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The coupling of epidermal growth factor receptor down regulation by 1alpha,25-dihydroxyvitamin D3 to the hormone-induced cell cycle arrest at the G1-S checkpoint in ovarian cancer cells

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Abstract

1alpha,25-dihydroxyvitamin D3, 1,25(OH)₂D₃, regulates gene expression through the vitamin D receptor. The present studies identify the epidermal growth factor receptor, EGFR, as a target gene suppressed by 1,25(OH)₂D₃ in human ovarian cancer cells. The suppression was detected at both mRNA and protein levels in vitamin D-sensitive human ovarian cancer cells. A novel vitamin D response element was identified in intron 1 of the EGFR genome, a known hotspot for its transcriptional regulation. Chromatin immunoprecipitations and reporter gene analyses showed that the intronic DNA element bound to vitamin D receptor and a co-repressor and was functional in mediating transcriptional suppression of EGFR promoter by 1,25(OH)₂D₃ under stable transfection conditions. Consistent with the EGFR down regulation, 1,25(OH)₂D₃ suppressed activation of the external signal regulated kinase by epidermal growth factors. Over expression of an active EGFR in vitamin D sensitive ovarian cancer cells caused resistance to 1,25(OH)₂D₃-induced growth suppression and diminished the hormonal regulation of cyclin D1, cyclin E, Skp2 and p27, a group of cell cycle regulators that mediate 1,25(OH)₂D₃-induced cell cycle arrest at G1-S checkpoint. Taken together, our studies demonstrate that 1,25(OH)₂D₃ suppresses the

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response of human ovarian cancer cells to mitogenic growth factors and couple the suppression to the cell cycle arrest at G1-S checkpoint by the hormone.

Keywords

Cell cycle; EGF; EGFR; Ovarian cancer; Vitamin D; Vitamin D receptor

Introduction

Epidemiological and experimental evidence support the concept that vitamin D deficiency or insufficiency contributes to the development of multiple human cancers including epithelial ovarian cancer (OCa) (Zhang et al., 2006). 1,25(OH)₂D₃ or its synthetic analogs suppress the growth of human OCa cell lines and ovarian tumor xenografts in mice (Jiang et al., 2004; Jiang et al., 2003; Li et al., 2004; Zhang et al., 2005a). The anti-tumor activity, as also shown in several other tumors (Blutt et al., 2000; Colston et al., 1992; Nakagawa et al., 2005), renders 1,25(OH)₂D₃ and its analogs the potential to fight against OCa, a malignancy with an overall 5-year survival rate of about 40%. Hormonal therapies are usually low cost and convenient to use. Studies of the mechanisms underlying 1,25(OH)₂D₃ actions on OCa cells may lead to the eventual development into clinics of active vitamin D compounds, either alone or in combination with other drugs, as new therapeutic and/or preventive methods for the management of human OCa.

Published studies have shown that 1,25(OH)₂D₃ induces cell cycle arrests and apoptosis in OCa cells (Jiang et al., 2004; Jiang et al., 2003; Li et al., 2004). These cellular effects are likely to be mediated through the nuclear vitamin D receptor (VDR) that binds vitamin D response elements (VDREs) and directly control the transcription of target genes. Consistently, a large number of VDR target genes have been identified in OCa cells (Zhang et al., 2005b) and several of them validated as mediators for the action of 1,25(OH)₂D₃ in OCa cells. Among the mediators, the catalytic subunit of telomerase reverse transcriptase was destabilized at the mRNA level by 1,25(OH)₂D₃ to cause apoptosis (Jiang et al., 2004) whereas GADD45 was identified as a primary target gene that accounted for the cell cycle arrest at the G2-M checkpoint (Jiang et al., 2003; Li et al., 2004). In addition, the p27 protein was stabilized by 1,25(OH)₂D₃, which was found to be responsible for the cell cycle arrest at the G1-S checkpoint but such an effect appears to be indirectly mediated through down regulation of Skp2 and cyclin E mRNA, which are known to act together to degrade p27 protein (Li et al., 2004). The primary vitamin D target gene responsible for this action of 1,25(OH)₂D₃ on p27 and G1-S checkpoint remains to be identified.

Cyclin D-dependent protein kinase complexes are known to couple mitogenic signals to the cell cycle progression. Mitogens such as the epidermal growth factor (EGF) stimulate cyclin D1 transcription, which initiates the self-reinforcing E2F transcriptional program together with p27 degradation, alleviating mitogen-dependency at G1-S and committing cells to S phase entry (Balmain and Cook, 1999; Sherr, 2000; Wu et al., 1996). In the present study, we present data supporting the concept that 1,25(OH)₂D₃ suppresses the response of OCa cells to EGF by down regulating the epidermal growth factor receptor (EGFR) transcription through a VDRE in intron 1 of its genome. The studies link the suppression of signaling through a mitogenic growth factor by 1,25(OH)₂D₃ to p27 accumulation and identify EGFR as the primary VDR target gene that mediates the effect of the hormone on cell cycle arrest at the G1-S checkpoint.

Materials and Methods

Chemical Reagents, Antibodies and Cell Cultures

1,25(OH)₂D₃ was purchased from Calbiochem (La Jolla, CA). EB1089 (seocalcitol) was generously provided by Leo Pharmaceutical Products. Antibodies against EGFR, ERK1, p-ERK1/2, cyclins E and D1 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); p-EGFR (Tyr1068) was from Cell Signaling (Danvers, MA), anti-p27 antibody was from Transduction Laboratories (Lexington, KY), anti-Skp2 and anti-Hsp60 antibodies were from Zymed Laboratories Inc. (South San Francisco, CA). cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Applied Biosystems (Foster City, CA). All oligonucleotides were synthesized by Invitrogen Inc (Carlsbad, CA).

All cell lines used in this study have been described previously (Zhang et al., 2005b). 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. For 1,25-(OH)₂D₃ and EB1089 treatment, the compounds were dissolved in ethanol, diluted to the desired concentration in culture medium, and added to the cells. For time course analyses, the hormones were added at different times to allow the treated cells to be harvested at the same time. For example, for an experiment with 9 days as the longest point of treatment, the group of zero day treatment was actually treated with vehicle for 9 days. Similarly, for the 3-day group, the cells were treated with vehicle for the first 6 days followed by the hormone for the last 3 days. For longer treatments, the medium was replaced with fresh medium containing 1,25-(OH)₂D₃ or vehicle every third day.

Northern Blot and Real Time PCR

Northern blot was used to determine the level of EGFR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as previously described (Jiang et al., 2003). Double-stranded oligonucleotides were end-labeled with ³²P using a T4 polynucleotide kinase labeling system (Invitrogen, Carlsbad, CA). The radiolabeled nucleotides were used to probe the membranes with transferred total RNA.

For quantitative PCR analyses, EGFR and 18S FAM-MGB probe premix were purchased from Applied Biosystems (Foster City, CA). EGFR mRNA and 18S rRNA levels were measured in triplicates with the ABI-7900T sequence detection system using the Taqman-based standard curve assay. C(t) value method was used for data analyses. The EGFR mRNA values were normalized with 18S rRNA and expressed as amounts relative to the control.

Cell Growth Assay, Flow Cytometry, and Statistical Analyses

To measure cell growth, OVCAR3 cells were plated in 96-well plates and treated with 1,25(OH)₂D₃ or ethanol (EtOH, vehicle). Colorimetric methylthiazole tetrazolium (MTT) assays were performed as previously described (Zhang et al., 2005a). Absorption at 595 nm (OD₅₉₅) was measured with an MRX microplate reader (DYNEX Technologies, Chantilly, VA). For each data point, eight samples were analyzed, and the experiment was reproduced three times.

To determine cell cycle distribution, cells were fixed with 70% ethanol, stained with 50 µg/ml propidium iodide, and analyzed on a FacScan (BD Biosciences). All cells were included in the analysis. No cells were “gated out,” which is considered the more robust manner for assessing cell cycle distribution. The samples were analyzed using ModFitLT from Verity Software House, which is an algorithmic-based modeling program that allows for manual

and automated cell cycle profiling. For each data point, duplicate samples were analyzed, and the experiment was reproduced three times. For cell cycle analyses and cell growth assays, statistical analyses were performed using independent sample t test. $p < 0.05$ was considered to be statistically significant.

Electrophoretic mobility shift assays (EMSA)

EMSA was performed as described previously (Jiang et al., 2003) with modifications. Briefly, double-stranded oligonucleotides were end-labeled with ^{32}P using a T4 polynucleotide kinase labeling system (Invitrogen). The sequences of the oligonucleotides for putative intronic VDRE are 5'-GGGAGAGTTGAATAAGTTGAGAA-3' and 3'-TTCTCAACTTATTCAACTCT-CCC-5'. 1 μl of radiolabeled probe (roughly 50,000 cpm) was mixed with 19 μl of DNA binding reaction mixture that contains 250 ng of VDR and/or 250 ng of RXR, 100 $\mu\text{g}/\text{ml}$ poly(dI-dC), 0.1 $\mu\text{g}/\mu\text{l}$ bovine serum albumin, 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ and EMSA buffer (Novagen). The mixture was incubated at room temperature for 30 min. For super shifting and competition experiments, VDR-RXR was pre-incubated with 2 μg of anti-VDR antibody, or 50 fold excess amounts of cold probes on ice, respectively for 20 min before the EMSA reaction. The reaction mixtures were resolved in a 5% non-denaturing polyacrylamide gel and protein-oligo complexes were revealed by autoradiography.

Immunoblotting Analyses and Chromatin Immunoprecipitation (ChIP) Assays

For immunoblotting analyses, cellular extracts were prepared in modified RIPA buffer containing 50 mM Tris (pH7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 2mM EDTA, 4 mM NaF, 2 mM Na_3VO_4 , 50 mM β -glycerol phosphate and protease inhibitor cocktail (Roche, Germany), separated on SDS-PAGE gels and blotted onto nitrocellulose membranes. Antibodies against EGFR, p-EGFR, skp27, p27, cyclin D1, cyclin E, p-ERK1, total ERK1, hsp60, and β -actin were used at the vendor-recommended dilutions. Proteins were detected using ECL.

For ChIP assays, OVCAR3 cells were treated with EtOH or 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ for 2 hrs and cross-linked with 1% formaldehyde. Then the cells were lysed in buffer (pH 8.0) containing 5 mM PIPES, 85 mM KCl, 0.5% Nonidet P-40 and protease inhibitor mixture. Cell nuclei were pelleted and re-suspended in buffer containing 50 mM Tris-Cl (pH 8.1), 10 mM EDTA, 1% SDS, and protease inhibitor mixture. Soluble chromatin was prepared by sonication and diluted in buffer containing 16.7 mM Tris-Cl (pH 8.1), 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, and protease inhibitor mixture. Immunoprecipitates were prepared with rat anti-VDR antibody (Sigma-Aldrich, St. Louis, MO), goat anti-NCoR (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-SMRTe antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and cognate IgG control antibodies. DNA was extracted from the immunocomplexes using a QIAquick spin kit (Qiagen, Valencia, CA). 2 μl out of 30 μl of DNA extracts were used for PCR. The sequence of primers for ChIP assays is 5'-TTCGACAGTACAGGCTTCCTGGTT-3' and 5'-TGTGTGTTTACAGTGGGTCAGGGT-3' for the intronic VDRE; 5'-ACCCGAATAAAGGAGCAGTTTCCC-3' and 5'-GTGCCCTGAGGAGTTAATTTCCGA-3' for the promoter VDRE.

Construction of Recombinant Luciferase Reporter Plasmid and Site-Directed Mutagenesis

The EGFR promoter-driven luciferase reporter has been described (Gonzalez et al., 2002). To construct pEGFR-intron-1-Luc of about 0.5 and 1 kb, respectively, EGFR intron-1 genomic DNA fragments from 81240 to 81825 and 81369 to 82415 bp (EGFR genomic sequence Genbank ID: AF288738) were amplified by PCR. The intron-1 sequences and were linked to the EGFR or SV40 promoter in a tandem manner.

To mutate in the intronic VDRE in the 1 kb intron-1-Luc reporter plasmid, the two nucleotides GA in the second half site were replaced with TT using PCR-based site-directed mutagenesis (QuikChangeH, Stratagene, La Jolla, CA). The forward (5'-GCCAGGGAGAGTTGAATAAGTTTTGAAATGTCATGTCGAAGCTATT-3') and reverse (5'-AATAGCTTCGACATGACATTTCAAACTTATTCAACTCTCCCTGGC-3') primers were designed according to manufacturer's instructions. Each 50 μ l PCR reaction mixture contained template DNA, 5 μ l 10 $\times$ mutagenesis buffer, 2 μ l of a 20 μ M solution of each mutant forward and reverse primer, 1 μ l of a 10 mM solution of dNTP, and 2.5 U Pfu DNA polymerase in sterile water. The PCR reaction was conducted for 95°C for 2 min followed by 18 cycles of 20 sec at 95°C, 10 sec at 60°C and 30 sec at 68°C and a final 5 min extension at 68°C. The PCR product was digested with 10 U DpnI for 1 h at 37°C before being transformed into the XL10-Gold ultracompetent cells. After transformed colonies were screened by PCR, the mutant plasmids produced from the clones were isolated, purified and sequenced.

Establishment of Stable Transfectants

An EGFRvIII plasmid in Bluescript was kindly provided by Drs. H.-J. Su Huang and Webster K. Cavenee, which was used to construct pcDNA3-EGFRvIII. OVCAR3 cells were transfected with 10 μ g of pcDNA3-EGFRvIII or intronic VDRE based reporter gene plasmids. Stable clones were obtained through selection with 100 μ g/ml G418 for a period of about 4 weeks and clonal isolation with glass cylinders. For each stable transfection study, at least two independent clones were analyzed and representative data are presented.

Transcription Studies

Transcription studies of transient transfections were conducted as previously described (Jiang et al., 2003; Lee et al., 2000). Briefly, OVCAR3 cells were transfected with the reporter constructs and treated with 1,25(OH)₂D₃ or vehicle for 36 h. Luciferase assays were performed using the Luciferase Assay System (Promega, Madison, WI) and the activity was normalized to cognate β -galactosidase (β -gal) activity. For stable transfectants, cells were treated with vehicle or 1,25(OH)₂D₃ for different times as specified in the figures. Luciferase activity was determined and normalized with protein amounts.

Results

1,25(OH)₂D₃ down regulated EGFR expression in OVCAR3 cells

We previously reported the profiling of changes in gene expression in human OCa cells induced by 1,25(OH)₂D₃ with U95Av2 and U133A Affymetrix chip (Zhang et al., 2005b). In the initial analysis with U95Av2 chips, EGFR was identified as being down regulated by 1,25(OH)₂D₃ at all time points. Further analyses with U133A chips showed a time-dependent down regulation. The U133A studies have three independent time course analyses with 12 data points, thus permitting statistical analyses. Analysis of the variance between groups (ANOVA) showed that the down regulation is statistically significant ($P < 0.05$). EGFR did not make the list of genes regulated by 1,25(OH)₂D₃ (Zhang et al., 2005b) because the degree of down regulation was less than 2-fold. However, our published studies suggest that the microarray analyses are reproducible but underestimate the degree of regulation (Zhang et al., 2005b). Therefore, we performed Northern blot analyses to investigate the EGFR down regulation even though the degree of change on microarray was modest. As shown in Fig. 1A, both 1,25(OH)₂D₃ and its synthetic analog EB1089 decreased EGFR mRNA expression in a time-dependent manner. EB1089 showed a stronger effect on EGFR mRNA than 1,25(OH)₂D₃. This is consistent with its stronger agonistic activity. Subsequent dosage analyses with quantitative RT-PCR analyses showed that 1,25(OH)₂D₃ and EB1089 significantly decreased EGFR mRNA at concentrations as low as 10⁻⁹

($p < 0.05$) and 10^{-11} ($p < 0.001$) M, respectively (Fig 1B). It is important to note that $1,25(\text{OH})_2\text{D}_3$ at 10^{-11} M increased EGFR mRNA. It remains to be determined whether the increase is somehow related to the reported growth stimulatory activity of the hormone at very low concentrations (Miettinen et al., 2004).

To test whether EGFR mRNA down regulation was due to changes in mRNA stability, OVCAR3 cells were treated with $1,25(\text{OH})_2\text{D}_3$ or vehicle in the presence of a RNA synthesis inhibitor, actinomycin D, and RNA was extracted and subjected to Northern blotting analyses. The signal was quantified and normalized to that of GAPDH. As shown in Fig 1C, $1,25(\text{OH})_2\text{D}_3$ did not decrease the half life of EGFR mRNA which is approximately 4 hrs in cells treated with vehicle or $1,25(\text{OH})_2\text{D}_3$. The studies reveal that the down regulation of EGFR by $1,25(\text{OH})_2\text{D}_3$ is likely to occur at the transcriptional level.

Identification of a novel functional VDRE in intron 1 of EGFR gene

Previous studies (McGaffin et al., 2004) described a putative vitamin D response element within EGFR promoter region which were reported to be functional in breast cancer cells (McGaffin and Chrysogelos, 2005). Thus, we transfected the EGFR promoter-based reporter gene into OVCAR3 cells and first tested its response to $1,25(\text{OH})_2\text{D}_3$ in transient transfection studies. We did not observe a negative effect by $1,25(\text{OH})_2\text{D}_3$ (data not shown). We then stably transfected the reporter into OVCAR3 cells and tested its response to $1,25(\text{OH})_2\text{D}_3$ in the context of chromatin. As shown in Fig 2A, the reporter gene was not decreased by $1,25(\text{OH})_2\text{D}_3$ treatment over a period of six days. The data suggest that the putative VDRE reportedly to be functional in breast cancer cells is not the VDRE element responsible for EGFR down regulation by $1,25(\text{OH})_2\text{D}_3$ in OCa cells.

To determine whether the EGFR gene contains additional VDREs, we searched for putative VDREs, including direct repeats with three intervening nucleotides (DR3), direct repeat with six intervening nucleotides and inverted palindromes with nine intervening nucleotides, in the EGFR gene (Toell et al., 2000) and identified a DR3 element in intron 1 of EGFR (Fig. 2B), a region known as a hotspot for the transcriptional regulation of EGFR (Buerger et al., 2000; Chrysogelos, 1993). Electrophoretic mobility shift assays (EMSA) showed that the identified DR3 element formed a protein complex in reactions containing recombinant VDR and RXR but not in reactions lacking VDR or RXR, suggesting that the VDR binds to the DNA element as a heterodimer (Fig 2C). The addition of an anti-VDR antibody caused a further up-shift of the DNA-protein complex, confirming the presence of VDR protein in the DNA complex. The signal of the DNA-protein complex was reduced by the addition of a 50 fold excess of cold probe, suggesting that the signal is specific to the DR3 element. ChIP assays (Fig. 2D) with anti-VDR and anti-corepressor antibodies showed that the VDR protein and NCoR, not SMRT, bound to the intronic VDRE *in vivo* in the presence of $1,25(\text{OH})_2\text{D}_3$. The VDR and corepressor did not occupy the promoter VDRE in parallel analyses, which is consistent with its lack of activity in reporter assays. The ChIP assays suggest that the hormone is likely to recruit VDR/corepressor protein complexes to the intronic DNA element to suppress EGFR expression.

To test the functionality of the intronic VDRE, an EGFR DNA fragment of about 500 bp in length containing the intronic VDRE was placed in front of the SV40 promoter of the pGL3-basic vector (Fig. 3A). The response of the resultant reporter gene to $1,25(\text{OH})_2\text{D}_3$ was tested in transfection studies. In transient transfections, $1,25(\text{OH})_2\text{D}_3$ treatment for periods up to 6 days in length failed to exert an effect on the reporter gene (Fig. 3A). After the reporter was stably integrated into the genome of the OVCAR3 cells, the promoter activity was suppressed by $1,25(\text{OH})_2\text{D}_3$ in a time-dependent manner (Fig 3A). OVCAR3 stable clones with pGL3 control vector that contains the SV40 enhancer did not show a reduction in luciferase activity by $1,25(\text{OH})_2\text{D}_3$ (data not shown). A similar degree of suppression by

1,25(OH)₂D₃ was detected with a stably integrated reporter gene in which a 1 kb intronic DNA fragment containing the DR3 element was placed in front of the EGFR promoter (Fig. 3B). More importantly, the mutation of the intronic VDRE at two key nucleotides of the 2nd half site eliminated the suppression by 1,25(OH)₂D₃. The data suggest that the intronic DR3 element is a functional VDRE in OCa cells and that the promoter VDRE is inactive either alone or in the context of the intronic DNA element.

Suppression of EGFR protein expression by 1,25(OH)₂D₃ and its involvement in the suppression of OCa cell growth

To determine whether the down regulation of EGFR mRNA translates into a decrease in EGFR protein expression and alteration of EGF signaling, we compared the level of EGFR protein expression as well as EGF-induced ERK activation in cells treated with 1,25(OH)₂D₃ and vehicle. Western blot analyses found that 1,25(OH)₂D₃ pre-treatment decreased the expression of EGFR protein in OVCAR3 cells in a time-dependent manner (Fig. 4A). Treatment of OVCAR3 cells with synthetic vitamin D analog EB1089 also decreased EGFR expression (data not shown). The suppression of EGFR expression by active vitamin D compounds was also observed in vitamin D-sensitive OV2008 cells but not in the vitamin D-resistant SKOV3, OVCAR8 and OVCAR10 cells (Fig 4B). The data show that the EGFR down regulation is not limited to OVCAR3 cells and correlates with vitamin D sensitivity. At the same time, pre-treatment with 1,25(OH)₂D₃ also decreased the basal and EGF-induced ERK1 activation (Fig. 4C), showing that the decreased level of EGFR expression is translated into a decrease in the capacity of the receptor to mediate the mitogenic effect of its ligand.

To assess the contribution of EGFR down regulation in 1,25(OH)₂D₃-induced growth suppression, a constitutively activated EGFR, EGFR variant III (EGFRvIII), which lacks 267 amino acids from its extracellular domain (Nishikawa et al., 1994; Wong et al., 1992), was stably transfected into OVCAR3 cells (Fig 5A). Then, the growth suppression induced by 1,25(OH)₂D₃ was compared between parental cells and the EGFRvIII stable transfectants. As shown in Fig. 5B, the EGFRvIII stably expressed in OVCAR3 cells is active as revealed by the increased level of auto-phosphorylation. The levels of VDR protein expression was not decreased by the active EGFR. 1,25(OH)₂D₃ treatment for 6 days reduced OVCAR3 cell density, an effect that is significantly reduced by the stable expression of EGFRvIII. Quantitative measurement of cell growth by MTT assay estimated that 1,25(OH)₂D₃ treatment for 6 days decreased the growth of parental OVCAR3 cells by more than 40%, a reduction that was largely diminished by the stable expression of EGFRvIII (Fig. 5C). The percentage of growth inhibition by 1,25(OH)₂D₃ is significantly different between OVCAR3 cells and the EGFRvIII clones at both 3 and 6-day treatments (P<0.01) These analyses argue that EGFR down regulation contributes significantly to the overall suppression of OCa cell growth by 1,25(OH)₂D₃.

Under our conditions, the EGFRvIII stable clone appears to grow slower than OVCAR3 cells, for which the exact reasons are unknown but may be simply an artifact of the EGFRvIII overexpression. The active EGFR has been reported to stimulate stress activated protein kinases such as JNK (Antonyak et al., 1998), which at high levels may have a negative effect on basal cell proliferation.

Coupling of EGFR down regulation to p27 induction and cell cycle arrest at G1/S by 1,25(OH)₂D₃

Mitogens such as EGF are known to exert their effects on cell growth by regulating cell cycle progression through the G1/S checkpoint. Our previous studies identified the stabilization of p27 as one of the mechanism underlying 1,25(OH)₂D₃-induced growth

suppression in OCa cells and linked the p27 stabilization to cell cycle arrest at G1/S (Li et al., 2004). To determine whether EGFR down regulation underlies the growth arrest at G1/S, parental OVCAR3 and the EGFRvIII stable transfectants were treated with 1,25(OH)₂D₃ for 6 days and the cell cycle distribution was determined by flow cytometry. In parental OVCAR3 cells, 1,25(OH)₂D₃ induced, in a time-dependent manner, the accumulation of cells in G1/G0 phases, which is accompanied by a reduction in the percentage of cells in the S and G2/M phases (Fig. 6A). The constitutive expression of EGFRvIII blunted the 1,25(OH)₂D₃-induced alteration in the distribution of cells in G1/G0, G2/M and S phases (Fig. 6A). EGFRvIII expression did not change the cell size as indicated by the side scatter analyses of cells treated with 10⁻⁷ M 1,25(OH)₂D₃ for 6 days (Fig 6B). Furthermore, constitutive expression of EGFRvIII effectively eradicated the increased expression of p27 (Fig. 6C), decreased expression of Skp2 and cyclin E, as well as the decreased expression of cyclin D1 (Fig. 6C). We previously reported that 1,25(OH)₂D₃ induced cell cycle arrest at G1/S through regulating p27, Skp2, Cyclin E protein, and Cyclin E-CDK2 complex activities (Li et al., 2004; Zhang et al., 2006). These analyses identify the 1,25(OH)₂D₃-induced EGFR down regulation as the primary event for the cell cycle arrest at G1/S induced by 1,25(OH)₂D₃.

Discussion

1,25(OH)₂D₃ exerts its growth suppression effects on OCa cells by acting on the G1/S and G2/M cell cycle checkpoints (Zhang et al., 2006). Signaling through CDK4/6-Rb-E2F axis is essential for progression through G1/S and we previously reported that 1,25(OH)₂D₃ up-regulated p27 by regulating the Skp2 and cyclin E-CDK2 complex (Li et al., 2004). However, the primary target genes for 1,25(OH)₂D₃ that initiates the changes along the axis are not identified. Current studies have shown that 1,25(OH)₂D₃ represses EGFR expression at the mRNA and protein levels as well as EGFR function. *In vitro* and *in vivo* binding assays have identified a functional intronic VDRE, suggesting that EGFR is a primary target of 1,25(OH)₂D₃. These studies couple the EGFR regulation by 1,25(OH)₂D₃ to the G1/S suppression by 1,25(OH)₂D₃, and lead to a better understanding about the mechanism underlying the regulation of G1/S transition by 1,25(OH)₂D₃ in OCa cells. Overall, the data support the model depicted in Figure 7 that, in OCa cells, 1,25(OH)₂D₃ directly suppresses EGFR transcription, resulting in sequential changes in the level of cyclins D1 and E, the activity of cyclin D1/CDK4/6 and cyclin E-CDK2, the level of Skp2, and the stability of p27 that efficiently suppresses OCa cell progression through G1/S checkpoint.

The effect of 1,25(OH)₂D₃ on EGFR has been previously described in several other cell lines. In osteoblast-like cells UMR-106-01, it was shown that 1,25(OH)₂D₃ increased EGFR levels by increasing its mRNA stability (Gonzalez et al., 2002). In A431 epidermoid cells, it was shown that 1,25(OH)₂D₃ impaired EGF-induced EGFR nuclear translocation and, consequently, its binding to AT-rich DNA sequences and the transcriptional activation of the cyclin D1 promoter (Cordero et al., 2002). However, the total level of EGFR in A431 cells was not altered. 1,25(OH)₂D₃ was found to down regulate EGFR expression in keratinocytes (Boisseau-Garsaud et al., 1996) and in breast cancer cells (Koga et al., 1988). In breast cancer cells, 1,25(OH)₂D₃ suppressed cell growth (McGaffin et al., 2004) and inhibited EGFR expression by acting through a VDRE located in the promoter region (McGaffin and Chrysogelos, 2005). In studies with UMR-106-01, the same EGFR promoter VDRE failed to mediate the effect of vitamin D on EGFR mRNA (Gonzalez et al., 2002). Our studies have established EGFR as a primary target gene that is directly suppressed by 1,25(OH)₂D₃ at the transcriptional level in OCa cells. Similar to the studies in UMR-106-01 cells, we showed that the promoter VDRE is not functional. Instead, a novel intronic VDRE has been identified by combinational analyses utilizing *in vitro* and *in vivo* approaches. In our studies, the EGFR promoter showed neither a response to 1,25(OH)₂D₃ itself nor a

synergistic effect with the intronic VDRE. Currently, it is unknown why the promoter VDRE is functional in breast cancer cells but not in OCa or UMR-106-01 cells. Considering all available information from different cell lines, it appears that the effects of $1,25(\text{OH})_2\text{D}_3$ on EGFR and its mechanisms of action are cell type specific.

Intron 1 in EGFR is a hotspot for transcriptional regulation (Chrysogelos, 1993), in which both positive and negative enhancer elements have been previously described (Haley and Waterfield, 1991; Maekawa et al., 1989). In addition, a polymorphic dinucleotide repeat located in intron 1 of EGFR was also found to regulate its transcription (Gebhardt et al., 1999), most likely through the control of transcriptional termination in the intron 1 region. Because the regulatory role of intron 1 in EGFR transcription is likely to occur through a conformational change mediated by chromatin remodeling, it is not surprising that only stable transfections of the luciferase reporter construct with the intronic VDRE yielded a response to $1,25(\text{OH})_2\text{D}_3$ whereas no response was observed in transient transfectants. The ChIP studies showed that VDR and NCoR bound to the intronic VDRE in the presence of $1,25(\text{OH})_2\text{D}_3$, suggesting ligand-induced VDR and corepressor recruitment as the mechanism underlying the repression. It is known that the transcriptional repression by nuclear receptors involves transcriptional co-repressors that form complexes with chromatin remodeling enzyme histone deacetylases. Further studies are needed to determine whether EGFR down regulation is associated with chromatin remodeling and changes in the histone acetylation status of the involved nucleosomes.

It is important to point out that the degree of EGFR mRNA down regulation by $1,25(\text{OH})_2\text{D}_3$ in Northern blot did not correlate exactly with the data from reporter gene and Western blot analyses. Besides the DR3 in intron 1, our sequence analyses revealed the presence of more than 20 DR3-type VDRE-like elements around EGFR genome (data not shown). Although our data ruled out the involvement of the promoter VDRE in EGFR down-regulation, other putative VDREs in the EGFR genome may cooperate with the intronic VDRE, providing a possible explanation for the slower change observed in the analyses with reporter genes that contains primarily the intronic VDRE. The fact that the decrease in EGFR protein occurs later than the decrease in the mRNA suggests the possible existence of additional mechanisms of EGFR regulation by $1,25(\text{OH})_2\text{D}_3$ at translational or posttranslational steps. The lack of exact correlation between changes in mRNA and protein levels has been noticed in our published studies with GADD45 (Jiang et al., 2003). The slower change in protein levels may explain why the hormonal effects on OCa cell growth is chronic and requires long time treatment.

Even though our data show that EGF-induced ERK activation is suppressed by $1,25(\text{OH})_2\text{D}_3$, it remains to be determined whether downstream changes in the expression of cyclin D1 and p27 is ERK-dependent. For example, EGFR is known to regulate p27 levels independent of MAPK/ERK signaling in A431 cells (Busse et al., 2000). Furthermore, EGFR has been shown to move to the nucleus and to bind adenine/thymidine-rich sequence (or ATRS) in the cyclin D1 promoter (Lin et al., 2001). In our studies, $1,25(\text{OH})_2\text{D}_3$ down regulated cyclin D1 expression while the constitutively activated EGFR blocked this action, suggesting that $1,25(\text{OH})_2\text{D}_3$ regulated cyclin D1 through the EGFR. It is possible that the regulation of p27 by EGFR is mediated through its transcriptional activity in the nucleus on cyclin D1 and that $1,25(\text{OH})_2\text{D}_3$ reduces the amount of EGFR binding to the cyclin D1 promoter thus increasing p27 expression, which subsequently decreases CDK4/6 activity and leads to a decrease in the levels of Skp2 and cyclin E, cyclin A/CDK2 activity and blocks the entry of cells in G1 to S phase. Furthermore, ERK is pleiotropic. Besides cell cycle regulation at G1/S checkpoint, ERK is known to cross talk with many signaling pathways. For example, ERK phosphorylates SMAD1 and regulates TGF β and BMP signaling (Yue et al., 1999). ERK also suppresses death receptor-mediated apoptosis by

suppressing the activation of the caspase effector machinery (Tran et al., 2001). Interestingly, TGF β and 1,25(OH) $_2$ D $_3$ converge on SMAD transcriptional coactivators in mammalian cells (Yanagisawa et al., 1999). Staurosporine suppresses the genomic action of 1,25(OH) $_2$ D $_3$ signaling through caspase-3 mediated VDR cleavage (Malloy and Feldman, 2009). Our previous studies revealed a negative effect of 1,25(OH) $_2$ D $_3$ on death receptor-mediated apoptosis (Zhang et al., 2005b). Thus, suppression of EGFR and ERK signaling by 1,25(OH) $_2$ D $_3$ will have a broad effect on cellular processes beyond p27 stabilization and G1/S checkpoint.

Our study is the first demonstration of an effect of 1,25(OH) $_2$ D $_3$ on OCa cell's growth response to external mitogenic stimuli, which is likely to be a major contributor to the chemopreventive effect of vitamin D. EGFR is highly expressed in OCa cells and is being actively investigated as a molecular target for treatment of OCa (Lassus et al., 2006; Niikura et al., 1997). Altered expression or activation of EGFR and the downstream molecular components of its signaling pathway linked to cyclins and p27 has been shown to contribute to OCa growth and progression (Maihle et al., 2002; Morishige et al., 1991). Particularly, the constitutively active EGFRvIII has been described to be expressed in 75% of human ovarian tumors (Moscatello et al., 1997). Since the present studies suggest that EGFR and its downstream signaling molecules play an essential role in 1,25(OH) $_2$ D $_3$ action, their alteration may negatively affect the growth suppression of OCa cells by 1,25(OH) $_2$ D $_3$, causing resistance of these cells to 1,25(OH) $_2$ D $_3$ treatment. Thus, drugs blocking EGFR signaling are potentially useful in sensitizing vitamin D-resistant ovarian tumors to 1,25(OH) $_2$ D $_3$ or in combinational treatment together with the hormone to achieve optimal therapeutic effects.

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The abbreviations used are

1,25(OH)$_2$D$_3$	1 α ,25-dihydroxyvitamin D $_3$
OCa	ovarian cancer
VDR	vitamin D receptor
EGFR	epidermal growth factor receptor
VDRE	vitamin D response element

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Fig. 1.

Regulation of EGFR expression by 1,25(OH)₂D₃ in OVCAR3 cells. (A) 1,25(OH)₂D₃ and its synthetic analog decrease EGFR mRNA in OVCAR3 cells. OVCAR3 cells were treated with 10⁻⁷ M 1,25(OH)₂D₃ (VD) or EB1089 (EB) for the indicated times. Total RNA was isolated and Northern blot analyses were performed as described in *Materials and Methods*. (B) Dosage effects of 1,25(OH)₂D₃ and EB1089 on EGFR mRNA. OVCAR3 cells were treated with 1,25(OH)₂D₃ and EB1089 at indicated concentrations for 3 and 6 days, respectively. EGFR mRNA C(t) values were normalized with that of the cognate 18s ribosomal RNA and expressed as amounts relative to the vehicle control (time zero as 1). Samples were analyzed in triplicates and the error bars stands for the standard error of means. Student t test was performed (* p<0.05, ***p<0.005). (C) Effect of 1,25(OH)₂D₃ on EGFR mRNA stability. OVCAR3 cells were treated with ethanol (EtOH) or 10⁻⁷ M 1,25(OH)₂D₃ (VD) for three days. The cells were washed and subsequently treated with 5 µg/ml actinomycin D (ActD) for the indicated times. Northern blot analyses were performed and signals quantified using Scion Image Beta 4.02 software. EGFR signals were normalized with the corresponding GAPDH signal and presented as the percentage of EGFR mRNA levels at time zero. A representative diagram of three independent experiments is shown.

Fig. 2.

Identification of a putative VDRE in EGFR intron 1 and its interaction with VDR *in vitro* and *in vivo*. (A) The lack of function of the putative VDRE in EGFR promoter in OVCAR3 cells. A schematic presentation of the EGFR promoter-based reporter construct is shown at the top. Luciferase activity of the reporter in stable OVCAR3 transfectants treated with 1,25(OH)₂D₃ (VD) for the indicated times was determined and normalized with total protein. The reporter assays were analyzed in duplicate and error bars stand for standard error of means. The assays were repeated three times. (B) Representative sketch of EGFR genomic sequence (genomic sequence GenBank ID: AF288738) showing position of a putative VDRE in intron 1. The sequence of human osteocalcin (hOC) VDRE, rat 24-hydroxylase (CYP24) proximal VDRE, rat parathyroid hormone-related protein (PRHrP) distal VDRE and the consensus DR3 sequences are listed for comparison. The sequences of the 3-base pair spacers are shown in small letters and the hexameric VDRE half sites in capital letters. (C) *In vitro* interaction of the putative intronic VDRE with recombinant VDR and RXR proteins. EMSA assays were performed in the presence of 10⁻⁷ M 1,25(OH)₂D₃ using putative EGFR intronic VDRE probes. Pre-incubation with 2 μ g of anti-VDR antibody (VDR Ab) or a 50-fold excess cold probes were included for super-shifting and competition purposes. (D) Recruitment of VDR and co-repressors to the intronic but not the promoter VDRE. Soluble chromatin was prepared from OVCAR3 cells treated with ethanol (-) or 10⁻⁷ M 1,25(OH)₂D₃ (+) for 2 hrs. Immunoprecipitations were performed with

control (IgG), anti-VDR, anti-NCoR or anti-SMRTe antibodies as indicated. EGFR intronic (Intronic) and EGFR promoter (Promoter) VDRE fragments in the precipitates were detected by PCR using corresponding primers.

Fig. 3.

The putative intronic VDRE mediates the transcriptional down regulation of EGFR by $1,25(\text{OH})_2\text{D}_3$ in OCa cells. (A) EGFR intronic DNA fragment (~500 bp) containing the putative VDRE was placed in front of SV40 promoter in pGL3-Basic vector. A schematic presentation of the reporter construct is shown at the top. OVCAR3 cells transiently transfected with 0.2 μg of the reporter construct and 0.3 μg of pCMVGal or stably transfected with the reporter were treated with 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ for the indicated times. Luciferase activity was determined in duplicate and normalized with cognate β -gal activity (for transient transfections) or total protein (for stable transfectants). Error bars stand for error of means. The reporter assays were repeated three times and with two independent stable clones. Student t test was performed and the decrease is significant starting at one-day treatment (*** $p < 0.005$) (B) The lack of synergy with the putative promoter VDRE. EGFR intronic DNA fragments (1.1 kb) containing the putative VDRE was sub-cloned into EGFR promoter-based pGL3 reporter gene. Schematic presentation of the reporter construct is shown at the top. OVCAR3 cells stably transfected with the reporter were treated with $1,25(\text{OH})_2\text{D}_3$ for the indicated times. Luciferase activity was determined and analyzed as in panel A. (D) Mutational analyses of the intronic VDRE. The 2nd half site of the intronic VDRE in the reporter gene described in panel B was mutated. The wild type (WT) and mutant (MT) reporters were stably transfected into OVCAR3 cells. The cells were treated and reporter assays analyzed as described in panel A.

Fig. 4.

1,25(OH)₂D₃ decreases the level of EGFR protein and suppresses ERK activation by EGF in OCa cells. (A) Suppression of EGFR protein expression by 1,25(OH)₂D₃ and EB1089 in sensitive cells. Cells were treated with ethanol (EtOH), 10⁻⁷ M 1,25(OH)₂D₃ (VD) or 10⁻⁸ M EB1089 (EB) for the indicated times. The levels of EGFR, β-actin and Hsp60 proteins were determined by immunoblotting. (B) Lack of a vitamin D effect on EGFR in vitamin D resistant OCa cells. Cells were treated and the levels of protein expression determined as in panel A. (C) Suppression of EGF-induced ERK1 activation by 1,25(OH)₂D₃ in OCa cells. OVCAR3 cells were treated with ethanol (EtOH) or 1,25(OH)₂D₃ (VD) for three days, serum-starved and treated with 60 ng/ml EGF for the indicated times. Levels of total and phosphorylated ERK1 were determined by immunoblotting.

Fig. 5.

Resistance to $1,25(\text{OH})_2\text{D}_3$ -induced growth suppression of OVCAR3 EGFRvIII stable transfectants. (A) Level of EGFR protein was determined by immunoblotting of parental OVCAR3 cells and stable EGFRvIII transfectants. (B) Parental OVCAR3 cells and EGFRvIII stable transfectants were treated with ethanol (EtOH) or 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ (VD) for 6 days. VDR levels were determined by Western blot. The levels of EGFR phosphorylation (p-EGFR) was determined by immune-precipitations with anti-EGFR followed by Western blot with a phospho-specific anti-EGFR antibody. Representative pictures of treated cells were also shown. (C) Parental OVCAR3 cells and EGFRvIII stable transfectants were treated with ethanol (EtOH) or $1,25(\text{OH})_2\text{D}_3$ (VD) for indicated times. Cell growth was determined by MTT assays. Eight samples were analyzed in parallel for each data point and the assays were reproduced three times. Error bars denote standard error of means. Student t test was performed (** $p < 0.01$; *** $p < 0.005$).

Fig. 6.

Suppression of 1,25(OH)₂D₃-induced cell cycle arrest at G1-S checkpoint by constitutively active EGFRvIII. (A) Parental OVCAR3 cells and stable EGFRvIII transfectants were treated with ethanol or 10⁻⁷ M 1,25(OH)₂D₃. Cell cycle distribution at G0/G1 and S phases was analyzed by flow cytometry. Samples were analyzed in duplicate. The error bars stands for standard error of means. Student t test was performed and the changes in G1, S and G2/M phases caused by 1,25(OH)₂D₃ in OVCAR3 cells is significant for both 6 and 9-day treatments. For EGFRvIII stable clone the changes in 9-day treatment is significant but the percentage of growth suppression is significantly less than in OVCAR3 cells ($p < 0.005$). (B) Parental OVCAR3 cells and stable EGFRvIII transfectants were treated with 10⁻⁷ M 1,25(OH)₂D₃ for 6 days. The side scattered data were shown, of which the height of the forward scatter (FSC) indicates no difference in cell size between the two groups. (C) The Parental OVCAR3 cells and stable EGFRvIII transfectants were treated with 1,25(OH)₂D₃. Levels of p27, Skp2, cyclin E and cyclin D1 protein expression were determined by immunoblotting with cognate antibodies. Blots were probed for β -actin as the loading control. Two independent stable clones were analyzed, which yielded the same information for all target proteins. The data from a representative clone are shown.

Fig. 7.

A working model depicting linkage of EGFR down regulation by 1,25(OH)₂D₃ to cell cycle arrest at G1-S checkpoint in OCa cells. 1,25(OH)₂D₃ works through the VDR transcription complex to suppress EGFR transcription, resulting in a sequential reduction of the level of cyclins D1 and E, the activity of cyclin D1/CDK4/6 and cyclin E/CDK2, and the amount of Skp2 ubiquitin ligase. These events lead to the accumulation of p27 that efficiently suppresses OCa cell progression through the G1-S checkpoint.