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1,25(OH)$_2$Vitamin D$_3$ induces elevated expression of the cell cycle inhibitor p18 in a squamous cell carcinoma cell line of the head and neck

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BACKGROUND: 1α,25-Dihydroxyvitamin D$_3$ [1,25(OH)$_2$ Vitamin D$_3$] induces growth inhibition in squamous cell carcinoma (SCC) cell lines of the head and neck by arresting the cells in the G0/G1 phase of the cell cycle, probably due to an enhanced expression of p21, which could be demonstrated in other cell lines (JPPA, SCC9) before. In SCC25, a SCC cell line isolated from tongue, growth inhibition but no overexpression of p21 was detected. The retinoblastoma gene, as a direct target of G1 cyclin–CDK complexes, showed an obvious shift from the hyperphosphorylated to the hypophosphorylated form under 1,25(OH)$_2$Vitamin D$_3$, which indicates that the growth inhibition takes place in the G0/G1 phase. To explore the possible pathway of growth inhibition in SCC25 we investigated other cell cycle inhibitors (p18, p19, p27).

METHODS: Synchronized cells were treated with 1,25(OH)$_2$Vitamin D$_3$ over 96 h. The cell cycle status and expression of cell cycle-regulating proteins was determined by fluorescence-activated cell sorting (FACS) and Western blotting.

RESULTS: An overexpression of p18 in 1,25(OH)$_2$Vitamin D$_3$ vs. ethanol-treated cells was determined until 30 h in SCC25. No influence was detectable on the expression of p27 and p19.

CONCLUSION: One mechanism by which 1,25(OH)$_2$ Vitamin D$_3$ controls cell growth might be the upregulation of p21. As p21 was unsusceptible to 1,25(OH)$_2$Vitamin D$_3$ in SCC25, other inhibiting proteins were necessary to be tested. The proven upregulation of p18 seems to be the responsible step for growth inhibition of 1,25(OH)$_2$Vitamin D$_3$ in SCC25.

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Introduction

Recently, the biologically active form of vitamin D$_3$, 1α,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$Vitamin D$_3$], has shown promising results as a potent antitumour agent. Beside regulation of calcium homeostasis regarded to clinical interests, 1,25(OH)$_2$Vitamin D$_3$ acts antiproliferative and induces differentiation in squamous cell carcinoma (SCC) cell lines of different origin *in vitro* (1, 2), which is achieved by an arrest of cells in the G0/G1 phase of the cell cycle (3). In addition, among various changes, 1,25(OH)$_2$Vitamin D$_3$ develops its growth-inhibiting effects on cancer cell lines probably through enhancement of cyclin-dependent kinase inhibitors (CDKIs), predominantly through an upregulation of p21. A 1,25(OH)$_2$Vitamin D$_3$-mediated enhanced expression of the cell cycle inhibitory protein p21 could be demonstrated in SCC of the head and neck (SCCHN; 4, 20).

This investigation was performed to find other inhibitors responsible for growth inhibition in a cell line with no p21 upregulation.

The molecules responsible for the control of the cell cycle are linked together to regulate the process of cell proliferation. One group of the proliferation-controlling proteins is the INK4 family, which consists of the cyclin-dependent kinases (CDKs) CDK4 and CDK6 and the CDKIs p18 and p19. CDKIs prevent CDKs from phosphorylating critical substrates, such as retinoblastoma gene (pRb). The pRb is one of the target proteins of G1–CDK complexes. Phosphorylation of pRb leads to its liberation, which induces the transcription of genes necessary for cell growth.

The CDKIs p15, p16, p18 and p19 have been suggested as candidates for tumour-suppressor genes. Their blocking of CDKs leads to growth inhibition and alteration of these CDKIs may cause abnormal cell cycle. Alteration or deletion of CDKIs leads to enhancement of CDKs (e.g. CDK4 and CDK6) and Rb gets phosphorylated (5, 6). Hyperphosphorylation of Rb causes E2F expression and ends in entering the S-phase of the cell cycle. Many genes important for cell proliferation have E2F receptors, e.g. c-myc, cdc2, DNA

polymerase α (5). Entering the S-phase results in activation of these genes and in cell proliferation, hypophosphorylation inhibits E2F and gene expression in S-phase (7). Another CDKI – which is known to play a role in the G1 phase of the cell cycle – p27, could be a regulator in linking extracellular growth controlling signals to progression to or exit from the cell cycle.

Materials and methods

Cell culture

SCC25, a human SCCHN cell line from the tongue, JPPA (laryngeal carcinoma), SCC9 (tongue carcinoma) and HaCaT (immortalized keratinocytes) were used. JPPA and HaCaT were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco Life Technologies Ltd, Paisley, UK), supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 1% 4-2-hydroxyethyl-1-piperazine-ethanesulfonic acid buffer (HEPES) and 1% penicillin/streptomycin (all from Gibco). SCC9 and SCC25 were maintained in RPMI 1640 (Gibco) supplemented with 10% FCS and 1% penicillin/streptomycin.

All cells were synchronized by keeping them in confluence for 48 h, harvested and, in order to avoid contact inhibition, half to 2 million cells were seeded on Petri dishes. Diluted in the corresponding medium $1,25(OH)_2$Vitamin D_3 was added in different concentrations (10^{-7} and 10^{-10} M). It is dissolved in ethanol (EtOH) and was kindly provided by Leo Pharmaceutical Products (Ballerup, Denmark).

Cell counting

Cells were seeded in culture dishes at a density of 0.5 to 1.5×10^6 for cell counting. After adhesion they were incubated with DMEM/RPMI with or without $1,25(OH)_2$Vitamin D_3 (10^{-7} and 10^{-10} M) as well as EtOH (Merck, Darmstadt, Germany; 10^{-3} M) and grown in an incubator at 37°C containing 95% humidity and 5% CO_2. The cultures were trypsinized, centrifuged, resuspended in media and counted by trypan blue staining in a Bürker-Türk chamber at indicated time points. Viability of the cells was permanently ≥90%, as determined by trypan blue exclusion.

Proliferation assay/ELISA

Cells were seeded in triplicate at a density of 6×10^4 cells per well in 96-well plates. After adhesion (24 h), $1,25(OH)_2$Vitamin D_3 (10^{-7} and 10^{-10} M) and EtOH (10^{-3} M) were added and remained for at least 72 h. Enzyme-linked immunosorbent assay (ELISA) was carried out with a commercially available kit for proliferation assays (MTT assay, Boehringer Mannheim, Germany). Medium was completely aspired and cells were pulsed with BrdU for 2 h. After supplementation of Anti-BrdU-Antibody and washing, BrdU incorporation was measured (ELISA-Reader, Anthos ht ll, Anthos Labtec Instruments, Salzburg, Austria).

Cell cycle analysis by FACS

Cell cycle measurements were performed with fluorescence-activated cell sorting (FACS) on all cell lines. Cells were seeded in culture dishes at a density of 2×10^6. After adhesion they were incubated with $1,25(OH)_2$Vitamin D_3(10^{-7} M) and EtOH (10^{-3} M). At indicated time points the cultures were trypsinized, centrifuged, resuspended in media and single cell suspensions in phosphate-buffered saline (PBS) were prepared. Ice-cold 70% EtOH was vigorously added and remained for at least 30 min in the ice-cold condition. Cells were harvested by centrifugation and resuspended in PBS. Then RNase (1 mg/ml; Sigma, St Louis, MO, USA) and Propidium iodide (400 μg/ml; Sigma) were added and cells were incubated at 37°C for 30 min. Samples were analysed using an argon-ion laser tuned to 488 nm and measured with forward and orthogonal light scattering and red fluorescence.

Western blot

Cell cycle inhibitors including p18, p19 and p27 were analysed at the protein level by Western blot. Cells were washed twice in cold PBS and lysed in ice-cold NP-40 buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulphate (SDS), 1% Nonidet P40, 10 mM *p*-nitrophenolphosphate, 250 U/l aprotinin, 40 mg/ml leupeptin, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 40 mM glycerophosphate and 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The whole lysates (30–50 μg) were resolved by SDS polyacrylamide gel electrophoresis (12.5%; 1 h) and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Blocked membranes were probed with anti-p18, anti-p19 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-p27 (Calbiochem, Oncogene Research Products, Darmstadt, Germany) monoclonal antibody. As loading control, antiactin rabbit antibody (Sigma) was used. All blots were developed by using the enhanced chemiluminescent (ECL) detection system (Amersham Pharmacia Biotech, Little Chalfont, UK).

Northern blot

Total RNA was extracted from growing cells at indicated time points with Trizol 15–30 μg of total RNA was separated on 1.2% agarose-formaldehyde gels, blotted onto positively charged nylon membranes and covalently bound by backing 30 min at 120°C. Membranes were pre-hybridized in Dig Easy Hyb (Boehringer Mannheim) for 3 h at 68°C and hybridization was performed in the same buffer with Dig-labelled riboprobes overnight at 68°C. Blots were detected with the Dig luminescence detection Kit (Boehringer Mannheim) and exposed to Kodak X-ray film (Kodak, New York, NY, USA.

Statistical analysis

After testing the presuppositions showing a normal distribution of values an adequate *a posteriori* test (unpaired Student's *t*-test) was performed using the STATGRAPHICS PLUS for Windows, Version 4.0.

Results

Proliferation behaviour under 1,25(OH)$_2$Vitamin D$_3$

Cells of immortalized keratinocytes (HaCaT) and the carcinoma cell lines JPPA, SCC9 and SCC25 were incubated for 72 h with or without 1,25(OH)$_2$Vitamin D$_3$ and EtOH.

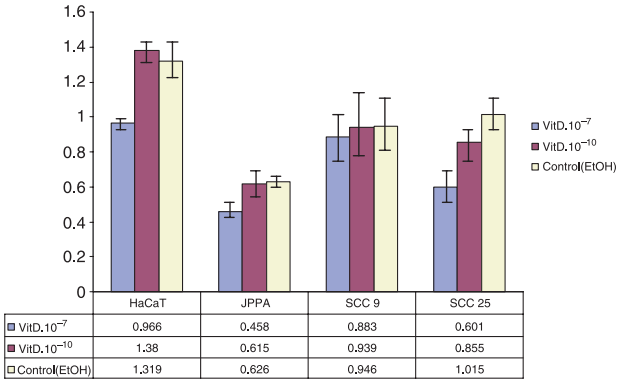
Growth reduction in SCC25, JPPA and HaCaT after incubation with 1,25(OH)$_2$Vitamin D$_3$ occurred after 48 h and was visible until 72 h, determined by trypan blue staining (Fig. 1). SCC9 cell count was decimated after treatment with 1,25(OH)$_2$Vitamin D$_3$ after 72 h. The viability of cells was permanently ≥90%.

BrdU uptake in ELISA proliferation assay experiments was significantly lower in cells cultivated with 1,25(OH)$_2$Vitamin D$_3$ than in EtOH-exposed cells in a dose-depending manner (Fig. 2).

All cell lines tested were vitamin D receptor (VDR)-positive investigated by FACS (data not shown). Additionally, VDR expression was enhanced after application of 1,25(OH)$_2$Vitamin D$_3$.

Cell cycle

The cells were synchronized by keeping the cells in confluence for 48 h and seeded in Petri dishes. 1,25(OH)$_2$Vitamin D$_3$ and EtOH were added and cells were prepared for FACS analysis and measured at indicated time points (0, 18, 24, 36, 48 and 72 h). In SCC25 an accumulation of cells in the G0/G1 phase under the influence of 1,25(OH)$_2$Vitamin D$_3$ was detected until 48 h with a significantly higher level in 1,25(OH)$_2$Vitamin D$_3$ exposed cells than in DMEM ± EtOH treated at the 48-h time point. JPPA cells showed G0/G1 arrest until 36 h (Table 1).



	HaCaT	JPPA	SCC 9	SCC 25
VitD.10^{-7}	0.966	0.458	0.883	0.601
VitD.10^{-10}	1.38	0.615	0.939	0.855
Control(EtOH)	1.319	0.626	0.946	1.015

Figure 2 BrdU uptake of HaCaT, JPPA, SCC9 and SCC25 after addition of 1α,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$; 10^{-7}, 10^{-10}] vs. control (EtOH) measured after 48 h.

Expression of p18

The influence of 1,25(OH)$_2$Vitamin D$_3$ on the expression of cell cycle regulating proteins in SCCHN was investigated by Western blot analysis. After addition of DMEM/RPMI ± 1,25(OH)$_2$Vitamin D$_3$ and EtOH the cells were prepared at 6-h time intervals up to 48 h and after 72 and 96 h. The blots were detected with anti-p18, anti-p19 and anti-p27 monoclonal antibodies. Antiactin rabbit antibody was used as a loading control.

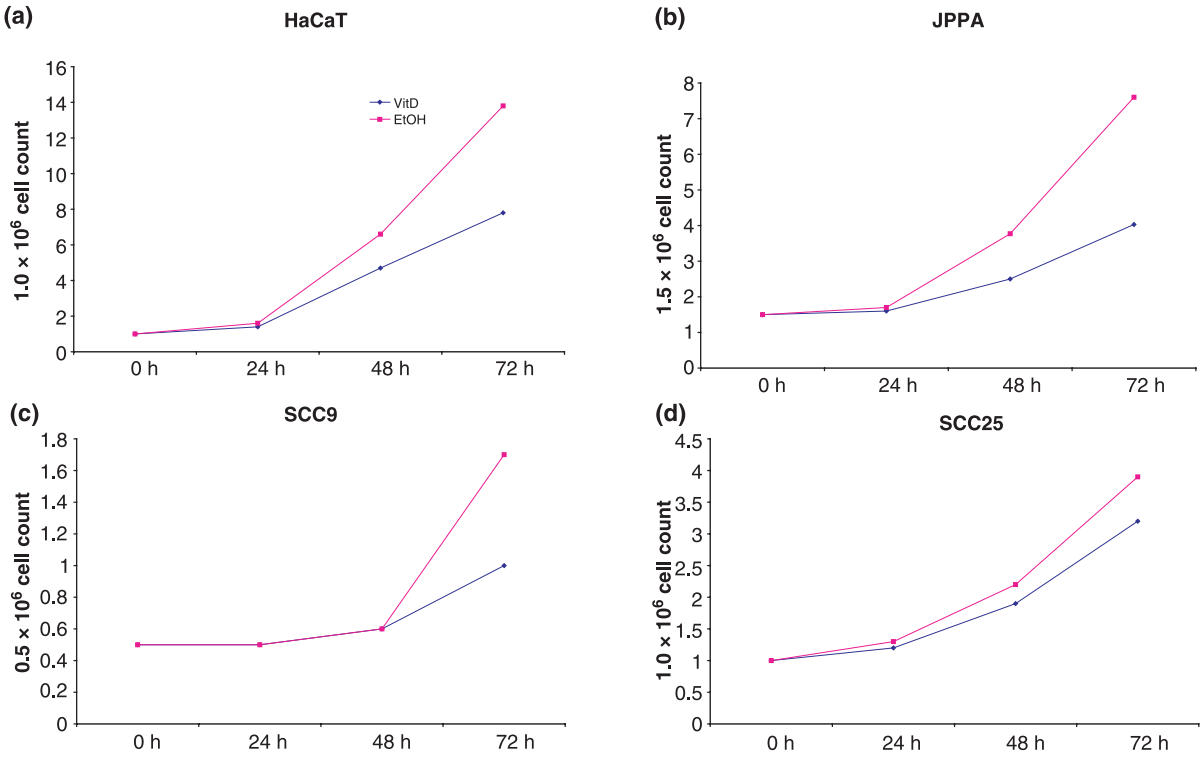


Figure 1 Proliferation behaviour under the influence of 1α,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$; 10^{-7}, 10^{-10}] HaCaT, JPPA, SCC9 and SCC25 were synchronized and cultured in growth medium with or without 1,25(OH)$_2$D$_3$ (10^{-7}, 10^{-10}) for 72 h. Ethanol (EtOH) was used as control.

Table 1 Cell cycle distribution of $1,25(OH)_2D_3$ (10^{-7}) treated SCCHN cell lines (in 24-h time intervals the DNA content was analysed by propidium iodide staining and FACS analysis)

	G0/G1	*S*	*G2/M*
HaCaT			
$1,25(OH)_2D_3$			
0 h	72.8 ± 5.0	4.05 ± 2.8	23.15 ± 0.8
24 h*	59.9 ± 0.7	24.25 ± 1.35	15.85 ± 0.6#
48 h*	61.85 ± 0.95	15.5 ± 1.7	22.65 ± 0.75
72 h*	65.15 ± 1.95	15 ± 1.2	19.75 ± 0.75
Control (EtOH)			
24 h	49.52 ± 3.8	30.75 ± 3.9	18.8 ± 2.0
48 h	51.15 ± 0.25	18.35 ± 0.65	30.5 ± 0.4
72 h	52.75 ± 1.45	19.8 ± 1.4	27.45 ± 0.5
JPPA			
$1,25(OH)_2D_3$			
0 h	73.95 ± 0.25	15.2 ± 2.65	11.55 ± 2.78
24 h*	66.95 ± 3.97	16.73 ± 4.1	16.32 ± 4.47#
48 h*	46.25 ± 4.84	30.15 ± 0.95	23.6 ± 5.3#
72 h*	65.4 ± 9.7#	18.66 ± 3.6#	15.81 ± 5.25#
Control (EtOH)			
24 h	57.38 ± 5.77	25.58 ± 6.48	17.2 ± 1.9
48 h	37.8 ± 1.6	32.8 ± 1.86	29.45 ± 3.4
72 h	60.7 ± 8.3	21.65 ± 6.38	18.25 ± 5.25
SCC9			
$1,25(OH)_2D_3$			
0 h	72.45 ± 0.75	3.45 ± 2.18	24.1 ± 4.2
24 h*	60.55 ± 7.55	21.05 ± 7.1#	18.4 ± 0.2#
48 h*	34.05 ± 0.25	63.8 ± 0.8	2.25 ± 0.85
72 h*	45.25 ± 3.85	39.75 ± 1.45	15.15 ± 5.14#
Control (EtOH)			
24 h	49.6 ± 0.9	25.35 ± 6.25	25.05 ± 7.25
48 h	25.5 ± 1.4	67.95 ± 3.85	6.55 ± 2.14
72 h	34.25 ± 2.45	49.15 ± 1.05	16.8 ± 3.2
SCC25			
$1,25(OH)_2D_3$			
0 h	79.7 ± 1.31	8.4 ± 1.48	11.9 ± 1.58
24 h*	79.6 ± 2.43#	7.4 ± 1.15	13.0 ± 0.37
48 h*	55.0 ± 1.15	38.2 ± 4.44	6.8 ± 1.58
72 h*	5.00 ± 1.72	80.8 ± 5.14	14.2 ± 1.67
Control (EtOH)			
24 h	79.7 ± 1.25	9.3 ± 0.66	11.1 ± 0.38
48 h	23.8 ± 0.63	56.2 ± 4.98	20.0 ± 1.64
72 h	47.17 ± 0.93	31.6 ± 3.99	21.2 ± 1.46

*$P < 0.05$ vs. control; #$P > 0.05$ vs. control made by STATGRAPHICS PLUS for Windows, Version 4.0.
$1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; SCCHN, squamous cell carcinoma of the head and neck; FACS, fluorescence-activated cell sorting.

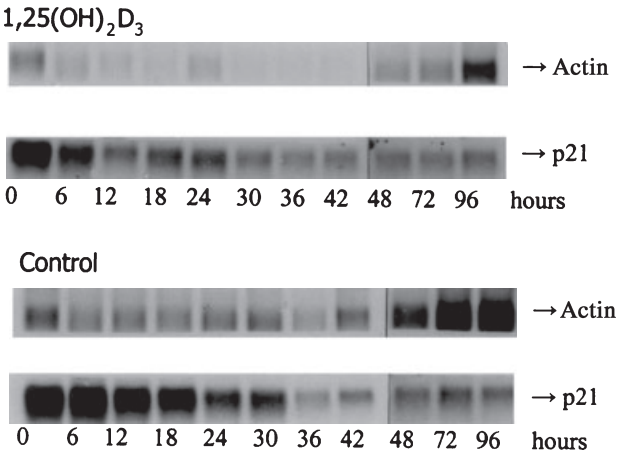


Figure 3 Time course of p21 mRNA in SCC25. SCC25 cells were cultured in the presence or the absence of 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] at 10^{-7} M. At indicated time points mRNA was prepared for Northern blot analysis, which was done with riboprobes directed against the coding region of p21. Equal amounts were loaded as determined by detecting the same blot with an actin probe.

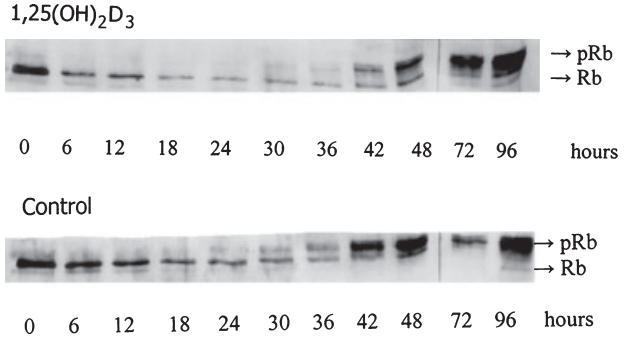


Figure 4 Western blot SCC25/phosphorylation status of retinoblastoma protein (pRb). SCC25 cells were treated with or without 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. Probes were prepared at indicated time intervals. The phosphorylation status of pRb was detected over 96 h.

The influence of $1,25(OH)_2$Vitamin D_3 on p21 was tested in preliminary studies. p21 and p27 were enhanced in SCC9, JPPA and HaCaT after addition of $1,25(OH)_2$Vitamin D_3. In SCC9, the upregulation of p21 and p27 after 30 and 36 h was followed by a G0/G1 phase block after 48 h and a reduced proliferation rate after 72 h. In JPPA and HaCaT, the overexpression started earlier and G0/G1 phase block was observed after 24 h, while growth inhibition occurred after 48 h (Figs 1 and 2).

In SCC25, the G0/G1 block and the growth inhibition were obvious, but the overexpression of p21 failed to appear, as determined by Northern blot analysis (Fig. 3).

Investigating the phosphorylation status of pRb, a shift from the hyperphosphorylated to the hypophosphorylated form could be observed from 36 h (Fig. 4). This was the evidence that CDKs were inhibited and we therefore investigated other cell cycle inhibitors of the G0/G1 phase.

SCC25 expressed all tested cell cycle-inhibiting proteins. At synchronization level (0 h) the level of p18 expression was equal in $1,25(OH)_2$Vitamin D_3 and control. After addition of $1,25(OH)_2$Vitamin D_3 (10^{-7}), p18 expression remained constant in a higher intensity than in the control experiment until the 30-h time point (Fig. 5). This overexpression was followed by a G0/G1 phase block and a reduced proliferation rate after 48 h (Fig. 1 and Table 1).

Similar expression patterns with an slight upregulation of p18 under $1,25(OH)_2$Vitamin D_3 at the time points 18 and 36 h could be detected in JPPA (Fig. 6).

Results obtained from Western blotting were scanned and examined by densitometric analysis (Quantity One®, Bio-Rad, Hercules, CA, USA). To obtain a semiquantitative assessment of protein expression, the

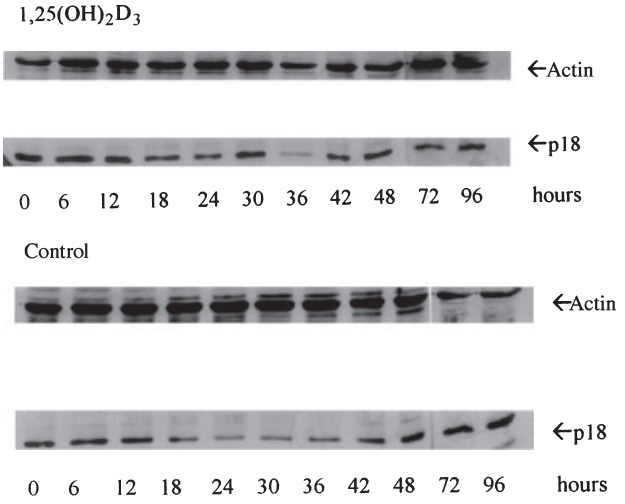


Figure 5 Time course of p18 protein expression under the influence of 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. SCC25 cells were synchronized and incubated in the presence or the absence of 1,25$(OH)_2D_3$ (10^{-7}) for 96 h. At indicated time points cells were lysed and prepared for Western blot analysis. Blots were detected with anti-p18 and actin was used as loading control.

data were normalized to the expression level of the corresponding housekeeping gene β-actin. Data are expressed as normalized ratios by comparing the integrated density values for the proteins in question with those for β-carotene.

Expression of p19 and p27

To determine the mechanism of G0/G1 arrest by 1,25$(OH)_2$Vitamin D_3, another inhibitor of CDK4 was tested to decode possible other pathways of growth inhibition than via p18 upregulation in SCC25. Bands of low intensity of p27 protein expression were seen in SCC25, whereby the control cell line (HaCaT) showed higher levels of protein expression. Adding 1,25$(OH)_2$Vitamin D_3 or EtOH did not change p27 expression in SCC25.

Expression of p19 protein was detected in JPPA, HaCaT, SCC9 and SCC25 in different quantity. In comparison with EtOH, 1,25$(OH)_2$Vitamin D_3 did not enhance the levels of the protein expression in one of these cell lines.

Discussion

To develop potential anticancer drugs in head and neck cancer, the influence of the biologically active metabolite of vitamin D, 1,25$(OH)_2$Vitamin D_3, on growth behaviour was discovered in the past. Growth-inhibiting, differentiation-inducing and metastazation-influencing effects have been detected in various cancer cells, such as breast cancer (8), leukaemia (10) and melanoma cells (11). We could determine a 1,25$(OH)_2$Vitamin D_3-mediated arrest of head and neck cancer cell lines in the G0/G1 phase of the cell cycle. This effect is due to a block in the transition of cells from the G0- to the S-phase and is mediated by the VDR. This arrest might be ascribed to an enhancement of a CDKI – p21 (4).

In one of the cell lines, isolated from a tongue carcinoma (SCC25) this upregulation of p21 could not be detected, although an accumulation of SCC25 cells in the G0/G1 phase was determined by FACS. Investigation of the phosphorylation status of the pRb showed a shift from the hyperphosphorylated form to the hypophosphorylated form, which was a distinct advice for

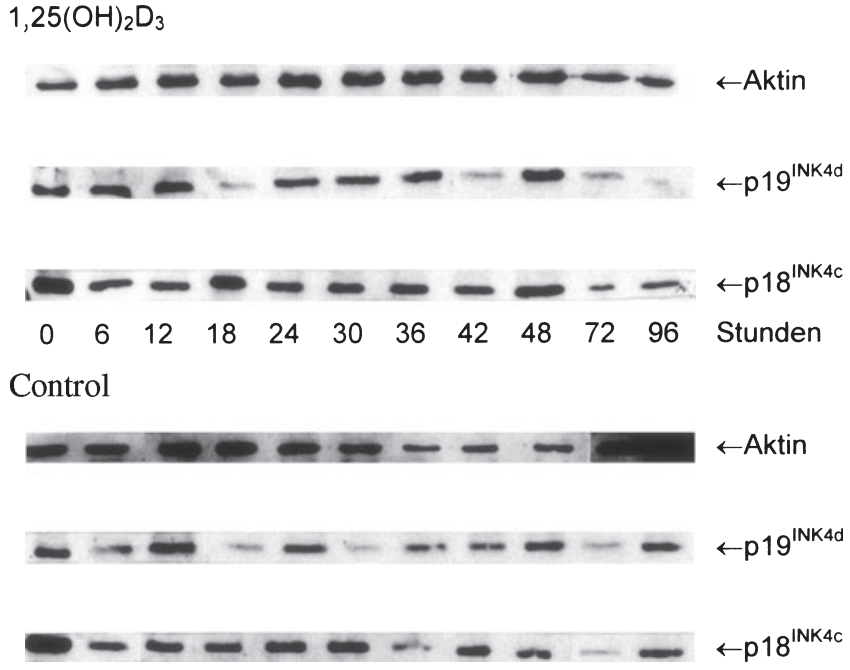


Figure 6 Elevation of p18 in JPPA. JPPA cells were treated with or without 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. Overexpression of p18 under 1,25$(OH)_2D_3$ was detected after 18 and 36 h.

a growth inhibition because of a G0/G1 phase block. Consequently, we investigated other CDKIs of the G0/G1 phase in this cell line. Therefore, we determined the expression of p18, p19 and p27 under the influence of $1,25(OH)_2$Vitamin D_3.

Cyclin-dependent kinase inhibitors were found in different quantities in all cell lines investigated. G0/G1 accumulation of cells was detected in JPPA and HaCaT after 24 h and in SCC25 and SCC9 after 48 h. The possible effect requiring G0/G1 arrest, p21 overexpression under $1,25(OH)_2$Vitamin D_3, was registered in JPPA and HaCaTs, but could not be identified in SCC25. To ascertain the $1,25(OH)_2$Vitamin D_3-mediated cell arrest in G0/G1 phase of the cell cycle in SCCHN, we investigated other CDKIs of this cell cycle phase. CDKIs of G0/G1 phase, which have been suggested as candidates for tumour-suppressor genes (11) – blocking CDK4 and CDK6 – are p18, p19 and p27. All of them were expressed in our cell lines, also including SCC25. Addition of $1,25(OH)_2$Vitamin D_3 resulted in an overexpression of p18 in SCC25 and was detected until the 30-h time point. Other CDKIs, such as p19 and p27 did not show an altered expression pattern after exposure to $1,25(OH)_2$Vitamin D_3.

The growth-inhibiting and differentiation-inducing effects of $1,25(OH)_2$Vitamin D_3 are described to be due to an enhancement of the cell cycle inhibitor p27 in SCCHN (12). The analogue of $1,25(OH)_2$Vitamin D_3 – EB1089 – also increases p27 by encoding the F-Box protein p45-SKP2, which is a marker for poor head and neck cancer prognosis (12). Prudencio et al. (13) demonstrated that $1,25(OH)_2$Vitamin D_3 and EB1089 are able to arrest proliferation in G0/G1 phase in SCCHN via upregulation of p27 and the DNA damage gene gadd45α. In addition, canine SCCs express VDR and treatment with $1,25(OH)_2$Vitamin D_3 and its analogues led to growth inhibition and differentiation (14).

It could be demonstrated that ras-transformed keratinocytes express 1α-hydroxylase, produce $1,25(OH)_2$Vitamin D_3 and suppress tumour growth and induce differentiation in an autocrine mechanism (15). Bikle and co-workers could demonstrate that skin cancer produces $1,25(OH)_2$Vitamin D_3 and responds to $1,25(OH)_2$Vitamin D_3 inducing 24-hydroxylase. No differentiation could be detected in response to $1,25(OH)_2$Vitamin D_3, which seems to be due to an overexpression of the DRIP complex leading to a block of gene induction for $1,25(OH)_2$Vitamin D_3 (16, 17). In addition, $1,25(OH)_2$Vitamin D_3 treatment requires a downregulation of angiopoietin-2 in endothelial cells in SCC and fibrosarcoma cells.

This supports the hypothesis that $1,25(OH)_2$Vitamin D_3 might act via inhibition of angiogenesis (18). Besides suppression of cell proliferation, cell cycle arrest, apoptosis and downregulation of angiogenetic factors e.g. vascular endothelial growth factor (VEGF), $1,25(OH)_2$ Vitamin D_3 enhances the sensitivity for chemotherapeutics-like cisplatin (19).

In conclusion, growth inhibition and accumulation of cells in the G0/G1 phase of the cell cycle through $1,25(OH)_2$Vitamin D_3 could be proven even in SCC25. The overexpression of p21 failed to appear, but we could demonstrate a distinct upregulation of p18 in SCC25 after adding $1,25(OH)_2$Vitamin D_3.

These results indicate that $1,25(OH)_2$Vitamin D_3 mediates its growth inhibition via two important CDK inhibitors in head and neck cancer cell lines. Enhancement of p21 and p18 seems to be responsible for cell arrest in the G0/G1 phase of the cell cycle. The inhibitor p21 plays a critical role in the $1,25(OH)_2$Vitamin D_3-mediated growth inhibition concerning the G0/G1 arrest of head and neck cancer cells in this phase. Absent overexpression of p21 in SCC25 and p18 overexpression makes it obvious that other inhibitors of the cell cycle might play a crucial role in the G0/G1 accumulation of cells under $1,25(OH)_2$Vitamin D_3.

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