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Differential biological effects of 1,25-dihydroxyVitamin D_3 on melanoma cell lines in vitro☆

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Abstract

1,25-DihydroxyVitamin D_3 and analogs have been shown to inhibit proliferation and to induce differentiation in different cell types, including human melanocytes. However, various tumor cell lines that fail to respond to the antiproliferative effects of Vitamin D analogs have also been reported. Using real-time PCR (LightCycler), we have compared mRNA expression of Vitamin D receptor (VDR), Vitamin D-25-hydroxylase (25-OHase), 25-hydroxyVitamin D-1α-hydroxylase (1α-OHase), and 1,25-dihydroxyVitamin D-24-hydroxylase (24-OHase) in a melanoma cell line that responds to antiproliferative effects of Vitamin D (MeWo) with a non-responsive melanoma cell line (SkMel5). Additionally, modulation of cell proliferation by calpain inhibitors, as well as regulation of mRNA expression of *VDR*, *1α-OHase*, and *24-OHase* genes by Vitamin D analogs were assessed in melanoma cell lines in vitro using a WST-1 based colorimetric assay and real-time PCR, respectively. RNA for VDR, 25-OHase, 1α-OHase, and 24-OHase was detected in melanoma cell lines. In contrast to SkMel5 cells, treatment of MeWo cells with calcitriol resulted in a dose-dependent increase in mRNA for VDR and 24-OHase as well as in a suppression of cell proliferation (up to approximately 50%). Our findings demonstrate that local synthesis or metabolism of Vitamin D metabolites may be of importance for growth regulation of MM and melanoma cell lines. Additionally, metastasizing MM represents a promising target for palliative treatment with new Vitamin D analogs that exert little calcemic side effects or for pharmacological modulation of calcitriol synthesis/metabolism in these tumors.

Keywords: Vitamin D; Cancer; Calpain; Malignant melanoma; Real-time polymerase chain reaction

1. Introduction

1,25-DihydroxyVitamin D_3 (Calcitriol, $1,25(OH)_2D_3$) and analogs have been shown to inhibit proliferation and to induce differentiation in various cell types, including human melanocytes [1,2]. However, tumor cell lines that fail to respond to the antiproliferative effects of Vitamin D analogs have also been reported. We here report two melanoma cell lines that respond differentially to Vitamin D-induced antiproliferative effects. It is well known that $1,25(OH)_2D_3$ acts via binding to a corresponding intranuclear receptor (VDR), present in target tissues [3,4]. VDR belongs to the superfamily of transacting transcriptional regulatory factors, which includes the steroid and thyroid hormone receptors as well as the retinoid-X receptors and retinoic acid receptors [5,6]. There are two principal enzymes involved in the formation of circulating $1,25(OH)_2D_3$ from Vitamin D, the hepatic microsomal or mitochondrial Vitamin D 25-hydroxylase (25-OHase) and the renal mitochondrial enzyme 1α-hydroxylase (1α-OHase) for Vitamin D and $25(OH)D_3$, respectively [7,8]. $1,25(OH)_2D_3$ is metabolized in target cells at least in part by 1,25-dihydroxyVitamin D 24-hydroxylase (24-OHase), resulting in a specific C-24 oxidation pathway to yield the biliary excretory product calcitroic acid. These hydroxylases belong to a class of proteins known as cytochrome P450 mixed function monooxidases. Recently, extrarenal activity of 1α-OHase has been reported in various cell types including macrophages, keratinocytes, prostate and colon cancer cells [9,10]. It has now been shown that mechanisms involved in Vitamin D mediated growth regulation, including apoptosis, are characterized by a pathway involving Ca^{2+} release from the endoplasmic reticulum and that Vitamin D induced apoptosis is mediated via calpain as the major execution protease [11,12]. The aim of this study was to answer the following questions:

(a) Do we find differences in the expression of VDR, 24-OHase or 1α-OHase in a melanoma cell line that

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responds to the antiproliferative effects of $1,25(OH)_2D_3$ (MeWo) as compared to a non-responding melanoma cell line (SkMel5)?

(b) Is calpain involved in antiproliferative effects of Vitamin D analogs in melanoma cell lines?

2. Methods

2.1. Cell culture

Human melanoma cell lines SkMel5 and MeWo were cultivated in RPMI (10% FCS, 37 °C, 5% CO_2) using 75 cm^2 culture flasks (Greiner, Germany) or 96-well plates. Semi-confluent cells were incubated with 5% fetal calf serum (charcoal-treated) and with or without 10^{-7} M 1,25-dihydroxyVitamin D3 or Seocalcitol (EB 1089). Vitamin D analogs were kindly provided by Lise Binderup (Leo Pharmaceuticals, Ballerup, Denmark). Calpain inhibitor I (ALLN, Sigma, Germany) and Calpain inhibitor II (ALLM, Sigma, Germany) were added 10 and 20 μM, respectively, after 96 h treatment with 1,25-dihydroxyVitamin D3.

2.2. RNA isolation

RNA isolation was carried out with RNeasy Kit (Qiagen, Hilden, Germany) according to manufactures manual. RT-PCR were carried out with Promega MMLV-reverse transcriptase, using polyd(T)15 primers and 2 μg RNA.

2.3. RNA analysis

Expression of *VDR*, *25-OHase*, *1α-OHase*, and *24-OHase* was analyzed in melanoma cell lines using real time PCR (LightCycler, 50 cycles) and gene specific primers (Table 1). We used 10-fold serial dilutions of cDNA as external standards. Expression levels were determined as ratios between target genes and the reference gene *β2-microglobulin*.

Table 1
Primers for PCR analysis

Gene	Sequence
VDR	5′-CCAGTTCGTGTGAATGATGG-3′ 5′-GTCGTCCATGGTGAAGGA-3′
1α-OHase	5′-TGTTTGCATTTGCTCAGA-3′ 5′-CCGGGAGAGCTCATACAG-3′
24-OHase	5′-GCAGCCTAGTGCAGATTT-3′ 5′-ATTCACCCAGAACTGTTG-3′
25-OHase	5′-GGCAAGTACCCAGTACGG-3′ 5′-AGCAAATAGCTTCCAAGG-3′
β2-microglobulin	5′-CCAGCAGAGAATGGAAAGTC-3′ 5′-GATGCTGCTTACATGTCTCG-3′

2.4. Proliferation assay

Cell proliferation analysis was performed in 96-well plates using a tetrazolium salt (WST-1) based colorimetric assay according to product specifications (Roche, Diagnostics, Mannheim, Germany; Cat. no. 1644807).

3. Results

3.1. RNA levels of 1α-OHase, 24-OHase, 25-OHase, and VDR in melanoma cell lines SkMel5 and MeWo

Real-time PCR analysis showed mRNA expression of *1α-OHase*, *24-OHase*, *25-OHase*, and *VDR* genes in both melanoma cell lines (Fig. 1).

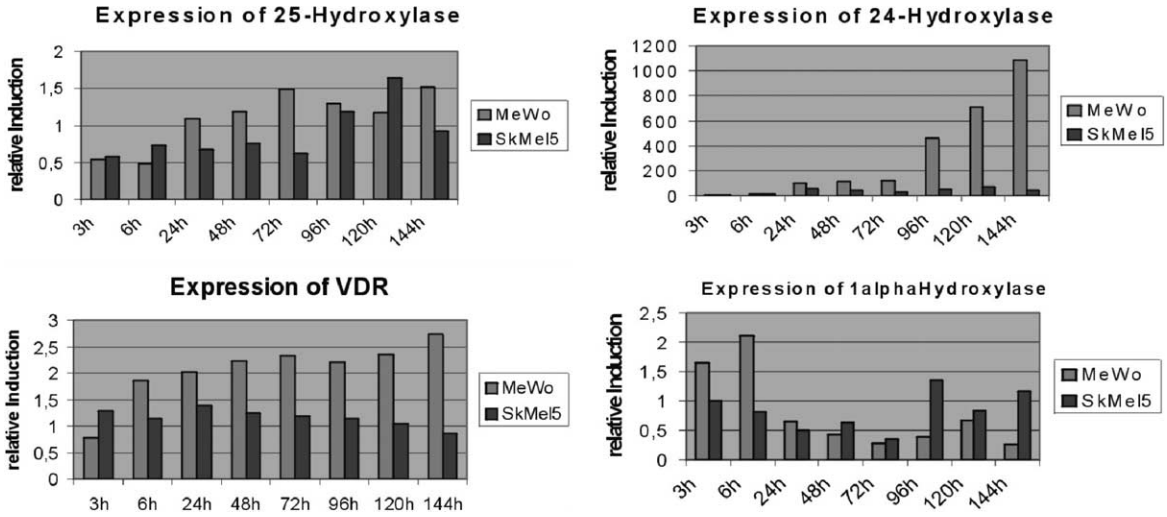

Fig. 1. Expression of *25-OHase*, *24-OHase*, *1α-OHase*, and *VDR* genes in $1,25(OH)_2D_3$ (10^{-7} M) treated MM cell lines. Median of target gene/*β2-microglobulin* ratio is shown. Note strong induction of 24-OHase mRNA in MeWo but not in SkMel5 cells.

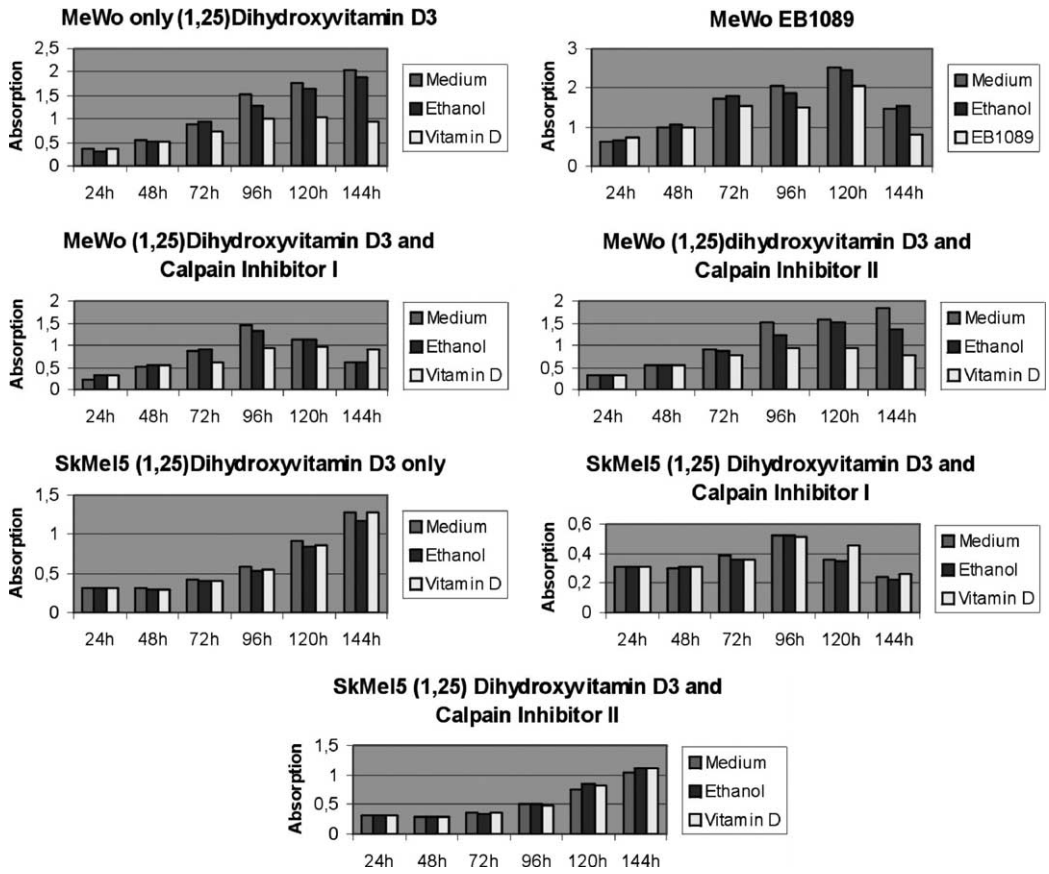

Fig. 2. WST-1 cell proliferation assay. Antiproliverative effect of $1,25(OH)_2D_3$ (10^{-7} M) on SkMel5 and MeWo cells. Note that $1,25(OH)_2D_3$ induces significant inhibition of cell proliferation in MeWo, but not in SkMel5 cells. Incubation of MeWo or SkMel5 cells with $1,25(OH)_2D_3$ (10^{-7} M) and calpain inhibitors I or II did not result in pronounced inhibition of cell proliferation as compared to incubation of cells with calpain inhibitor alone.

3.2. MeWo cells respond to the antiproliferative effects of Vitamin D analogs while SkMel5 cells fail to respond

Incubation of MeWo cells with calcitriol (10^{-7} M) or EB 1089 (10^{-7} M) resulted in a significant inhibition of cell proliferation that was most pronounced after 144 h (Fig. 2). In contrast, SkMel5 cells were unresponsive to the antiproliferative effects of calcitriol.

3.3. Differential induction of VDR and 24-OHase mRNA in MeWo and SkMel5 cells

As shown by real-time PCR, RNA for VDR and 24-OHase is strongly induced by calcitriol in MeWo cells (Fig. 1). In contrast, induction of RNA for VDR and 24-OHase was not shown in SkMel5 cells, indicating a functional defect of VDR-mediated transcription in this cell line.

3.4. Vitamin D analogs do not inhibit cell proliferation in MeWo or SkMel5 cells in the precence of calpain inhibitor

Incubation of MeWo or SkMel5 cells with Vitamin D analogs and calpain inhibitors I or II did not result in pronounced inhibition of cell proliferation as compared to incubation of cells with Vitamin D analogs alone (Fig. 2).

4. Discussion

We here report that melanoma cell lines show a differential response to the antiproliferative effects of Vitamin D analogs (MeWo-responsive; SkMel5-unresponsive). Interestingly, incubation with calcitriol induced mRNA for 24-OHase and VDR in MeWo but not in SkMel5 cells. In conclusion, it can be speculated that the underlying mechanisms that cause unresponsiveness of SkMel5 cells to the antiproliferative effects of Vitamin D analogs are related to a defect in VDR-mediated transcription. The regulation of the abundance of VDR is an important mechanism that modulates cellular responsiveness to $1,25(OH)_2D_3$. The mechanisms underlying the regulation of VDR abundance have been shown to include alterations in the rate of transcription of the VDR gene and/or the stability of VDR mRNA. Vitamin D response elements have been identified in numerous genes involved in cellular growth,

differentiation, apoptosis, invasion and metastasis of tumor cells; i.e. cell cycle regulators such as the human *p21/WAF1*, *cyclin A* and *cyclin E* genes, the human *nm23.H1* gene, the human *c-fms*, *c-fos*, *c-jun*, and *c-myc* genes, the human retinoblastom gene, the murine fibronectin gene, the human plasminogen activator inhibitor 2 gene, the human laminin and laminin receptor (α6) genes, and the chicken β3-integrin gene (review in [13,14]). We speculate that unresponsiveness of SkMel5 cells to the antiproliferative effects of Vitamin D analogs is caused by a defect in VDR-mediated transcription that may involve these signalling pathways.

Additionally, our findings indicate that calpain may be involved in Vitamin D-induced suppression of cell proliferation. Calpain, also named calcium activated neutral protease (CAPN), represents an ubiquitious intracellular cytoplasmic non-lysosomal cysteine endopeptidase that requires calcium ions to exert its activity. Two major isoenzymes are known, μ-calpain (CAPN1) and m-calpain (CAPN2), requiring micromolar and millimolar calcium concentrations for activation, respectively [15]. So far no difference in the substrate specificity of the two isozymes has been found. The enzymes are composed of two subunits: an 80 and a 30 kDa subunit. The latter is common to the two forms of the enzyme [16]. Calpain is usually present in an inactive form and is activated by calcium and phospholipids. The process of activation is usually, but not necessarily, accompanied by limited autoproteolysis. It has been shown that the activation of calpain involves the dissociation of the enzyme into subunits and that calpain functions as a monomer of the 80 kDa subunit [17]. The activation process is enhanced by the translocation of calpain to the cell membrane. The in situ activity of calpain is regulated by the level and cellular compartmentalization of the enzymes and their endogenous specific inhibitor, calpastatin [18]. Recently, it has been shown that calpains are frequently activated in apoptosis models involving elevated intracellular free calcium [19,20]. Moreover, it was demonstrated that calpain-induced Bax-cleavage product is a more potent inducer of apoptotic cell death than wild-type Bax [21]. It is now evident that Vitamin D-mediated apoptosis is mediated via calpain as the major execution protease [11,12]. We have recently detected strong calpain expression in malignant melanoma and metastases of malignant melanoma [22]. Taken all these data together, an increasing body of evidence now indicates that calpain may be the major execution protease in Vitamin D-induced apoptosis and is involved in Vitamin D-induced suppression of cell proliferation in melanoma cells. Our findings support the concept that metastasizing MM represents a promising target for palliative therapy with new Vitamin D analogs that exert little or no calcemic side effects. Pharmacological modulation of Vitamin D synthesis, application of prodrugs (i.e. 25-hydroxyVitamin D), the development of specific 24-OHase inhibitors or the combination of Vitamin D analogs with chemotherapeutics or cytokines may be concepts for the palliative treatment of metastasizing MM.

References

[1] C. Danielsson, K. Fehsel, P. Polly, C. Carlberg, Differential apoptotic response of human melanoma cells to 1-alpha,25-dihydroxyVitamin D_3 and its analogues, Cell Death Differ. 5 (11) (1998) 946–952.
[2] C. Danielsson, H. Torma, A. Vahlquist, C. Carlberg, Positive and negative interaction of 1,25-dihydroxyVitamin D_3 and the retinoid CD437 in the induction of human melanoma cell apoptosis, Int. J. Cancer 81 (3) (1999) 467–470.
[3] W.E. Stumpf, M. Sar, F.A. Reid, Y. Tanaka, H.F. DeLuca, Target cells for 1,25-dihydroxyVitamin D3 in intestinal tract, stomach, kidney, skin, pituitary and parathyroid, Science 209 (1979) 1189–1190.
[4] A.R. Baker, D.P. McDonnell, M. Hughes, Cloning and expression of full-length cDNA encoding human Vitamin D receptor, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 3294–3298.
[5] S.A. Kliewer, K. Umesono, D.J. Mangelsdorf, R.M. Evans, Retinoic X receptor interacts with nuclear receptors in retinoic acid, Nature 355 (1992) 446–449.
[6] R.M. Evans, The steroid and thyroid hormone receptor superfamily, Science 240 (1988) 889–895.
[7] H.L. Henry, Vitamin D hydroxylase, J. Cell Biochem. 49 (1) (1992) 4–9.
[8] D.W. Nebert, F.J. Gonzalez, *P450* genes: structure, evolution, and regulation, Ann. Rev. Biochem. 56 (1987) 945–993.
[9] G. Jones, H. Ramshaw, A. Zhang, R. Cook, V. Byford, J. White, M. Petkovich, Expression and activity of Vitamin D-metabolizing cytochrome P450s (CYP1α and CYP24) in human non-small cell lung carcinomas, Endocrinology 140 (1999) 3303–3310.
[10] V. Tangpricha, J.N. Flanagan, L.W. Whitlatch, C.C. Tseng, T.C. Chen, P.R. Holt, M.S. Lipkin, M.F. Holick, 25-HydroxyVitamin D-1alpha-hydroxylase in normal and malignant colon tissue, Lancet 26 (357(9269)) (2001) 1673–1674.
[11] I.S. Mathiasen, I.N. Sergeev, L. Bastholm, F. Elling, A.W. Norman, M. Jaattela, Calcium and calpain as key mediators of apoptosis-like death induced by Vitamin D compounds in breast cancer cells, J. Biol. Chem. 277 (34) (2002) 30738–30745.
[12] J. Reichrath, M. Rech, M. Seifert, Vitamin D-induced apoptosis and melanoma: does calpain represent the major execution protease rather than caspases? J. Pathol. 201 (2003) 335–336.
[13] J. Reichrath, Will analogs of 1,25-dihydroxyVitamin D_3 (calcitriol) open a new era in cancer therapy? Onkologie 24 (2001) 128–133.
[14] C.M. Hansen, L. Binderup, K.J. Hamberg, C. Carlberg, D. Vitamin, Cancer: effects of $1,25(OH)_2D_3$ and its analogs on growth control and tumorigenesis, Front Biosci. 6 (2001) 820–848.
[15] D.E. Croall, G.N. Demartino, Calcium-activated neutral protease (calpain) system: structure, function, and regulation, Physiol. Rev. 71 (1991) 813–847.
[16] H. Sorimachi, S. Ishiura, K. Suzuki, Structure and physiological function of calpains, Biochem. J. 328 (1997) 721–732.
[17] T.C. Saido, H. Sorimachi, K. Suzuki, Calpain: new perspectives in molecular diversity and physiological–pathological involvement, FASEB J. 8 (1994) 814–822.
[18] R.D. Lane, D.M. Allan, R.L. Mellgren, A comparison of the intracellular distribution of m-calpain, m-calpain and calpastatin in proliferating human A431 cells, Exp. Cell. Res. 203 (1992) 5–16.
[19] T. Nakagawa, J. Yuan, Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis, J. Cell. Biol. 150 (4) (2000) 887–894.
[20] S.K. Ray, M. Fidan, M.W. Nowak, G.G. Wilford, E.L. Hogan, N.L. Banik, Oxidative stress and Ca^{2+} influx upregulate calpain and induce apoptosis in PC12 cells, Brain Res. 852 (2) (2000) 326–334.

[21] H. Toyota, N. Yanase, T. Yoshimoto, M. Moriyama, T. Sudo, J. Mizuguchi, Calpain-induced Bax-cleavage product is a more potent inducer of apoptotic cell death than wild-type Bax, Cancer Lett. 189 (2) (2003) 221–230.

[22] M. Hettrich, J. Reichrath, C. Welter, T. Mitschele, A. Kerber, V. Meineke, W. Tilgen, M. Seifert, Differential expression patterns of calpain isozymes 1 and 2 (CAPN 1 and 2) in cutaneous malignancies, Arch. Dermatol. Res. 294 (1/2) (2002) 81.