1,25(OH)₂ Vitamin D₃ Induces Elevated Expression of the Cell Cycle-regulating Genes P21 and P27 in Squamous Carcinoma Cell Lines of the Head and Neck

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INTRODUCTION

In addition to its classical role as a regulator of calcium and phosphate homeostasis, the biologically active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], has antiproliferative and differentiation-inducing effects. This has been described in a variety of cells, including keratinocytes, thymocytes and lymphocytes, as well as in malignant cells of mammary, skeletal, intestinal and myeloid origin (1±3). These biological effects of 1,25(OH)₂D₃ are predominantly mediated via its own nuclear hormone receptor, the vitamin D receptor (VDR), which directly modulates the transcription of vitamin D₃ target genes (1). In human myelomonocytic leukemic cells (U-937), 1,25(OH)₂D₃ induces terminal differentiation (4) and in T lymphocytes an inhibition of proliferation and activation both in vivo and in vitro could be demonstrated (5). Incubation of cultured human epidermal keratinocytes with 1,25(OH)₂D₃ resulted in a time- and dose-dependent inhibition of the proliferation and induction of differentiation (6). We recently demonstrated in squamous cell carcinoma cell lines of the head and neck (SCCHN) that 1,25(OH)₂D₃ is able to inhibit proliferation and induce differentiation due to an arrest of cells in the G0/G1 phase of the cell cycle (7).

The cell cycle of eucaryotic cells is regulated by a family of cyclin-dependent kinases (cdks), which promote orderly progression through the cell cycle by phosphorylating their subsequent substrates (8). The cdks exist as complexes with the cyclins and their corresponding inhibitors, which modulate cdk function (9). Additionally, the expression of cyclins and inhibitors is regulated by external signals (9). According to the recent literature 1,25(OH)₂D₃ directly influences the expression of cell cycle regulatory proteins functioning in the transition of cells from the G1 to S phases in prostate, breast and myeloid leukemic cells (10, 11). SCCHN also express the VDR, which mediates the G1/S block induced by 1,25(OH)₂D₃ (7). However, the molecular mechanisms underlying these VDR-mediated effects remain unknown. Therefore we investigated the influence of 1,25(OH)₂D₃ on the proliferation, cell cycle status and expression of proteins directly involved in the transition of cells from the G1 to S phases in the cell cycle of SCCHN cell lines as well as in human immortalized keratinocytes [HaCaT] (12). At various time intervals we studied the protein and mRNA expression patterns of the possible vitamin D₃ target proteins cyclin D1 and the corresponding inhibitors p21 and p27. Additionally, as a downstream event of G1 cyclin–cdk com-
plexes, we investigated the phosphorylation status of the retinoblastoma protein (pRb).

MATERIALS AND METHODS

Cell culture

Human immortalized keratinocytes (HaCaT) and the laryngeal carcinoma cell line (JPPA) were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Gibco Life Technologies Ltd., Paisley, UK) supplemented with 200 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% (v/v) fetal calf serum (FCS). The tongue carcinoma cell line (SCC 9) was cultured in RPMI 1640 (Gibco) supplemented with penicillin/streptomycin and 10% FCS. All cell lines were grown in an incubator at 37°C containing 5% CO₂. All cells were synchronized by keeping them for 48 h in confluence, harvested by trypsinization (trypsin/EDTA; Gibco) and seeded on Petri dishes in the corresponding medium by the addition of 10⁻⁷ M 1,25(OH)₂D₃ (dissolved in ethanol; kindly provided by Leo Pharmaceuticals Products, Ballerup, Denmark) or 10⁻³ M ethanol as control. At indicated time intervals cells were simultaneously prepared for FACS analysis and Western and Northern Blotting experiments.

Cell counting

Cells (1–2 million) were seeded in the appropriate medium with additive, which was changed after 48 h. At indicated times cells were trypsinized, centrifuged, resuspended in medium and counted in a Bürker–Türk chamber. Viability of cells (>90%) was determined by trypan blue exclusion.

Cell cycle analysis by flow cytometry

Cell cycle analysis was performed on all cell lines incubated with either 10⁻⁷ M 1,25(OH)₂D₃ or 10⁻³ M ethanol. At indicated time intervals cells were harvested, resuspended in 2 ml PBS and fixed in 2 ml ice-cold 70% (v/v) ethanol at 4°C. The cells were harvested by centrifugation and resuspended in 800 μl PBS before staining with 100 μl propidium iodide (40 μg/ml; Sigma, St. Louis, MO) and 100 μl RNAse A (2 μg/μl; Boehringer Mannheim, Mannheim, Germany) for 1 h at 37°C. The DNA content was measured on 1 million cells using a FACSscan after prior calibration with a DNA quality control particle kit (Becton Dickinson, San Jose, CA). Calculation of percentage distribution in different phases of the cell cycle was performed with CellFit software (Becton Dickinson) using a sum of broadened rectangles fit.

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 EDTA, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P40, 10 mM p-nitrophenol phosphate, 250 U/l aprotinin, 40 mg/ml leupeptin, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 40 mM glycophosphate and 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Whole lysates (30–50 μg) were resolved by SDS–polyacrylamide gel electrophoresis (7.5% and 12.5%) and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Blocked membranes were probed with anti-p21(CIP1/WAF1) mouse monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-p27(KIP1) mouse monoclonal antibody (Pharmingen, San Diego, CA), anti-cyclin D1 mouse monoclonal antibody (Santa Cruz Biotechnology) and anti-actin rabbit antibody (Sigma), which is routinely used as a loading control. To determine the phosphorylation status of the pRb (110–116 kDa) a mouse monoclonal antibody recognizing all forms of pRb (Oncogene Research Products, Cambridge, MA) was used. All blots were developed by using the enhanced chemiluminescent (ECL) detection system (Amersham Pharmacia Biotech, Little Chalfont, UK).

RT-PCR and RNA probes

Total RNA of isolated cells was prepared using Tri- zol (Gibco) according to the manufacturer’s instructions. Total RNA (1 μg) was used as template for first-strand cDNA synthesis with an oligo-dT primer (RT-PCR kit; Perkin Elmer, Branchburg, NJ). Ten percent of the cDNA reaction products was subjected to a hot-start PCR protocol using primers specific for either the p21, p27 or β-actin genes, which were used for quantification. The thermal cycles were: 94°C for 10 min, followed by 30 cycles at 94°C for 30 s, 55°C for 25 s and 72°C for 55 s. The amplified products were separated on 1.5% agarose gel, transferred to a positively charged nylon membrane (Boehringer Mannheim) and hybridized with a digoxigenin three-tailed internal oligonucleotide. The product was detected using an alkaline phosphatase-labeled anti-digoxigenin antibody and nitrobluetetrazolium salt (Dig detection system, Boehringer Mannheim).

Riboprobes for Northern Blotting were directed against the coding region of the corresponding mRNA and were generated by incorporating the T7 promoter sequence into the 3’ primer for PCR. The gel-purified PCR products were used as a template
Table I. Proliferation behavior and cell cycle distribution of 1,25(OH)$_2$D$_3$-treated SCCHN cell lines: number of cells ($\times 10^6$) as determined by cell counting. Representative example of one of the independent experiments.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>HaCaT</th>
<th>JPPA</th>
<th>SCC 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>24</td>
<td>1.4</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>48</td>
<td>4.7</td>
<td>2.7</td>
<td>0.6</td>
</tr>
<tr>
<td>72</td>
<td>7.8</td>
<td>4.3</td>
<td>1.0</td>
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<tr>
<td>Control</td>
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<tr>
<td>24</td>
<td>1.6</td>
<td>1.6</td>
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<td>48</td>
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<tr>
<td>72</td>
<td>13.8</td>
<td>8.0</td>
<td>1.7</td>
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</tbody>
</table>

for in vitro transcription of an antisense RNA probe, which was labeled with Dig-UTP according to the manufacturer's instructions (Boehringer Mannheim).

Northern Blot

Total RNA was extracted from growing cells at the indicated time intervals with Trizol. Total RNA (15–30 µg) was separated on 1.2% agarose–formaldehyde gel, blotted onto positively charged nylon membranes and covalently bound by baking for 30 min at 120°C. Membranes were prehybridized in Dig Easy Hyb (Boehringer Mannheim) for 1–3 h at 68°C and hybridization was performed in the same buffer with Dig-labeled riboprobes overnight at 68°C. Blots were detected with the Dig luminescence detection kit (Boehringer Mannheim) and exposed to Kodak X-ray film.

RESULTS

Cell numbers and analysis of the cell cycle

In preliminary experiments we investigated the proliferation behavior and cell cycle status under the influence of 1,25(OH)$_2$D$_3$ in immortalized keratinocytes (HaCaT) and in SCCHN cell lines (JPPA and SCC 9). Synchronized cells were seeded and cultured in the presence or absence of 10$^{-7}$ M 1,25(OH)$_2$D$_3$ for 3 d. At 24-h intervals cells were counted and prepared for FACS analysis. Viability of cells was always > 90%. In rapidly growing cells (HaCaT and JPPA) the number of 1,25(OH)$_2$D$_3$-treated cells decreased markedly after 48 h and in SCC 9 after 72 h compared with control cells (Table I). In addition, an accumulation of cells in the G0/G1 phase of the cell cycle was detectable in HaCaT and JPPA after 24 h (after 48 h in SCC 9), which remained constant throughout the observation period (Table II).

Elevated expression of p21 and p27 induced by 1,25(OH)$_2$D$_3$

The influence of 1,25(OH)$_2$D$_3$ on the expression of cell cycle-regulating proteins acting in the G1 phase of the cell cycle in SCCHN was investigated by Western Blot analysis (Fig. 1). After synchronization (0 h time point), 1,25(OH)$_2$D$_3$ (10$^{-7}$ M)-treated and untreated cells were prepared at 6 h time intervals up to 48 h, as well as after 72 and 96 h. Blots were detected with anti-cyclin D1, anti-p21 and anti-p27 antibodies, while actin was used as loading control. In HaCaT as well as in JPPA and SCC 9 an influence of 1,25(OH)$_2$D$_3$ on the expression of the inhibitors p21 and p27, but not cyclin D1, could be detected. In SCC 9, which we used as an example of a SCCHN cell line, the time course of cyclin D1 expression was similar for both control cells and 1,25(OH)$_2$D$_3$-treated cells (Fig. 1). In the control the p21 protein was highly expressed in synchronized cells and decreased rapidly when cells started to divide (0–12 h time intervals). In contrast, the expression of the p27 protein was not elevated at 0 h. Levels of both inhibitors rose again after 72 and 96 h, respectively, when the cells were almost confluent (Fig. 1). In 1,25(OH)$_2$D$_3$-treated cells all cell lines showed a marked overexpression of both inhibitors compared with the control. In SCC 9 cells an elevated expres-

Table II. Proliferation behavior and cell cycle distribution of 1,25(OH)$_2$D$_3$-treated SCCHN cell lines: DNA content (%). Representative example of one of the independent experiments.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>HaCaT</th>
<th>JPPA</th>
<th>SCC 9</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>S</td>
<td>G2/M</td>
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Hypophosphorylation of pRb under the influence of 1,25(OH)₂D₃

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. We therefore additionally examined the phosphorylation status of pRb in HaCaT, JPPA and SCC9 exposed to 1,25(OH)₂D₃.

In untreated SCC9 cells—shown as an example—only the unphosphorylated form of pRb was detectable at the 0 h time point (Fig. 1). With the initiation of growth, pRb became phosphorylated and shifted to bands with higher molecular weight (12–24 h). From 30 to 72 h pRb was phosphorylated to a high degree in control cells (Fig. 1). In 1,25(OH)₂D₃-treated cells pRb shifted back to the hypophosphorylated form after 36 h. From 48 to 96 h a distinct amount of pRb was unphosphorylated compared to control cells. This time course of an induction of the hypophosphorylated form of pRb under the influence of 1,25(OH)₂D₃ was visible in all investigated cell lines and occurred simultaneously with the induced expression of the p21 protein. This could be confirmed in short-time experiments showing that the alterations in p21 overexpression were accompanied by changes in the phosphorylation status of the pRb (Fig. 2).

1,25(OH)₂D₃ induces the expression of p21 mRNA

In order to reveal an influence of 1,25(OH)₂D₃ at the transcriptional level of p21 and p27 expression, cells were seeded—in parallel to the experiments above—for Northern Blot analysis. In all cell lines tested and, as shown for SCC 9, in control cells, p21 mRNA was highly expressed at 0 h but decreased rapidly as cells started to divide (up to 12 h; Fig. 3). At 36 and 42 h p21 mRNA was again slightly induced. The time course of p21 mRNA expression in 1,25(OH)₂D₃-treated cells showed a marked upregulation which was initially detectable after 24 h and remained nearly constant over the period of investigation when compared to the control (Fig. 3). Under the influence of 1,25(OH)₂D₃ an increase in p27 mRNA could also be detected (data not shown). Furthermore, short-
1,25-vitamin D₃ induces p21/p27 in SCCHN

**Fig. 3.** Induction of p21 mRNA in SCCHN under the influence of 1,25(OH)₂.D₃. SCC 9 cells were cultured for 96 h in the presence or absence (control) of 1,25(OH)₂.D₃ at 10⁻⁷ M. At several time intervals—in parallel with protein expression analysis—mRNA was prepared and Northern Blot analysis 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.

time experiments in JPPA revealed alterations in the time course of p21 mRNA overexpression (data not shown), which has already been observed at the protein level.

**DISCUSSION**

The antiproliferative effect of the biologically active 1,25(OH)₂.D₃ has been reported for a variety of malignant and non-malignant cells. Human epidermal keratinocytes (6) and SCCHN cell lines (7) respond to 1,25(OH)₂.D₃ by inhibition of their proliferation and stimulation of differentiation in a time- and dose-dependent manner. These effects are due to a block in the transition of cells from the G0 to S phases in the cell cycle and occur, like the majority of 1,25(OH)₂.D₃-mediated biological actions, through the nuclear VDR. The VDR, a member of the steroid receptor family, binds to specific vitamin D response elements, which are located in the promoter region of vitamin D₃ target genes regulating their transcription (1, 10).

To further delineate our results we performed time course experiments in 2 SCCHN cell lines (JPPA, SCC 9) as well as in immortalized keratinocytes (HaCaT) under the influence of 1,25(OH)₂.D₃. In all cell lines investigated 1,25(OH)₂.D₃ caused an accumulation of cells in the G0/G1 phase at a concentration of 10⁻⁷ M and, in consequence, a reduction in the proliferation rate. To study this antiproliferative activity of 1,25(OH)₂.D₃ in SCCHN more closely we first investigated the expression behavior of cyclin D1 and p21, 2 G1 cell cycle-regulating proteins, which have been predominantly implicated in the control of cell proliferation in a complex with a cdk4 protein (13). Cyclin D1, a putative oncogene, is synthesized by growth factor stimulation (14). Its corresponding inhibitor p21, defined as a tumor suppressor, inhibits the catalytic activity of cyclin D–cdk complexes and is involved in numerous important cellular processes, including DNA replication, mitogenesis, DNA repair and differentiation (15). In SCCHN an amplification and/or increased expression of p21 (16), as well as of the cyclin D1 protein (17), have been reported.

Under the influence of 1,25(OH)₂.D₃ the distribution of the cyclin D1 protein was not significantly altered. However, for p21 a marked overexpression induced by 1,25(OH)₂.D₃ was detectable in all cell lines. The time course of the elevated expression correlated with the proliferation behavior of the corresponding cell line, as p21 induction started at later time intervals in slow-growing cells. Furthermore, the initiation of the induced expression of p21 was consistent with the subsequent initiation of the G0/G1 phase block, as determined by FACS analysis. Short time course experiments revealed changes in the course of p21 overexpression, suggesting that p21 induction by 1,25(OH)₂.D₃ is not continuous. Marked overexpression, as well as alterations in the p21 expression, were also detectable at the mRNA level, pointing to a transcriptional regulation. Induction of p21 mRNA often occurs in a p53-dependent manner (18), p53 being a well-characterized tumor suppressor protein. Although deletions or mutations of the p53 gene are common in SCCHN the SCC 9 cell line is also deficient in p53 protein expression (19). Therefore the induction of the p21 protein suggests a transcriptional regulation of 1,25(OH)₂.D₃ by an interaction of the VDR with a vitamin D response element, representing a possible p53-independent mechanism (20).

Another potent inhibitor of cyclin–cdk complexes in the G1 phase of the cell cycle is the p27 protein, which has sequence similarity to p21 (21). In contrast to p21, p27 has not been defined as a tumor suppressor in human cancers analyzed so far. Our investigations into the expression behavior of p27 under the influence of 1,25(OH)₂.D₃ in SCCHN cell lines, as well as in HaCaT, revealed elevated expression of p27, which was detectable at the protein and mRNA levels. The distinct overexpression of both G1 cell cycle inhibitors p21 and p27 in SCCHN is consistent with recent published data concerning various cell
lines investigated under the influence of 1,25(OH)\(_2\)D\(_3\). In a myelomonocytic cell line, 1,25(OH)\(_2\)D\(_3\) enhanced the expression of p21 (10) and p27 in HL-60 cells (22). Further studies in MCF-7 cells showed that 1,25(OH)\(_2\)D\(_3\) induced an arrest of cells in the G0/G1 phase of the cell cycle, associated with a rapid accumulation of p21 and p27 (11).

The cell cycle inhibitors p21 and p27 predominantly bind to cyclin D–cdk4/6 and cyclin E–cdk2 complexes (18), which are necessary in order to phosphorylate pRb (15), a nuclear protein, the phosphorylation level of which determines its DNA binding properties. pRb phosphorylation leads to its inactivation and the subsequent transcription of genes responsible for cell growth. pRb was identified as a tumor promoter in retinoblastomas (23). Mutations have not been found yet in SCCHN (24). Under the influence of 1,25(OH)\(_2\)D\(_3\), in SCCHN as well as in HaCaT, the phosphorylation of pRb shifted to a large extent from the hyper- to the hypophosphorylated form, simultaneous with the increased expression of p21 and p27, pointing to a direct inhibition of G1 cyclin–cdk complexes.

These results indicate that, in SCCHN, 1,25(OH)\(_2\)D\(_3\) mediates its growth inhibitory effects via at least two important cdk inhibitors acting at a key regulatory point of the cell cycle. Deletions or mutations of G1 cell cycle-regulating proteins are frequent in SCCHN. For example, the loss of p16 protein function may be a critical event in the development of SCCHN (25). Such a lack of an important G1 cell cycle inhibitor could be restored by increased expression of p21 and/or p27 induced by 1,25(OH)\(_2\)D\(_3\). A protein- and/or RNA-based detection system for the expression of cell cycle-regulating proteins might then give useful information concerning the status of growth control pathways in the cell. In addition, 1,25(OH)\(_2\)D\(_3\) provides an alternative pathway for the control of aberrant proliferation resulting from mutated or deleted p53. However, for clinical application in the therapy of SCCHN, analogues having the same antiproliferative activity but less hypercalcemic effect are indispensable (11), in order to insert them in synergistic combinations with cytotoxic drugs and/or other biological response modifiers. Furthermore, investigations at the molecular level of 1,25(OH)\(_2\)D\(_3\)-mediated effects are necessary, so that 1,25(OH)\(_2\)D\(_3\) may in future represent an important substance for the control of cell growth and in the treatment of SCCHN malignancies.

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REFERENCES


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