abrogation of G₁ arrest eliminates one-third of the growth inhibition. The data suggest that 1,25(OH)₂D₃-induced G₁ arrest as a result of Skp2 and p27 alteration contributes to the growth arrest by 1,25(OH)₂D₃ in OVCAR3 cells.

1,25(OH)₂D₃ Decreases the Expression Level of Genes That Function Downstream of p27 to Control S Phase Entry—It is well established that DNA synthesis in S phase is controlled by E2F-regulated genes such as cyclin A, which binds and activates CDK2 (34). p27 inhibits CDK4/6 activity and decreases Rb phosphorylation, resulting in the activation of Rb and the inactivation of E2F. If 1,25(OH)₂D₃ inhibits cells in G₁ through p27 accumulation, 1,25(OH)₂D₃ would be expected to decrease the level of cyclin A as well as the CDK2 activity associated with it. Therefore, the expression level of CDK2 and its activity were measured in cells treated with vehicle or 1,25(OH)₂D₃. As shown in Fig. 9, 1,25(OH)₂D₃ decreased both the level of cyclin A protein as well as the activity of its associated CDK2. 1,25(OH)₂D₃ at 9 days post treatment eliminated more than 90% of cyclin A and its associated CDK2 activity. These data provide additional support for our conclusion that 1,25(OH)₂D₃ inhibits S phase entry through its action on p27.

DISCUSSION

Studies in multiple types of cancer cells have shown that 1,25(OH)₂D₃ alters the level of cell cycle regulators working along the CDK2-Rb-E2F axis, which controls cell cycle progression through the G₁/S checkpoint (35). However, very little is known about the proteins involved in 1,25(OH)₂D₃ action in regulating the cell cycle at the G₁/S checkpoint in OCa cells. The present report shows that 1,25(OH)₂D₃ regulates the expression of Skp2, p27, and cyclins E and A in OCa cells, suggesting the following sequence of actions leading to 1,25(OH)₂D₃-induced G₁ arrest (Fig. 10). 1,25(OH)₂D₃ decreases the expression of cyclin E and Skp2. The decrease in cyclin E abundance results in a decrease in CDK2 activity and p27 phosphorylation on Thr₁⁶⁷, which in turn decreases the affinity of p27 for Skp2. Decreased Skp2 abundance further limits the amount of Skp2 available to interact with p27. The combined effects of the decrease in the affinity of p27 for Skp2 and the decrease in the amount of Skp2 leads to a reduction in
the number of p27 molecules bound to Skp2. The end result is the increased stability and accumulation of p27 protein in the cell, which then induces accumulation of cells arrested at the G1 phase.

Our studies using OVCAR3 stable clones that overexpressing Skp2 and genetically engineered MEFs clearly demonstrate that p27 is the mediator for 1,25(OH)2D3-induced cell cycle arrest in G1, which is consistent with the conclusion reached by a contemporary study (33). The comparison between controls and the Skp2 stable clones in MTT assays shows that the arrest of OVCAR3 cells at the G1/S checkpoint contributes to about one-third of the overall growth inhibition by 1,25(OH)2D3. Unlike the steady induction of p27 by 1,25(OH)2D3 in MEFs observed in our study, Wade et al. (33) reported a rapid and transient induction of p27 in Swiss mouse MEFs. The exact reason for the discrepancy is not clear but likely due to the fact that the MEFs used in the studies are derived from different mouse strains. Studies in our laboratory showed that MEFs from various mouse strains respond very differently to 1,25(OH)2D3.

It is apparent that the inhibition of OCa cell growth by 1,25(OH)2D3 is a slow process. One possible explanation would be that 1,25(OH)2D3 is metabolized by 24-hydroxylase in a way similar to what has been described in DU145 prostate cancer cells (36). Our preliminary microarray analysis has shown that 1,25(OH)2D3 increased 24-hydroxylase by more than 50-fold in OVCAR3 cells (data not shown). This regulation of 24-hydroxylase was recently reported by others (37). However, the growth inhibition of OVCAR3 cells by the synthetic 1,25(OH)2D3, EB1089, that is not metabolized by 24-hydroxylase is also a slow process even though the effective concentration is 10 times lower than 1,25(OH)2D3 (data not shown). This indicates that the slower response may not be due to the metabolism of the hormone. In our unpublished microarray analysis, most of the genes in OVCAR3 cells were regulated modestly by 1,25(OH)2D3 at mRNA level (data not shown). In a recent study (13), we showed that the accumulation of GADD45 protein becomes apparent only after 1,25(OH)2D3 treatment for more than 3 days, whereas the mRNA induction is detected within 2 h. The data suggest that a clear explanation for the slow growth response of epithelial cancer cells to 1,25(OH)2D3 may have to rely on a clear understanding of the mechanism of 1,25(OH)2D3 action in OCa cells, particularly the way by which the relevant target genes for 1,25(OH)2D3 are regulated. Further studies are needed to provide a clear answer for this important question.

Our findings that 1,25(OH)2D3 regulates the level of Skp2 mRNA and that the regulation precedes the decrease in cyclins E and A (Fig. 6C) suggest that Skp2 is likely the 1,25(OH)2D3 response gene that initiates the p27 stabilization process, which is responsible for the early change in CDK2 activity before cyclin E levels start to decrease. The finding that cyclin E levels decrease later than the increase in p27 indicates that this phenomenon and the decrease in its associated CDK2 activity are likely secondary to the hormone-induced increase in p27 levels. Decreased cyclin E, through feedback, further enhances the effect of 1,25(OH)2D3 on p27 stability by decreasing Thr187 phosphorylation. Similar to cyclin E, the decrease in cyclin A levels also occurs later than the change in p27, suggesting that such a change may also be secondary to p27 accumulation. In contrast to that of cyclin E, the decrease in cyclin A abundance is not related to p27 stability. Cyclin A, instead, likely acts downstream of p27-Rb-E2F to directly control cell cycle progression through S phase. Further studies are needed to establish whether the effect of 1,25(OH)2D3 on the expression of cyclins E and A depends on p27 accumulation.

In thyroid carcinoma cells, natural and synthetic 1,25(OH)2D3 analogs induce p27 hypophosphorylation and diminish association of p27 with Skp2 partly through PTEN phosphatase and the subsequent inhibition of protein kinase B signaling (38). In pituitary tumors, 1,25(OH)2D3 decreases the interaction of p27 with Skp2 and CDK2 (39), but the effect appears to be independent of PTEN phosphatase. Synthetic 1,25(OH)2D3, EB1089, has been reported very recently to decrease Skp2 mRNA levels in head and neck squamous carcinoma cells (40). At variance with this contemporary study, our investigation suggests that the increased p27 stability induced by 1,25(OH)2D3 is due to its combined effect on both p27 hypophosphorylation and Skp2 reduction. Because 1,25(OH)2D3 decreases the expression of cyclin E and the associated CDK2 activity, which explains the decreased phosphorylation of p27 induced by the hormone, our findings obviously differ from those in thyroid tumors (38), which implied that the effect on p27 phosphorylation is due to the alteration of PTEN phosphatase by the hormone. Consistent with our conclusion, however, 1,25(OH)2D3 did not decrease the activity of protein kinase B in OVCAR3 cells (data not shown).

p21 has been shown to be transcriptionally up-regulated in leukemia cells by 1,25(OH)2D3 through a vitamin D response element identified in the p21 promoter (28). As shown in Fig. 2, p21 protein levels were not changed in OCa cells by 1,25(OH)2D3. The reason that vitamin D receptor/retinoic X receptor is clearly transcriptionally active in OVCAR3 cells but fails to mediate the transcriptional up-regulation of p21 remains to be elucidated. It is interesting to note that a decrease in p27, but not p21, levels has been frequently detected in OCa tissues (41) and that the decrease in p27 abundance is mainly due to changes in protein stability (42). More interesting, a recent report (43) showed that amounts of p21 increased together with that of cyclin E, whereas amounts of p27 decreased in OCa derived from 7,12-dimethylbenzanthracene-treated in rats, suggesting the existence of intrinsic differences between the function of the two CDK inhibitors in OCa cells.

In addition to the decrease in p27 levels, cyclin E expression is increased in OCa cells (44). Studies investigating cyclin gene amplification and overexpression in breast and ovarian cancers have yielded evidence for the selection of cyclin D1 in breast cancers and cyclin E in ovarian tumors (44). A recent study also suggests that the Skp2 levels are increased in ovarian epithelial cancers as compared with benign tumors and low malignant potential neoplasms (45). It remains to be determined whether the treatment of OCa with 1,25(OH)2D3 will restore these alterations to the level in normal cells. Overall, the results of the current study together with those of a previous one that defined the mechanism of G2/M arrest induced by 1,25(OH)2D3 in OCa cells (13) provide a strong rationale for further investigating 1,25(OH)2D3 and its synthetic analogs as a possible chemo-preventive and/or therapeutic drugs for OCa, a gynecological cancer associated with an otherwise dismal prognosis.
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