Induction of Ovarian Cancer Cell Apoptosis by 1,25-Dihydroxyvitamin D₃ through the Down-regulation of Telomerase*

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The maintenance of telomere length is required for continued cell proliferation, and ~85–90% of human cancers, including ovarian epithelial cancers (OCas), show high activity of telomerase. In this study we present evidence to support the hypothesis that 1,25-dihydroxyvitamin D₃ (1,25(OH)₂VD₃) induces OCa cell apoptosis by down-regulating telomerase activity. Quantitative reverse transcription-PCR analysis shows that 1,25(OH)₂VD₃ decreases the level of human telomerase reverse transcriptase (hTERT) mRNA, the catalytic subunit of telomerase. The decrease is not due to transcriptional repression through the putative vitamin D response element present in the 5′ regulatory region of hTERT gene. Instead, 1,25(OH)₂VD₃ decreases telomerase activity by 1,25(OH)₂VD₃-induced growth suppression. Although the cell cycle progression of these clones stably expressing hTERT is inhibited by 1,25(OH)₂VD₃ to a similar degree as that of the parental cells, these clones are more resistant to apoptosis induced by 1,25(OH)₂VD₃. In contrast to parental cells, which lose proliferation potential after the 1,25(OH)₂VD₃ treatment, hTERT-expressing clones resume rapid growth after withdrawal of 1,25(OH)₂VD₃. Overall, the study suggests that the down-regulation of telomerase activity by 1,25(OH)₂VD₃ and the resulting cell death are important components of the response of OCa cells to 1,25(OH)₂VD₃-induced growth suppression.

Vitamin D is a lipophilic hormone, and its effects are mediated by the vitamin D receptor (VDR)1 (1), which is a member of the steroid/thyroid nuclear receptor superfamily. 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. The VDR acts as a ligand-inducible transcription factor that in most cases forms heterodimers with the retinoid X receptor (RXR). The activated receptors bind vitamin D response elements (VDREs) to regulate the expression of the target genes through activation or repression of transcription.

In addition to classic effects on calcium homeostasis, bone density, and mineral metabolism, the active metabolite of vitamin D, 1,25(OH)₂VD₃, modulates cellular proliferation (2, 3), differentiation (4), and apoptosis (5) of both normal and malignant cells. Our recent studies have shown that the growth of multiple OCa cell lines is suppressed by 1,25(OH)₂VD₃ (6), suggesting that active vitamin D compounds are potential therapeutic agents for OCa treatment and prevention. Molecular analyses have identified GADD45 as a primary target gene that mediates the effect of 1,25(OH)₂VD₃ on G₂/M arrest (7) and the p27 CDK inhibitor as the mediator for the arrest at G₁/S checkpoint (6).

The ends of chromosomes, telomeres, are subject to progressive shortening in normal somatic cells, leading ultimately to irreversible growth arrest, known as senescence (8). In contrast, the telomere length in cancer cells is stabilized by telomerase, an enzyme that catalyzes the synthesis of telomeric DNA repeats. Telomerase is a ribonucleoprotein complex containing three essential components, the telomerase RNA, which contains a sequence complementary to the telomeric TTAGGG repeat, the catalytic subunit, telomerase reverse transcriptase, which catalyzes the reverse transcription of TTAGGG repeats in the telomerase RNA, and the telomerase-associated protein 1, which helps the reverse transcription. Whereas all human somatic cells express the telomerase RNA constitutively (9, 10), the level of hTERT is increased in most human cancers, and its expression determines the cellular activity of telomerase (11). Thus, the expression of the hTERT gene appears to be the rate-limiting factor for telomerase activity in human cancer cells.

The reactivation of telomerase has been shown to be one of the three events required to transform a normal human epithelial cell into a cancer cell (12). An anti-apoptotic function of telomerase has been described in human fibroblasts and neuronal cells (13–15). Studies in epidermoid tumor cells indicate that inhibition of telomerase in tumor cells, whose telomeres are already short, leads to chromosomal damage, which in turn triggers apoptotic cell death (16). Thus, telomerase activation is most likely a causal event for tumorigenesis instead of just a marker for neoplasia, making telomerase an attractive target for discovery of anticancer drugs.

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1 The abbreviations used are: VDR, vitamin D (VD) receptor; RXR, retinoid X receptor; VDRE, vitamin D response element; OCa cells, ovarian epithelial cancer cells; hTERT, human telomerase reverse transcriptase; 1,25(OH)₂VD₃, 1,25-dihydroxyvitamin D₃; EMMA, electrophoresis mobility shift assay; ChIP, chromatin immunoprecipitation; MTT, methylthiazole tetrazolium; TRAT, telomere repeat amplification; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase(s).
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Similar to other cancer cells, there was a significant correlation of telomerase activity in OCa cells with the expression hTERT mRNA but not with the expression of the other two components of telomerase (17, 18). OCa cells containing wild type hTERT are highly tumorigenic, whereas cells expressing dominant negative hTERT fail to form tumors in nude mice (12), supporting hTERT as a potential target for anti-OCa drugs. The work presented here provides experimental evidence that 1,25(OH)2VD3 induces apoptosis in OCa cells, which involves the down-regulation of telomerase activity through degradation of hTERT mRNA. Further studies show that the down-regulation of hTERT and associated apoptosis are major contributors to suppression of OCa cell growth induced by 1,25(OH)2VD3 as well as to the suppressive effect of the hormone on the ability of OCa cells to resume proliferation after hormonal withdrawal.

EXPERIMENTAL PROCEDURES

Materials—pBabe-hTERT (19), hTERT reporter construct pGL3–3328LuC (20), CMV/gal, and p91023B-VDR (7) have been described previously. 1,25(OH)2VD3 was from Calbiochem. Baculovirus-expressed human VDR protein, human RXRα protein, and anti-RXRα antibody were from Affinity BioReagents Inc. (Golden, CO). Anti-VDR antibody was from Chemicon International (Temecula, CA). Anti-FLAG M2 antibody and anti-β-actin antibody were from Sigma. Oligonucleotides were synthesized by Invitrogen. The sequence of the oligonucleotides in sense orientation for producing complementary double-strand oligos for electrophoresis mobility shift assay (EMSA) is 5′-caacagtgtggagcgtgatgtc-3′ (putative hTERT VDRE); capital letters are used to indicate the hexameric core binding motifs in the VDRE primers. Human OC and VDRE-C has been described in a previous study (7). The sequences of PCR primers for chromatin immunoprecipitation (ChIP) assays are 5′-catcgtggtcggctcgc-3′ (upstream) and 5′-gtagagagggg-5′ (downstream).

Cytometry and Flow Cytometry—To measure cell growth, OVCAR3 cells or the hTERT stable clones were plated at 1 × 105 cells/well in 12-well plates and treated with vehicle or 1,25(OH)2VD3 or vehicle. MTT assays were performed as described (21). A570 was read on a MRX microplate reader (DYNEX Technologies, Chantilly, VA).

To determine the apoptosis, cells were harvested by trypsin digestion and washed with phosphate-buffered saline. Cell suspensions were incubated with annexin V-fluorescein isothiocyanate and propidium iodide for 15 min and then subjected to flow cytometry.

Cell cycle analyses were done as described (7). Statistical analysis was performed using the t test. p < 0.05 was considered to be statistically significant.

Telomerase Activity Assays—Telomerase activity was measured by the telomeric repeat amplification protocol using the telomerase PCR-enzyme-linked immunosorbent assay kit (Roche Applied Science) according to the manufacturer’s instructions. Telomerase activity was expressed as absorbance values at 450 nm, measured by a microtiter plate reader with a reference wavelength of 595 nm. All assays were performed in duplicate, and a series of dilutions of control telomerase was examined in parallel to obtain a titration curve used to normalize experimental variations. A PCR-TRAP assay performed with various amounts of protein indicated that 100 ng of protein extract was optimal for quantitative and reproducible assay for telomerase activity in OVCAR3 cells.

PCR Analyses—PCR primers and probes were chosen with the assistance of the Primer Express (PerkinElmer Life Sciences). To avoid amplification of contaminating genomic DNA in reverse transcription–coupled PCR (RT-PCR), upstream and downstream primers were placed in different exons. The forward primer of hTERT probe set 1 (1909F) and FAM/TAMRA probe 1 in exon 4, whereas the reverse primer (2017R) spans exons 4 and 5. The forward primer of hTERT probe set 2 (3081F) spans the junction of exons 13 and 14, and TAMRA/TAMRA probe 2 (3162R) is in exon 14. The nucleotide sequences are as follows: 1909F, 5′-gtcaggtgtgctgctgtaac-3′; 2017R, 5′-gagaagctctgtgctgctgtaac-3′; 3081F, 5′-ttcagagttgctgctgctgtaac-3′; 3162R, 5′-attagagggtaactgtgctgctgtaac-3′; and TAMRA/TAMRA probe 2, 5′-gatacagggtaactgtgctgctgtaac-3′.

Analyses of hTERT and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression by RT-PCR were performed as previously described (22). RNA was isolated with Trizol (Invitrogen) according to the manufacturer’s protocol. hTERT primers 1784 (5′-ggagaggttctgccag-3′) and 1910 (5′-gtatgccagttgctgact-3′) were chosen to amplify a 137 bp of genomic DNA, whereas hTERT, GAPDH transcript was amplified in parallel using primers 5′-ctcagatcattgctgctgctgtaac-3′ and 5′-gtatgccagttgctgactgatac-3′. PCR products were subjected to electrophoresis in 15% polyacrylamide gel and stained with SYBR green.

For real-time PCR analyses, RNA was prepared using the RNeasy RNA isolation kit (Qiagen) and digested with DNase on-column using RNase-free DNase (Qiagen). An RNA pool was generated by mixing aliquots of RNA from cells treated with vehicle or 1,25(OH)2VD3 for various time points. Concentrations of the pooled RNA ranging from 0 (buffer alone) to 50 ng/μl were used in the PCR analysis to generate the standard curve for each gene. The cycle threshold value was generated by 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 cycle threshold values, concentrations of the hTERT and GAPDH mRNAs were calculated from the equations. Each sample was analyzed at two different concentrations (50 and 2 ng/μl), and the result from the linear portion of the standard curve was presented. Samples were analyzed in triplicate at each concentration, and hTERT levels were normalized to the corresponding GAPDH.

EMSA, ChIP Assays, and Transcriptional Analysis—EMSA and ChIP assays were performed as described (7). For transfection studies, OCa cells were plated on 12-well plates and treated with 1,25(OH)2VD3 for 42 h. The cells were harvested and lysed in lysis buffer.

Establishment of hTERT Stable Clones and Measurement of the Telomerase Activity—For the establishment of stable clones expressing hTERT or reporter genes, OVCAR3 cells were transfected with 10 μg of pBabe-hTERT or pGL3–3328LuC plasmids together with 0.5 μg of pcDNA3. The stable clones were obtained through selection with 100 μg/ml G418 for a period of about 4 weeks and isolated by cloning using glass cylinders.

Telomere length was measured using a nonradioactive chemiluminescent assay (Roche Applied Science). 5 μg of genomic DNA was digested overnight with 20 units of HindI and RsaI and separated on a 0.8% agarose gel. After denaturation and neutralization, DNA was transferred to a nylon membrane and hybridized with digoxigenin-labeled (TTAGGG)10 oligonucleotides (Roche Applied Science). The signal was visualized by the digoxigenin luminescence detection. The average telomere length was determined by comparing the signals relative to a molecular weight standard.

RESULTS

Prolonged 1,25(OH)2VD3 Treatment Causes Apoptosis of OCa Cells and Diminishes Their Ability to Re-grow After Withdrawal of Hormone—Recent studies from our laboratories have shown that the growth of multiple OCa cell lines is suppressed by 1,25(OH)2VD3 through cell cycle arrest at both G1/S (6) and G2/M (7) checkpoints. Abrogation of the cell cycle arrest at specific checkpoints only partially relieves the growth-suppressing activity of the hormone (6, 7), suggesting the existence of additional mechanisms. To determine whether 1,25(OH)2VD3 induces apoptosis in OCa cells, OVCAR3 cells were treated with vehicle or 1,25(OH)2VD3 at 10−7 M for 9 days, and cell apoptosis was determined by flow cytometry after staining with annexin V. As shown in the representative profiles (Fig. 1, panel A), 1,25(OH)2VD3 increased the fraction of apoptotic cells. The quantification, based on flow cytometry in two independent experiments, showed that 1,25(OH)2VD3 did not induce apoptosis until the cells were treated for a minimum of 6 days (data not shown), indicating that apoptotic induction, similar to G2/M arrest (7), is also a late event. These analyses suggest that the growth suppression by 1,25(OH)2VD3 is likely due to the combined effect of the hormone on both cell cycle progression and cell death.
To test whether 1,25(OH)2VD3 exerts a long-lasting effect on the growth potential OCa cells, OVCAR3 cells were pretreated with the vehicle or the hormone for different numbers of days and re-plated, and their ability to grow in the absence of 1,25(OH)2VD3 was tested in MTT assays. As shown in Fig. 1, panel C, the ability of OVCAR3 cells to re-grow was decreased as the time of pretreatment with 1,25(OH)2VD3 increased. Pretreatment for 1 or 3 days had minor effects, whereas treatments for more than 6 days diminished the ability of the cells to re-grow after 1,25(OH)2VD3 withdrawal. These data suggest that 1,25(OH)2VD3 exerts a long-lasting effect in OCa cells.

Because the loss of ability to re-grow correlates with apoptosis, it is likely that these two processes are related.

1,25(OH)2VD3 Decreases Telomerase Activity and the Level of hTERT mRNA in OCa Cells—Because an anti-apoptotic function of telomerase has been described for other cells and the cells undergoing apoptosis are associated with decreased telomerase activity (24), we considered the possibility that 1,25(OH)2VD3 may induce apoptosis in OCa cells by down-regulating telomerase. To determine whether 1,25(OH)2VD3 decreases telomerase, OVCAR3 cells were treated with 1,25(OH)2VD3 and the telomerase activity was measured by the PCR-based telomeric repeat amplification protocol. As shown in Fig. 2, panel A, OVCAR3 cells expressed a constitutive level of telomerase activity that was decreased by 1,25(OH)2VD3 treatment in a time-dependent manner. The activity was decreased to about 50% of the control after 3 days of treatment. Only about 15% of the activity was retained in cells treated for 6 and 9 days. Because telomerase down-regulation preceded the apoptotic induction, the data are consistent with the idea that decreased telomerase activity might be responsible for the cell death induced by the hormone.

Because the level of hTERT is rate-limiting for the telomerase activity in cancer cells and the VDR is a ligand-regulated transcription factor, it is conceivable that the decreased telomerase activity is due to the down-regulation of the hTERT transcription by 1,25(OH)2VD3. To test this idea, the level of hTERT mRNA in OVCAR3 cells treated with vehicle or 1,25(OH)2VD3 was determined by RT-PCR. Compared with vehicle control, 1,25(OH)2VD3 at 10−7 M decreased the level of hTERT mRNA in a time-dependent manner (Fig. 2 B). The decrease began after 1 day of treatment and became significant after treatment for 3 days or longer (Fig. 2B), which correlated to the decrease in telomerase activity (Fig. 2A). The level of GAPDH mRNA was not altered, demonstrating the specificity for hTERT mRNA. To quantify the degree of the decrease in
hTERT mRNA, real-time PCR was performed using total RNA from OVCAR3 cells treated with vehicle or 10⁻⁷ M 1,25(OH)₂VD₃. As shown in Fig. 2, panel C, hTERT mRNA was decreased by half after treatment for 3 days and to one-fourth after 9 days. The down-regulation of hTERT mRNA persisted in the presence of cycloheximide, an inhibitor of protein synthesis (Fig. 2D), indicating that the down-regulation does not require new protein synthesis. Thus, hTERT is, thus, potentially a primary target regulated by 1,25(OH)₂VD₃ in OCa cells.

The Putative VDRE in the hTERT Promoter Is Not Functional in OCa Cells—A recent study reported the identification of a putative VDRE in the 5’ regulatory region upstream of the hTERT promoter (25). This element mediated the down-regulation of hTERT in prostate cancer cells by 1,25(OH)₂VD₃ in combination with 9-cis-retinoic acid but not by 1,25(OH)₂VD₃ alone. To test the possibility that the down-regulation of hTERT mRNA by 1,25(OH)₂VD₃ in OCa cells is mediated through this putative VDRE, the in vitro binding of VDR and RXR to such a response element was tested in EMSAs. As shown in Fig. 3, panel A, the putative VDRE of hTERT bound recombinant VDR/RXR proteins in vitro. The binding is specific for the VDR/RXR heterodimer since neither VDR nor RXR alone formed a detectable complex with the VDRE probes. The complex formation was blocked by excess amounts of cold VDRE derived from human osteocalcin gene but not by a non-specific DNA element as shown in our previous studies (7). The complex was super-shifted by the addition of an anti-RXR antibody but not by an unrelated antibody, M2, showing that the binding is specific for the receptors.

To determine whether the VDRE mediates the down-regulation of hTERT expression in OCa cells, OVCAR3 cells were transfected with a reporter gene containing a 3.3-kb 5’ regulatory region of hTERT including the putative VDRE (25) and treated with vehicle or 1,25(OH)₂VD₃. Contrary to our expectation, 1,25(OH)₂VD₃ slightly increased the activity of the reporter (Fig. 3B). To test whether the lack of repression was an artifact of the transient transfection assay, the reporter was stably transfected into OVCAR3 cells. Similar to the result from transient transfection, 1,25(OH)₂VD₃ did not decrease the reporter activity in the stable clones after treatment for 6 days (Fig. 3C).

The results from EMSA and the reporter analyses necessitated further investigation as to whether the putative VDRE interacts with the VDR in vivo. ChIP assays were performed with soluble chromatin prepared from OVCAR3 cells treated with vehicle or 1,25(OH)₂VD₃. As shown in Fig. 3, panel D, in response to 1,25(OH)₂VD₃, the anti-VDR antibody precipitated the GADD45 exonic region containing the functional VDRE (7). It did not precipitate the hTERT fragment containing the putative VDRE, showing that the VDR is not recruited by the hormone to the putative VDRE of hTERT in vivo. From these experiments we concluded that the putative VDRE in hTERT promoter is not functional in response to 1,25(OH)₂VD₃ in OVCAR3 cells, although it binds the VDR/RXR in vitro in EMSA assays.

Because 1,25(OH)₂VD₃ has been shown to inhibit the c-myc oncogene (26), which in turn can activate telomerase through two c-Myc binding sites in the hTERT promoter (27), there is a possibility that the repression of telomerase by 1,25(OH)₂VD₃ is mediated by c-Myc. However, this is not likely because the reporter constructed with the 3.3-kb hTERT region containing the c-Myc regulatory elements did not respond to inhibition by 1,25(OH)₂VD₃ (Figs. 3, B and C). Overall, the data suggest that the decreased hTERT mRNA is not due to transcriptional repression through either the putative VDRE or the c-Myc sites.
The putative hTERT VDRE is not functional in OVCAR3 cells, and 1,25(OH)₂VD₃ decreases the mRNA stability of hTERT.

A, EMSAs were performed in the presence of 10⁻⁷ M 1,25(OH)₂VD₃ using putative hTERT VDRE probes. Preincubation with 2 μg of anti-RXRβ (RXR-Ab) or 100-fold molar excess of cold human ovarian cancer cell VDRE (Cold hOC) was performed for the supershift and competition experiments, respectively. Specificity of the interaction was demonstrated by competition with a 100-fold molar excess of unlabeled human ovarian cancer cell VDRE oligo or a specific competitor and by the lack of competition with the GADD45 (VDRE-C) that does not interact with VDR/RXR (7). 2 μg of anti-FLAG M2 monoclonal antibody were used as a nonspecific antibody control for the super-shifting with anti-RXR antibody.

B, OVCAR3 cells were transfected with 0.2 μg of pGL3–3328Luc together with 0.1 μg of CMVβgal and 0.1 μg of p91023B-VDR. The transfected cells were treated with 10⁻⁷ M 1,25(OH)₂VD₃ or EtOH as indicated. Luciferase activity was determined and normalized with corresponding β-galactosidase activity.

C, pGL3–3328Luc was stably transfected into OVCAR3 cells. The 3328Luc-OVCAR3 cells were treated with 10⁻⁷ M 1,25(OH)₂VD₃ for 6 days and luciferase activity (RLU) was determined.

D, ChIP assays. Soluble chromatin was prepared from OVCAR3 cells treated with EtOH or 10⁻⁷ M 1,25(OH)₂VD₃ for 60 min. ChIP assays were performed with control (rat IgG) or anti-VDR antibody.

E, the stability of hTERT mRNA is decreased by 1,25(OH)₂VD₃. OVCAR3 cells were treated with 10⁻⁷ M 1,25(OH)₂VD₃ or EtOH for 3 days followed by treatment with 5 μg/ml actinomycin D. Total RNA was extracted at 0, 2, 8, and 12 h and subjected to real-time PCR analysis using probe set 1 and probe set 2.
1,25(OH)₂VD₃ Decreases the Stability of hTERT mRNA—Because the putative VDRE is not functional in OCa cells, the down-regulation of hTERT mRNA may be due to increased degradation of the mRNA. To examine whether 1,25(OH)₂VD₃ alters the stability of hTERT mRNA, real-time PCR analyses were performed in the presence of the RNA synthesis inhibitor actinomycin D. As evaluated by two sets of probes and normalized by GAPDH, the level of hTERT mRNA decreased more rapidly in the cells treated with 1,25(OH)₂VD₃ than in vehicle-treated cells (Fig. 3E). In comparison to vehicle-treated cells, 1,25(OH)₂VD₃ decreased the half-life of hTERT mRNA from 29.7 to 10.8 h and from 22.6 to 6.5 h as measured with probe sets close to the 5' and the 3' ends of the hTERT mRNA, respectively. Similar data were obtained with two different amounts of total RNA in two independent real-time PCR experiments. These analyses show that the decrease in the level of hTERT mRNA induced by 1,25(OH)₂VD₃ is due at least in part to a decrease in mRNA stability.

Stable Expression of hTERT in OCa Cells Increases Telomerase Activity, Prolongs Telomere Length, and Selectively Affects 1,25(OH)₂VD₃-induced Apoptosis over Cell Cycle Arrest—We next evaluated the contribution of the down-regulation of telomerase to the induction of apoptosis. Because hTERT is the limiting factor for telomerase activity, a hTERT expression vector (19) was stably transfected into OVCAR3 cells to increase telomerase activity. As shown in Fig. 4, panel A, telomerase activity in OVCAR3 cells was 2-4 fold higher than the activity in parental OVCAR3 cells. Consistent with results from previous studies (28, 29), the average telomere length was about 3 kb in OVCAR3 cells but was more than 6 kb in hTERT-OVCAR3 cells (Fig. 4B). These analyses show that the ectopic expression of hTERT in OVCAR3 cells increases telomerase activity and prolongs telomere length.

The next question was whether 1,25(OH)₂VD₃ decreases telomerase activity and telomere length. As shown in Fig. 4, panels C and D, neither telomerase activity (Fig. 4C) nor
telomere length (Fig. 4D) in hTERT-OVCAR3 stable clones was decreased by treatment with 1,25(OH)₂VD₃ for 6 or 9 days, as compared with vehicle controls. Because telomere length in OVCAR3 cells is already short (about 3 kb) and it is likely the minimal length required for survival, the further shortening of telomere length could not be detected due to apoptotic death of the cells induced by 1,25(OH)₂VD₃.

The above analyses showed that the ectopic expression of hTERT functionally eliminated the effect of the 1,25(OH)₂VD₃ on telomerase activity in the stable clones and that hTERT was no longer the rate-limiting factor for telomerase activity. Thus, these stable clones were analyzed by annexin V-based flow cytometry to assess if overexpression of hTERT protects the cells from 1,25(OH)₂VD₃-induced apoptosis. As shown in Fig. 5, panel A, in contrast to the dramatic increase in the apoptotic index induced in parental cells after 9 days treatment with 1,25(OH)₂VD₃, hTERT stable clones showed little apoptotic induction by the hormone. In two independent experiments, the apoptotic index of the parental cells was increased from about 20 to 70% (about 3.5-fold induction) on average. In contrast, the apoptotic index of the two stable clones was increased from about 20 to 30% on average from the two experiments (Fig. 5B). This shows that the ectopic expression of hTERT rescues, at least partially, OVCAR3 cells from 1,25(OH)₂VD₃-induced cell death.

In contrast to apoptosis, the cell cycle arrest at G₁/S and G₂/M checkpoints was induced to a similar degree in the parental cells and the hTERT clones, as assessed by flow cytometry.
etry (Fig. 5C). Together, these analyses revealed that hTERT down-regulation is specifically involved in the 1,25(OH)₂VD₃-induced apoptosis but not in the 1,25(OH)₂VD₃-induced cell cycle arrest. The effect on apoptosis was similar in two stable clones, indicating that it is not due to the potential artifact of clonal expansion during the selection with antibiotics.

**Stable Expression of hTERT Reduces 1,25(OH)₂VD₃-induced Growth Suppression and Enhances OCa Cell Ability to Re-grow after Hormonal Withdrawal**—The above analyses have shown that the stable expression of hTERT selectively protects 1,25(OH)₂VD₃-induced apoptosis but not cell cycle arrest. Thus, these clones were analyzed to determine if down-regulation of hTERT with the associated apoptosis contributes to the overall growth suppression of OCa cells by 1,25(OH)₂VD₃. As shown in Fig. 6, the growth of the parental OVCAR3 cells and the hTERT stable clones, both, was inhibited by 1,25(OH)₂VD₃. However, the degree of inhibition in hTERT-expressing clones was about 40% less than that of the parental cells after 9 days of treatment. In addition, the growth of parental cells halted after 6 day of treatment, whereas the cells of stable clones continued growing in the presence of 1,25(OH)₂VD₃.

Besides a decreased growth rate, OVCAR3 cells lost their ability to re-grow after the removal of 1,25(OH)₂VD₃ if they had been treated continuously for longer than 6 days (Fig. 1). This suggests that 1,25(OH)₂VD₃ induces a long-lasting inhibitory effect on the growth of the cells. Because apoptosis is a process that becomes irreversible after cells become committed, it is possible that the down-regulation of hTERT and the associated apoptosis may contribute to this long-lasting effect. To test this hypothesis, we compared the ability of the cells of expressing hTERT to re-grow after 1,25(OH)₂VD₃ withdrawal to that of parental OVCAR3 cells. After the treatment with 1,25(OH)₂VD₃ for 9 days, the cells were plated in 96-well plates with fresh medium without the hormone and cultured for additional 12 days in the absence of the hormone. As shown in Fig. 7, pretreatment with 1,25(OH)₂VD₃ diminished the ability of OVCAR3 cells to re-grow, whereas the cells treated with the vehicle grew exponentially. This is consistent with the result shown in Fig. 1. In contrast, the growth rate after the pretreatment with 1,25(OH)₂VD₃ of the two hTERT stable clones was not different from the same cells pretreated with vehicles. Their growth rate was similar to that of parental cells (Fig. 7). Our analysis suggests that down-regulation of telomerase in OCa cells and the resulting apoptosis contribute to both the direct growth suppression by the hormone as well as its long-lasting inhibitory effect on re-growth after hormonal withdrawal.

**DISCUSSION**

Progressive shortening of telomere associated with cell divisions limits the life span of normal cells and eventually leads to senescence. To become immortal, human cancers including OCa are invariably associated with activation of mechanism that maintains telomere length. Approximately 85–90% of cancers show reactivation of telomerase. The present study shows that telomerase in OCa cells is down-regulated by 1,25(OH)₂VD₃. Down-regulation of telomerase is due to decreased stability of hTERT mRNA rather than VDRE-mediated transcriptional repression through the putative VDRE present in the regulatory region of the hTERT gene (25). A role for RNA molecules, such as microRNA, in regulating mRNA degrada-
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