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ROLE OF 24-HYDROXYLASE IN VITAMIN D_3 GROWTH RESPONSE OF OVCAR-3 OVARIAN CANCER CELLS

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Vitamin D and its analogues are potent regulators of cell growth and differentiation both *in vivo* and *in vitro*. We studied the effects of 25-hydroxyvitamin D_3 [25(OH)D_3], 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2D_3$] and vitamin D analogue, EB 1089, on the growth of a human ovarian cancer cell line, OVCAR-3. We also studied the expression of vitamin D metabolising enzymes 24-hydroxylase (24OHase) and 1α-hydroxylase (1αOHase). Our results showed that high concentrations (10 and 100 nM) of 1,25(OH)$_2D_3$ inhibited a cell proliferation, whereas low concentration (0.1 nM) stimulated growth of the OVCAR-3 cells. In the concentration range of 10–500 nM a prohormone, 25(OH)D_3, stimulated growth. An amount of 1 nM EB 1089 and 100 nM 1,25(OH)$_2D_3$ inhibited growth with an equal magnitude. The expression of 24OHase was strongly induced by 1,25(OH)$_2D_3$ and EB 1089 in OVCAR-3 cells, and analysis of vitamin D metabolites showed the functionality of 24OHase. An inhibition of 24OHase activity with a novel 24OHase inhibitor enhanced growth-inhibiting effects of 1,25(OH)$_2D_3$ and suppressed the growth stimulation of 100 nM 25(OH)D_3. We also report the expression of a vitamin D activating enzyme, 1αOHase, in 7 ovarian cancer cell lines. The production of 1,25(OH)$_2D_3$ in OVCAR-3 cells was low, possibly due to an extensive activity of 24OHase or a low 1αOHase activity. These results suggest that in ovarian cancer cells vitamin D metabolizing enzymes might play a key role in modulating the growth response to vitamin D. The possible mitogenic effects of vitamin D should be considered when evaluating treatment of ovarian cancer with vitamin D.

Key words: *vitamin D; EB 1089; 1α-hydroxylase; 24-hydroxylase; ovary; cell growth*

Both epidemiologic and *in vitro* studies suggest that vitamin D may be an important factor in the development and progression of cancer.[1–4] Geographic data suggest that sunlight might be a protective factor for ovarian cancer.[5,6] Since the major source of vitamin D is through sunlight-induced synthesis in the skin, it has been hypothesized that vitamin D may mediate the protective effects observed. In addition, a case-control study in Mexico reports inverse association between vitamin D content of the diet and a risk of ovarian cancer.[7]

The action of vitamin D is mediated via its nuclear receptor, vitamin D receptor (VDR). Two different studies have reported that 43–50% of ovarian cancers express VDR.[8,9] In cell culture studies, vitamin D has been shown to inhibit the growth of various cancer cell lines.[4] We and others have shown that high 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2D_3$] concentrations inhibit the growth of a human ovarian cancer cell line, OVCAR-3, which expresses VDR.[8–10]

Because of its inhibitory growth effects, vitamin D is an attractive molecule in the anticancer drug development. The clinical use of 1,25(OH)$_2D_3$ in the treatment of cancer patients is limited due to its hypercalcemic side effects. Because of its less calcemic nature, a pro-hormone 25(OH)D_3 could be used in the cancer therapy in higher concentrations than 1,25(OH)$_2D_3$. Vitamin D analogues have been developed to separate the growth-regulating effects from the calcemic effects. EB 1089 is one of these analogues generated by altering the side-chain structure of the parent hormone.[11]

The effect of vitamin D is modulated by vitamin D metabolising enzymes, 24-hydroxylase (24OHase) and 1α-hydroxylase (1αOHase) expressed predominantly in kidney. The pro-hormone 25-hydroxyvitamin D_3 [25(OH)D_3] is converted to an active 1,25(OH)$_2D_3$ form by an enzyme 1αOHase. In addition to kidney, the expression of 1αOHase has been shown in skin, intestine, pancreas, adrenal medulla, brain and placenta.[12] The enzymatic activity of 1αOHase has also been detected in both normal and neoplastic prostate and in lung and colon cancer cells.[13–17] Some studies suggest that the expression of 1αOHase might change during the cancer development and progression.[14–16] Knockout studies in mice have shown the important role of 1αOHase in normal ovarian development, since smaller ovaries and impaired folliculogenesis are observed in 1αOHase null mutant mice.[18]

24OHase is a mitochondrial enzyme that catalyses the hydroxylation of 1,25(OH)$_2D_3$ to 1,24,25(OH)$_3D_3$ while 25(OH)D_3 is converted to 24,25(OH)$_2D_3$. 24OHase might also catalyse further hydroxylation steps of vitamin D metabolism.[19,63] These metabolites have been considered as inactivation products, which do not have a clear biologic function, but some studies have shown that vitamin D metabolites may also have specific effects in target cells.[20,21] Besides kidney, 24OHase enzyme has been detected in prostate, intestine, ovary and many other organs expressing vitamin D receptor.[22–28]

Although high concentrations of 1,25(OH)$_2D_3$ have been shown to be a potent growth inhibitor of many neoplastic cell lines, mitogenic effects have been reported with low concentrations of 1,25(OH)$_2D_3$ in several cell culture models.[21,29–33] In this study, we report the concentration-dependent growth modulation with 1,25(OH)$_2D_3$, 25(OH)D_3 and EB 1089 in the ovarian cancer cell line, OVCAR-3. The addition of 24OHase inhibitor (VID400) enhances growth-inhibiting effects of 1,25(OH)$_2D_3$ and converts growth-promoting effects of 25(OH)D_3 to inhibiting effects. We also show the expression of 1αOHase in ovarian cancer cell lines and the induction of 24OHase mRNA by vitamin D compounds in OVCAR-3 cells.

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MATERIAL AND METHODS

Cell culture

The human ovarian adenocarcinoma cell line, OVCAR-3 (ATCC, Manassas, VA) was maintained, as recommended by the supplier, in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO) supplemented with 10% FBS, 10 μg/ml insulin, 0.25% glucose and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin). Human ovarian adenocarcinoma cell lines UT-OC-1, UT-OC-2, UT-OC-3, UT-OC-4, UT-OC-5[34] and SK-OV-3 and a human keratinocyte cell line HaCaT were grown in DMEM (Sigma Aldrich) with 10% FBS and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin), and monkey kidney COS cells were maintained in DMEM/F12 (Sigma Aldrich) with 5% FBS. All cell lines were kept at 37°C in a humidified 95% air/5% CO_2 incubator.

Cell growth assay

When OVCAR-3 cells were on the logarithmic growth phase (70% confluent) the growth assay was started. For cell growth assay, 2,000 cell/200 μl/well were plated on 96-well culture plates. One day after plating, the medium (RPMI 1640, [Sigma Aldrich] supplemented with 10% FBS, 10 μg/ml insulin, 0.25% glucose and antibiotics) was changed and indicated concentrations of $1,25(OH)_2D_3$, $25(OH)D_3$, EB 1089 (kindly provided by Leo Pharmaceutical Products, Ballerup, Denmark), VID400 (specific 24OHase inhibitor, kindly provided by Dr. Anton Stuetz, Novartis Research Institute, Vienna, Austria) or combination of VID400 and $1,25(OH)_2D_3$ or $25(OH)D_3$ were added (day 0). Ethanol was used as a vehicle, and it was also included in the control. The medium containing ethanol vehicle and/or hormones were changed to a fresh one every third day. Cell growth samples were taken 0, 1, 3, 5, 7, 9 and 11 days after the treatment. Preliminary studies showed that during this period cells were at a logarithmic growth phase.

Relative cell numbers were quantified as described previously.[35] Cells were fixed on the bottom of the wells by addition of 10 μl of 11% glutaraldehyde solution in 0.1% phosphate buffer to 100 μl of medium. The plate was shaken 500 cycles/min for 15 min, washed 3 times by submersion in de-ionised water and air-dried. Fixed cells were stained with 0.1% solution of crystal violet dissolved in de-ionised water. After 20 min incubation, excess dye was removed by carefully washing with de-ionised water. The plate was air-dried prior to a bound dye solubilisation in 100 μl of 10% acetic acid. Relative cell number was given as absorbance units by measuring the optical density (590 nm) from each well using Victor 1420 multilabel counter (Wallac, Turku, Finland). Six determinations were used to calculate the mean optical density ± SD in each concentration at each time point. The absorbance value of day 0 (an overnight culture of 2,000 cells/well) was set as 0 by subtracting it from each value obtained from adjacent time-point measurements (days 1–11), and based on these values growth curves were created. Experiments were repeated 3–5 times. Day 11 was used to compare the effect of hormone treatments and 24OHase inhibitor. Statistical analyses were done using Student's *t*-test.

Detection of 24- and 1α-hydroxylase mRNAs

When cell culture bottles were grown to 70% confluence, the old medium was removed and replaced with medium containing 100 nM $1,25(OH)_2D_3$, $25(OH)D_3$ or EB 1089. Ethanol was used as a vehicle, and it was also added to the control cells. For RNA extraction, the cells were collected 4, 6 and 24 hr after the treatment with vitamin D compound or vehicle. RNA extractions were done with TRIZOL reagent (GIBCO Invitrogen Corporation, Paisley, UK). The integrity of RNA samples was confirmed on gel electrophoresis.

The expression of 24- and 1αOHase messenger RNA was detected using a reverse transcription-polymerase chain reaction (RT-PCR). To perform the RT-PCR, specific oligonucleotide primers were synthesised by Amersham Bioscience (Amersham, UK) (Table I). The reactions for 24-hydroxylase were performed in the LightCycler instrument (Roche Diagnostics, Basel, Switzerland) from 300 ng total RNA. PBGD (human porphobilinogen deaminase) mRNA was used as an external control. A master mix of the following components was prepared in a 20 μl volume: 0.5 μM PBGD primers or 0.3 μM 24OHase primers and 3.5mM Mn^{2+} for PBGD or 3.25 mM Mn^{2+} for 24OHase. Nucleotides, *Tth* DNA polymerase (DNA polymerase and reverse transcriptase activity), SYBR Green I and reaction buffer were included in the LightCycler-RNA Master SYBR Green I kit (Roche Diagnostics). For preparing the standard curve, total RNA from HaCaT cells, which express 24-hydroxylase mRNA,[28] was amplified in the same run as samples. The RT-PCR protocol was as follows: 20 min reverse transcription at 61°C and 30 sec denaturation at 95°C followed by 45 cycles with a 95°C denaturation for 1 sec, 62°C for PBGD or 57°C for 24OHase annealing for 7 sec and 72°C extension for 12 sec. Detection of fluorescent product was performed at the end of the extension step of each cycle. To verify the specific products, melting curve analysis and gel electrophoresis were done. The data were quantified by the Fit Points method with LightCycler Data Analysis software. The amplification efficiency and the relative expression ratio of 24OHase were calculated according to Pfaffl.[36] Hormone treatments and RT-PCR were done twice.

A normal RT-PCR was used for the detection of 1αOHase mRNA. RT-PCR (RobusT RT-PCR Kit, Finnzymes, Espoo, Finland) was performed according to the manufacturer's instructions from 1 μg total RNA. A negative control reaction (reactions without reverse transcriptase enzyme) was done from each sample. The RT-PCR protocol was as follows: 30 min reverse transcription at 48°C and 2 min denaturation step at 94°C followed by 30 cycles with 94°C denaturation for 30 sec, 54°C annealing for 30 sec and 72°C extension for 30 sec. The final extension after cycles was at 72°C for 7 min. Total RNA (0.5 μg) from monkey kidney COS cells transfected with human 1αOHase cDNA (kindly donated by Dr. S. Kato, Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan) was used as a positive control. A transfection was done according to the manufacturer's instructions with 1αOHase ORF cDNA in pcDNA3 mammalian expression vector by a lipofection (Lipofectamine, Life Technologies). A functional control reaction (MS2 RNA and primers for amplification of 1100 bp sequence) was included in the kit, and it was carried out with the same run as other samples. After gel electrophoresis, RT-PCR products were extracted from the gel and sequences were verified by hybridisation with ^{32}P-labelled RNA-probe made from 1αOHase cDNA.

TABLE I – OLIGONUCLEOTIDE PRIMER SEQUENCES FOR RT-PCR

Gene (accession no.)	Base pairs	Oligos	Sequence	Product size (bp)
1αOHase	1241–1261	F	5′-GTCAAGGAAGTGCTAAGACTG-3′	303
(AB005038)	1524–1543	R	5′-TGTTAGGATCTGGGCCAAAG-3′	
24OHase	833–852	F	5′-TGATCCTGGAAGGGGAAGAC-3′	212
(L13286)	1023–1044	R	5′-CACGAGGCAGATACTTTCAAAC-3′	
PBGD	695–714	F	5′-AAGTGCGAGCCAAGGACCAG-3′	298
(X04808)	969–992	R	5′-TTACGAGCAGTGATGCCTACCAAC-3′	

F, forward primer; R, reverse primer.

Metabolic analysis of 25(OH)D_3

OVCAR-3 cells (1.5×10^6 cell/flask) were plated on T25 culture flasks. One day after plating, cells were treated with 500 nM 25(OH)D_3 in RPMI 1640 medium supplemented with 10% FBS, 10 μg/ml insulin, 0.25% glucose and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin). After 0, 3 or 24 hr, the medium was collected and the cell monolayer was extracted with 1 ml methanol. After 15 min incubation at room temperature, the methanol was transformed into the same tube than the sample medium. The samples for the measurement of the 25(OH)D_3 metabolites were purified using the acetonitrile-C18 Sep-Pak (Waters Corporation, Milford, MA) procedure[37] followed by separation of the metabolites by a high-performance liquid chromatography. The concentrations of 24,25$(OH)_2D_3$ were quantified by a competitive protein binding assay[38] and 1,25$(OH)_2D_3$ by a radioreceptor assay.[39] The second measurement was done following the same procedure except dextran charcoal-treated FBS was used instead of FBS, 24OHase inhibitor (200 nM VID400) was used with the 500 nM 25(OH)D_3 treatment, and the samples were collected only after 0 and 24 hr.

RESULTS

Regulation of OVCAR-3 cell growth by different vitamin D compounds

Figure 1*a* illustrates the concentration-dependent stimulation of the cell growth with 25(OH)D_3 in OVCAR-3 cell line. An amount of 10 nM 25(OH)D_3 treatment stimulated growth by 32%, 50 nM stimulated growth by 41%, 100 nM by 39%, 200 nM by 35% and 500 nM 25(OH)D_3 by 11% when compared to the control (Fig. 1*a*). All differences were statistically significant when compared to the control ($p < 0.05$).

When high concentrations were used, 1,25$(OH)_2D_3$ inhibited growth of the OVCAR-3 cell line (Fig. 1*b*). An amount of 100 nM 1,25$(OH)_2D_3$ inhibited growth by 74% ($p < 0.001$) and 10 nM by 8% ($p < 0.0001$) when compared to the control. An amount of 0.1 nM 1,25$(OH)_2D_3$ stimulated growth by 14% ($p < 0.0001$), whereas 1 nM 1,25$(OH)_2D_3$ did not have an effect on the cell growth.

EB 1089 inhibited growth when 1 and 100 nM concentrations were used (Fig. 1*c*). When compared to the control, 100 nM EB 1089 inhibited growth by 84% and 1 nM by 73% ($p < 0.0001$). At 1 nM concentration, EB 1089 was as potent a growth inhibitor as 100 nM EB 1089. The growth inhibition was almost equal to 100 nM 1,25$(OH)_2D_3$ and 1 nM EB 1089 (74% *vs.* 73% of the control).

Expression of 1α-hydroxylase and 24-hydroxylase

To test whether enzymes 1α-hydroxylase and 24-hydroxylase might be involved in the metabolism of vitamin D compounds in the OVCAR-3 cell line, we studied the expression of these enzymes at mRNA level. We also studied whether the expression of 24OHase mRNA could be modulated by 25(OH)D_3, 1,25$(OH)_2D_3$ or EB 1089.

Our data indicate that the OVCAR-3 cell line expresses 1αOHase (Fig. 2). A single 303 bp band can be seen in 1αOhase-transfected COS sample (lane 3) and in both ethanol-treated control (lanes 7 and 8) and 100 nM 1,25$(OH)_2D_3$-treated (lanes 9 and 10) OVCAR-3 samples. A hybridisation with P^{32}-labelled probe showed that the 1αOHase sequence is amplified in RT-PCR. 1αOHase mRNA was also expressed in 6 other ovarian cancer cell lines (UT-OC-1–5 and SK-OV-3; data not shown).

Also 24OHase is expressed in OVCAR-3 cells and the expression of 24OHase is regulated by EB 1089 and 1,25$(OH)_2D_3$ almost equally. After 6 hr treatment, the expression of 24OHase mRNA (Fig. 3) was induced 650-fold with 100 nM 1,25$(OH)_2D_3$ and 600-fold with 100 nM EB 1089. After 24 hr, the expression levels were further increased. When compared to the control, 1,25$(OH)_2D_3$ treatment induced the expression by 1,100-fold and

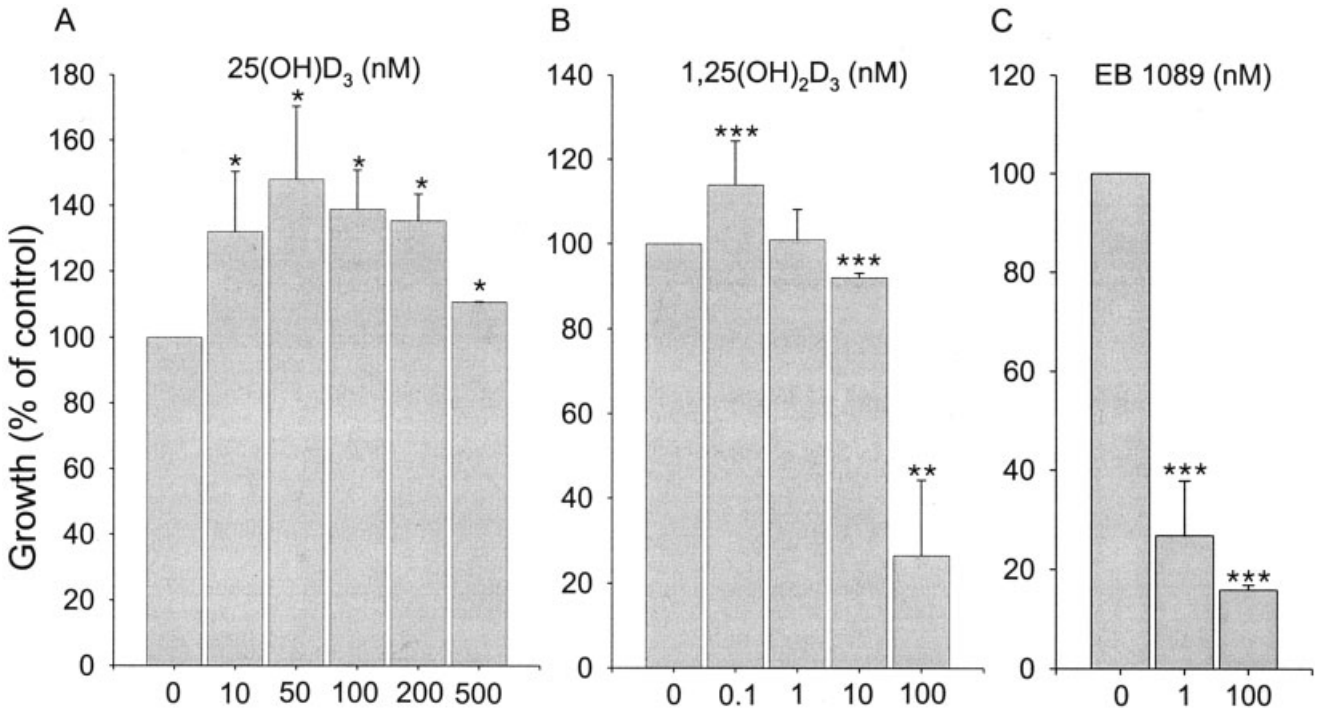

FIGURE 1 – Regulation of the cell growth by vitamin D compounds. The effect of 25(OH)D_3 *(a),* 1,25-$(OH)_2D_3$ *(b)* and EB 1098 *(c)* on the growth of OVCAR-3 cells. Cells were treated with indicated hormone concentrations for 11 days. Growth medium and hormones were changed to a fresh one every third day. After the treatment period, cells were fixed, stained with crystal violet, and the optical density (590 nm) was determined. The cell growth is presented as a percentage of ethanol-treated cells. The values represent the mean of 3–5 separate experiments ± SD. (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$, Student's *t*-test).

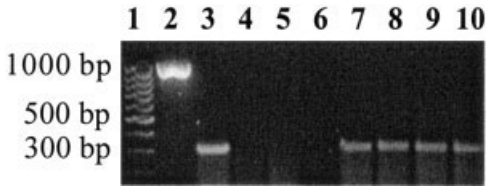

FIGURE 2 – The expression of 1αOHase in OVCAR-3 cells. A RT-PCR was used for the detection of 1αOHase mRNA from OVCAR-3 cells. A 303 bp band can be seen in the 1αOhase-transfected COS sample (lane 3) and in both ethanol-treated (lanes 7 and 8) and 100 nM 1,25-$(OH)_2D_3$-treated (lanes 9 and 10) OVCAR-3 samples. In lane 4, there is a negative control for the ethanol-treated sample, and lane 5 represents a negative control for the 1,25-$(OH)_2D_3$-treated sample. Lane 1 is a 100 bp marker, lane 2 is a RT-PCR functional control (1100 bp), and lane 6 is empty.

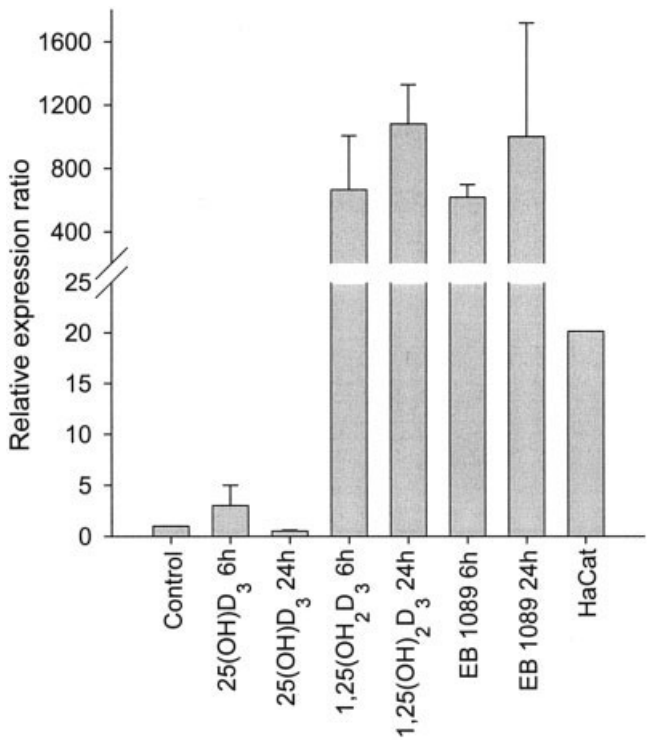

FIGURE 3 – The relative expression ratios of 24OHase mRNA in OVCAR-3 cells after 6 or 24 hr treatment with 100 nM 1,25-$(OH)_2D_3$, 25$(OH)D_3$, EB 1098 or ethanol (vehicle). A quantitative RT-PCR was done using 0.3 μg total RNA. The human keratinocyte cell line, HaCaT, was used as an expression control of 24OHase. The values represent the mean of 2 independent experiments ± SD.

EB 1089 by 1,000-fold. After 6 hr treatment, the expression in 25$(OH)D_3$- (100 nM) treated cells was slightly increased (3-fold) but returned to a basal level or even slightly downregulated (0.5 fold) after 24 hr treatment. The human keratinocyte cell line, HaCaT, was used as a control for the expression of 24Ohase, and our data indicate that the basal expression level is 20 times higher in HaCaT than in OVCAR-3 cells.

Metabolism of 25(OH)D_3 in OVCAR-3 cells

Since the both enzymes, 24OHase and 1αOHase, are expressed in the OVCAR-3 cell line, we studied the functionality of these enzymes. Analysis of metabolites generated from 25$(OH)D_3$ are shown in Table II. In the first experiment, the amount of 24,25$(OH)_2D_3$ was 4 times higher after 3 hr incubation than it was

TABLE II – METABOLITES OF 25$(OH)D_3$

	24,25$(OH)_2D_3$ (nM)		1,25$(OH)_2D_3$ (pM)	
	I	II[1]	I	II[1]
500 nM 25$(OH)D_3$, 0 hr	6	1	23	<20
500 nM 25$(OH)D_3$, 3 hr	24	ns	37	ns
500 nM 25$(OH)D_3$, 24 hr	112	27	33	28
500 nM 25$(OH)D_3$ + 200 nM VID400, 24 hr	ns	8	ns	27

[1]Cells were grown in RPMI 1640 supplemented with 10% dextran charcoal-treated FBS instead of FBS. ns, not studied.

when the experiment started (0 hr). After 24 hr, the production was further increased (18-fold). The basal level of 1,25$(OH)_2D_3$ was 23 pM, and after 3 hr incubation, the concentration was increased to 37 pM. After 24 hr, the concentration was almost equal or slightly decreased (33 pM).

In the second experiment, we supplemented RPMI 1460 medium with dextran charcoal-treated FBS instead of normal FBS. In this experiment, the concentration of 24,25$(OH)_2D_3$ was increased 27 times after 24 hr. When 24OHase inhibitor was used, the production reduced to one-third when compared to 500 nM 25$(OH)D_3$ treatment alone. At the beginning of the experiment (0 hr), the concentration of 1,25$(OH)_2D_3$ was undetectable, but after 24 hr we could detect 28 pM concentration of 1,25$(OH)_2D_3$. 24OHase inhibitor did not have an effect on production of 1,25$(OH)_2D_3$.

Effect of 24OHase inhibitor on growth response of 1,25(OH)$_2D_3$ and 25(OH)D_3

Because the metabolic measurements showed an extensive production of 24,25$(OH)_2D_3$ and an enzymatic activity of 24OHase, we decided to test the effect of 24OHase inhibitor, VID400, on the growth response of 1,25$(OH)_2D_3$ and 25$(OH)D_3$. As shown in Figure 4*a* and *b*, 200 nM VID400 alone had a growth-inhibitory effect on cells. The inhibition was 8% ($p < 0.05$) when compared to the control. In these experiments, 100 nM 25$(OH)D_3$ stimulated growth by 18% (Fig. 4*a*), but the difference was not statistically significant when compared to the control. When 100 nM 25$(OH)D_3$ was combined with 200 nM VID400, the stimulatory growth effect was converted to an inhibitory (14%, $p < 0.001$ when compared to the control).

We also studied the effect of 24OHase inhibitor on the growth response of 1,25$(OH)_2D_3$ (Fig. 4*b*). In these experiments, 1 nM 1,25$(OH)_2D_3$ alone did not have an effect on the cell growth. However, when it was combined with 200 nM VID400, it inhibited the growth by 27% ($p < 0.0001$) when compared to the control. An amount of 10 nM 1,25$(OH)_2D_3$ alone inhibited the growth by 26%, but a combination of 10 nM 1,25$(OH)_2D_3$ and 200 nM VID400 inhibited growth by 77%.

DISCUSSION

The growth inhibition of OVCAR-3 cells by 100 nM 1,25$(OH)_2D_3$ observed in our study is in an agreement with previous results on OVCAR-3 cells[8–10] and many other cancer cell lines.[4,40,41] Results of cell culture studies showing a growth inhibition are usually obtained with high 1,25$(OH)_2D_3$ concentrations (10–100 nM). However, there are also reports concerning the stimulation of cell proliferation with low 1,25$(OH)_2D_3$ concentrations. *In vitro* studies suggest that in normal tissues 1,25$(OH)_2D_3$ might have a role in maintaining the balance between proliferating and differentiating cell populations.[31–33] There are also reports that low concentrations of 1,25$(OH)_2D_3$ may stimulate the growth of neoplastic cell lines. Mitogenic effects of 1,25$(OH)_2D_3$ have been reported with low 1,25$(OH)_2D_3$ concentrations in prostate[41] and other cancer cell lines,[29,30] as well as in the ovarian cancer cell line in our study.

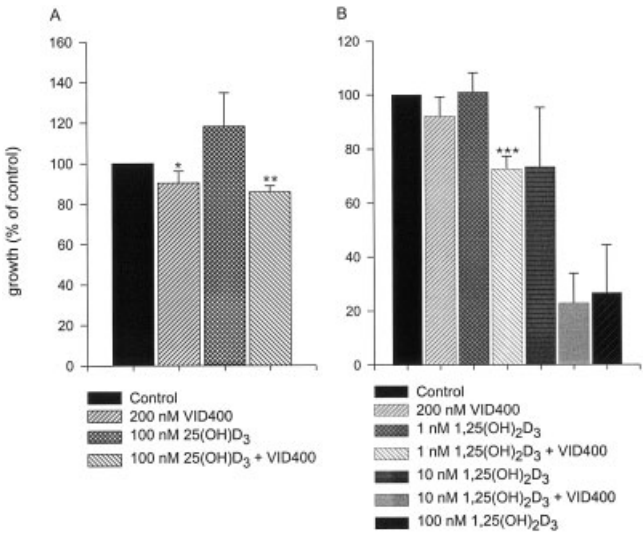

FIGURE 4 – The effect of 24OHase inhibitor on the cell-growth response to $25(OH)D_3$ *(a)* and $1,25\text{-}(OH)_2D_3$ *(b).* Cells were treated with indicated hormone concentrations or combinations of hormone and 24OHase inhibitor (VID400) for 11 days. The growth medium and hormones were changed to a fresh one every third day. After the treatment period, cells were fixed, stained with crystal violet, and the optical density (590 nm) was determined. The cell growth is presented as a percentage of ethanol-treated cells. The values represent the mean of 3 separate experiments ± SD. (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$, Student's *t*-test).

Vitamin D analogue, EB 1089, has been shown to be more potent in the tumour cell growth inhibition than $1,25(OH)_2D_3$ in both *in vivo* and *in vitro* experiments.[42–45] In our study, EB 1089 was a potent inhibitor of OVCAR-3 cell growth already at 1 nM concentration. Studies on other cancer cell types have suggested that EB 1089 may be 50–200 times more potent than $1,25(OH)_2D_3$ in regulation of cell growth,[46] which is in line with our result showing similar growth inhibition with 1 nM EB 1089 and 100 nM $1,25(OH)_2D_3$ in OVCAR-3 cells. Similar results have been observed with prostate cancer cell line LNCaP, where 100 nM $1,25(OH)_2D_3$ was as potent a growth inhibitor as 1 nM EB 1089.[47] EB 1089 has been tested in phase I trials in breast and colorectal cancer patients.[48] Because it could be used in lower concentrations than the parent hormone to achieve a growth inhibition, calcemic and toxic effects observed with $1,25(OH)_2D_3$ treatment might be avoided.

The relatively high concentrations of $1,25(OH)_2D_3$ required to obtain an inhibitory growth response may be due to an enzyme 24OHase. The ability of $1,25(OH)_2D_3$ to induce 24OHase through a vitamin D receptor-dependent process is well known and used as a marker of $1,25(OH)_2D_3$ action.[49–51] Previously, induction of specific activity of 24OHase by $1,25(OH)_2D_3$ has been shown in ovarian cancer cells.[25] 24OHase was highly inducible by $1,25(OH)_2D_3$ and EB 1089 (1,100- and 1,000-fold, respectively) in OVCAR-3 cells. 24OHase has been considered as an inactivating enzyme of $1,25(OH)_2D_3$. Metabolites generated by 24OHase pathway may still have their own distinct effects on cell growth, but they are poorly known. $1,24,25(OH)_2D_3$ and a further oxidized product, $1,25(OH)_2$-24-oxo-vitamin D_3, might have growth-promoting effects.[21,52] Recently a selective inhibitor of 24OHase, VID400, has been developed.[53] Our results show that inhibition of 24OHase enhances the growth inhibition of $1,25(OH)_2D_3$. In breast cancer, 24OHase has been described as a candidate oncogene, whose overexpression may give a growth advantage to cancer cells, since these cells may escape from the vitamin D-mediated growth control.[24] The chromosomal region 20q12-q13 is amplified in 54% of ovarian cancers,[54] and it is the same region in which the gene locus of 24OHase is mapped.[55] The growth-promoting role of 24OHase makes it an even more powerful oncogene, since it does not only degrade the most growth-inhibitory form of vitamin D, $1,25(OH)_2D_3$, but also converts it to a growth-stimulatory metabolite. The strong induction of 24OHase by $1,25(OH)_2D_3$, possible because of a genomic amplification, might be a reason why calcitriol therapy in ovarian cancer has not been successful.[56]

The induction of 24OHase by EB 1089 has been shown previously in rat kidney and intestine[26] and in human head and neck squamous cell carcinoma cells.[57] Although in our study on OVCAR-3 cells EB 1089 upregulated 24OHase mRNA levels by 1,000-fold, the metabolism of EB 1089 may not involve 24OHase. Because of its side-chain double-bond structure, it is rather hydroxylated in distal C26 and C26a sites.[58] Differences in metabolic pathways between EB 1089 and $1,25(OH)_2D_3$ might explain why EB 1089 inhibited growth already at a 1 nM concentration, whereas 1 nM $1,25(OH)_2D_3$ did not have any effect on the cell growth.

$25(OH)D_3$ has been considered as a pro-hormone. Previous studies have shown an inhibition of cell proliferation by $25(OH)D_3$ in cultured prostate cells[15,59,60] and in colon tissue *in vivo.*[61] However, our results indicate that in the range of 10 nM to 500 nM, $25(OH)D_3$ promotes ovarian cancer cell growth. The effect of $25(OH)D_3$ on cell growth might be cell and tissue-type specific. Some growth-modulating effects of $25(OH)D_3$ might be mediated via a direct binding to VDR, although the relative binding affinity of $25(OH)D_3$ to VDR is about 700-fold lower than that of $1,25(OH)_2D_3$.[62] Besides the direct binding to VDR, both of the vitamin D metabolising enzymes, 1αOHase and 24OHase, might regulate the cellular responses to $25(OH)D_3$. Many cell types are reported to express 1αOHase and might be able to convert $25(OH)D_3$ to an active metabolite $1,25(OH)_2D_3$.[12–17] Our finding that 1αOHase mRNA is expressed in the human ovarian cancer cell lines suggests that $1,25(OH)_2D_3$ could be generated locally. In the early phase of the human colorectal cancer genesis, the expression of 1αOHase and VDR mRNA are upregulated, whereas in poorly differentiated late-stage carcinomas, only low levels of the respective mRNAs can be detected.[14] In prostate cancer cells, the activity of 1αOHase was reported to be 10- to 20-fold lower than in normal prostate cells, leading to a reduced antiproliferative action of $25(OH)D_3$.[15] As shown in our study, the activity of $25(OH)D_3$ 1αOHase in these cells may be so low that only minimal amounts of $1,25(OH)_2D_3$ is produced, and low $1,25(OH)_2D_3$ concentrations was shown to be growth stimulatory in our study.

24OHase converts $25(OH)D_3$ to $24,25(OH)_2D_3$ and also catalyses further hydroxylation reactions.[63] The role of these products in the regulation of the cancer cell growth has not been studied extensively, but $24,25(OH)_2D_3$ is believed to contribute to the bone formation and the fracture healing.[20] Although we could detect only a slight induction of 24OHase mRNA after 6 hr treatment and even a downregulation after 24 hr, the metabolite analysis showed the extensive production of 24-hydroxylated product, $24,25(OH)_2D_3$. In our cell growth studies, the inhibition of 24OHase activity converted the growth stimulation to the growth inhibition, which suggests that the mitogenic effects of $25(OH)D_3$ might be mediated partially through 24-hydroxylated products.

We conclude that in ovarian cancer, high vitamin D concentrations may be required to acquire a beneficial inhibitory growth effect. Small amounts of vitamin D may stimulate the growth of ovarian cancer cells as does low concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ in the OVCAR-3 cell line. Because of the growth stimulation, the role of vitamin D and its metabolising enzymes, 24OHase and 1αOHase, needs to be established in more detail in ovarian cancer development and progression.

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