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VDR microRNA expression and epigenetic silencing of vitamin D signaling in melanoma cells☆

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ABSTRACT

Malignant melanoma cells express the vitamin D receptor (VDR). However, some melanoma cell lines fail to respond to the antiproliferative effects of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$). We reported previously that out of seven melanoma cell lines analyzed, three cell lines (MeWo, SK-Mel28, SM) respond to the antiproliferative effects of 1,25$(OH)_2D_3$, while the others (SK-Mel5, SK-Mel25, IGR, Meljuso) are resistant. It was the aim of this study to investigate whether epigenetic mechanisms are of importance for the abrogation of vitamin D signaling in vitamin D resistant melanoma cells. We used the histone deacetylase inhibitor (HDACI) trichostatin A (TSA) and the DNA methyltransferase inhibitor (DNMTI) 5-azacytidine (5-Aza) to elucidate the effects of protein acetylation and of DNA hypermethylation on 1,25$(OH)_2D_3$-induced effects on cell proliferation, respectively. Additionally we analyzed the expression of VDR microRNA in 1,25$(OH)_2D_3$-responding and resistant melanoma cells. TSA and 5-Aza exerted dose- and time-dependent antiproliferative effects on melanoma cell lines. Interestingly, combination therapy with 1,25$(OH)_2D_3$ and TSA exerted synergistic antiproliferative effects in a 1,25$(OH)_2D_3$-resistant melanoma cell line (IGR) ($p < 0.05$). Combination therapy with 1,25$(OH)_2D_3$ and 5-Aza resulted in synergistic (MeWo after 72 h; $p < 0.05$) or additive (other melanoma cell lines analyzed) antiproliferative effects. Additionally, we could show that VDR mRNA expression is relatively high in two of three 1,25$(OH)_2D_3$-responsive melanoma cells as compared to resistant cells, moreover this relatively high VDR expression is associated with low expression of miRNA125b in MeWo and SK-Mel28 cells. Our results suggest that the endogenous VDR mRNA level is inversely associated with expression of miRNA125b in melanoma cell lines analyzed. Moreover, miRNA125b may be involved in the regulation of VDR expression and in the resistance against 1,25$(OH)_2D_3$ in melanoma cells. It can be speculated whether miRNA125b may be of prognostic importance and/or may represent a therapeutic target for malignant melanoma. Drugs that influence epigenetic mechanisms might be promising therapeutics for the treatment of metastasized malignant melanoma, alone or in combination with antiproliferative or cytotoxic agents such as 1,25$(OH)_2D_3$.

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1. Introduction

In past decades, incidence of malignant melanoma has dramatically increased worldwide. Due to its high capacity to metastasize, malignant melanoma is recognized as one of the most aggressive malignancies [1]. Moreover, no effective treatment of metastasized melanoma is available. At present, dacarbazine has the best efficacy with a response rate ranging from 5% to 29% and a short 4-month median response duration [2]. The limited success of available treatments underlines the needs to develop new therapeutic and preventive approaches for melanoma [3]. The vitamin D endocrine system has been implicated in the pathogenesis and progression of various malignancies, including malignant melanoma [4–6]. Moreover, 1,25$(OH)_2D_3$, the biologically most active natural vitamin D metabolite that acts *via* binding to its corresponding intranuclear receptor (VDR), present in target tissue cells [7] has been considered as a potential anticancer agent due to its antiproliferative and pro-differentiating effects. Malignant melanoma cells express the vitamin D receptor (VDR) [4,6]. However, some melanoma cell lines fail to respond to the antiproliferative effects

Abbreviations: 5-Aza, 5-azacytidine; DNMTI, DNA methyltransferase inhibitor; HDACI, histone deacetylase inhibitor; microRNA, micro messenger ribonucleic acid; mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; RXR, retinoid-X receptor; TSA, trichostatin A; VDR, vitamin D receptor; VDRE, vitamin D response element.

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of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) [4,6]. We reported previously that out of seven melanoma cell lines analyzed, three cell lines (MeWo, SK-Mel28, SM) respond to the antiproliferative effects of 1,25$(OH)_2D_3$, while the others (SK-Mel5, SK-Mel25, IGR, Meljuso) are resistant [6]. The reason for 1,25$(OH)_2D_3$-resistance in melanoma cell lines is unknown. It was the aim of this study to investigate whether epigenetic mechanisms are of importance for the abrogation of vitamin D signaling in vitamin D resistant melanoma cells using the histone deacetylase inhibitor (HDACI) trichostatin A (TSA) and the DNA methyltransferase inhibitor (DNMTI) 5-azacytidine (5-Aza) to elucidate the effects of protein acetylation and of DNA hypermethylation on 1,25$(OH)_2D_3$-induced effects on cell proliferation, respectively. Additionally we aimed to analyze the expression of VDR microRNA in 1,25$(OH)_2D_3$-responding and resistant melanoma cells.

2. Materials and methods

2.1. Cell culture

Human melanoma cell lines IGR, SK-Mel28, SK-Mel25, SK-Mel5, MelJuso, SM, and MeWo were cultured in PRMI (10% fetal calf serum, 37 °C, 5%CO_2) using 100, 0/20 mm tissue culture dishes or 96 well plates (Greiner, Frickenhausen, Germany) as described previously [6]. 1,25$(OH)_2D_3$ and TSA (Sigma–Aldrich, Taufkirchen, Germany) were dissolved in ethanol as a stock solution and stored in the dark in −20 °C, 5-Aza (Sigma–Aldrich) was dissolved in PBS. Cells were plated in 96 well (1×10^3 cells per well). Media containing varying concentrations of 1,25$(OH)_2D_3$, TSA or 5-Aza and their combinations were added to a final volume of 100 μl per well and plates were incubated for 96 h, with re-dosing on a daily basis.

2.2. Proliferation assay

Cell proliferation was estimated in 96 well plates using a colorimetric immunoassay, based on the measurement of BrdU incorporation during DNA synthesis (BrdU ELISA kit Roche Diagnostics, Mannheim, Germany). In brief, BrdU (10 mM 5-bromo-2′-deoxyuridine) is added to the cells after 24 h of each daily treatment before cells are fixed, DNA is denaturated and a peroxidise labelled anti-BrdU-antibody is added. Immune complexes are detected by the subsequent substrate (tetramethyl-benzidine) reaction, which is quantified by measuring the absorbance values that directly correlate to the amount of DNA synthesis and therefore the number of proliferating cells.

2.3. RNA, microRNA analysis

RNA isolation was performed using RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's manual. MicroRNA was isolated using miRNeasy Kit (Qiagen, Germany). Expression of VDR mRNA and miRNA125b [8] were analyzed in melanoma cell lines using real time PCR (LightCycler, Roche, Mannheim, Germany, 50 cycles) with a modification of the techniques published previously [6,8].

2.4. Calculation of the effect of drugs on cell proliferation

The inhibitory effect of each drug used in the treatment was calculated as follows, as published previously [9,10]. Proliferation% = (proliferation in treated wells/proliferation in control wells) × 100. Theoretical additive effect $I_{ab} = 100 \times [1 - (1 - I_a/100) \times (1 - I_b/100)]$, where I_{ab} is the additive theoretical inhibitory effect, expressed as % inhibition, I_a and I_b are the measured inhibitory effects (%) of each agent acting alone as compared with those of controls. Synergistic effect is that with an experimental value significantly greater than the theoretical value, and additive effect is that where the experimental value does not significantly differ from the theoretical value. All analyses were compared using the Student's *t*-test.

3. Results

3.1. Treatment of melanoma cell lines with 1,25$(OH)_2D_3$, TSA, 5-Aza and their combination

Treating melanoma cells with 1,25$(OH)_2D_3$ (10^{-6} M, 10^{-7} M, and 10^{-8} M) every 24 h. resulted in dose- and time-dependent antiproliferative effect in some melanoma cell lines (MeWo and SK-Mel28) while the remaining (IGR, SK-Mel5) were resistant (Fig. 1). Treatment with HDAC-inhibitor TSA or DNA-methyltransferase inhibitor 5-Aza exerted dose- and time-dependent antiproliferative effects on melanoma cells (Figs. 2 and 3). We asked the question whether the resistance against 1,25$(OH)_2D_3$ could be restored by co-treatment with TSA or 5-Aza. Combination therapy with 1,25$(OH)_2D_3$ and TSA exerted synergetic antiproliferative effects in a 1,25$(OH)_2D_3$-resistant melanoma cell line (IGR) after 24 h. 1,25$(OH)_2D_3$ (10^{-8} M) and TSA (15 ng) alone each inhibited IGR cells after 24 h 5% (±4%) and 33% (±1.4%), respectively, while the combination showed 41% (±1.8%) inhibition ($p < 0.05$). However, after 48 h and 72 h IGR proliferation presented no statistical significances comparing TSA alone with the combination (Fig. 2). In contrast, antiproliferative effects of TSA alone in SK-Mel28 were stronger as compared to combination of TSA and 1,25$(OH)_2D_3$, indicating a protective effect of 1,25$(OH)_2D_3$, against the antiproliferative effects of TSA (Fig. 2). Combination therapy with 1,25$(OH)_2D_3$ and 5-Aza exerted synergistic (MeWo only after 72 h, $p < 0.05$ but not after 24 h and 48 h) or additive antiproliferative effects in most melanoma cell lines analyzed (Fig. 3).

3.2. Melanoma cell lines expression of VDR mRNA and miRNA125b

We investigated the VDR mRNA expression as compared to expression of miRNA125b. Interestingly, we could show that VDR mRNA expression is more pronouncedly increased in two of three 1,25$(OH)_2D_3$-responsive melanoma cells as compared to resistant cells. Additionally, this relatively high VDR expression was associated with low expression of miRNA125b in MeWo and SK-Mel28 cells. Our results suggest that the endogenous VDR mRNA level is inversely associated with expression of miRNA125b in melanoma cell lines analyzed (Fig. 4).

4. Discussion

In the present study, we confirm previous findings demonstrating an abrogation of 1,25$(OH)_2D_3$-mediated signaling in most malignant melanoma cell lines [6]. The reason for 1,25$(OH)_2D_3$-resistance in melanoma cell lines is unknown. We here show that both 1,25$(OH)_2D_3$-resistant and -responsive melanoma cells respond in dose- and time-dependent manner against the antiproliferative effects of HDAC-inhibitor TSA and DNA-methyltransferase inhibitor 5-Aza. As has been shown by other studies, deacetylase and DNA-methyltransferase inhibition are known to be effective tools in many cancer therapy regimes [11–13]. Interestingly, antiproliferative effects of 1,25$(OH)_2D_3$ on some melanoma cell lines were in this study pronounced in a synergistic or additive manner, when it was combined with 5-Aza or TSA. Additionally, we could show that VDR mRNA expression is relatively high in two of three 1,25$(OH)_2D_3$-responsive melanoma cells as compared to resistant cells, additionally this relatively high

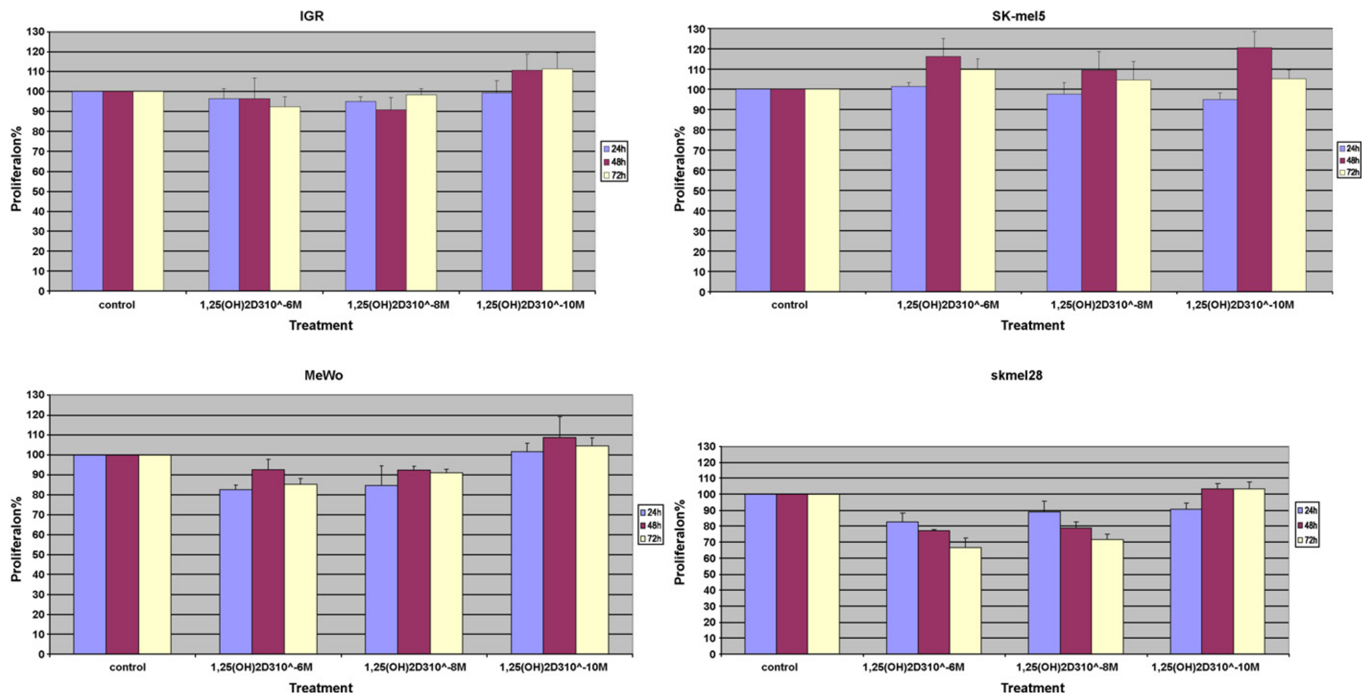

Fig. 1. Treatment of melanoma cells with $1,25(OH)_2D_3$ in three different concentrations (10^{-6} M, 10^{-8} M and 10^{-10} M) every 24 h. Each data point represents the mean of three separated experiments. Some melanoma cell lines respond dose- and time-dependent to antiproliferative effects of $1,25(OH)_2D_3$, while others are resistant.

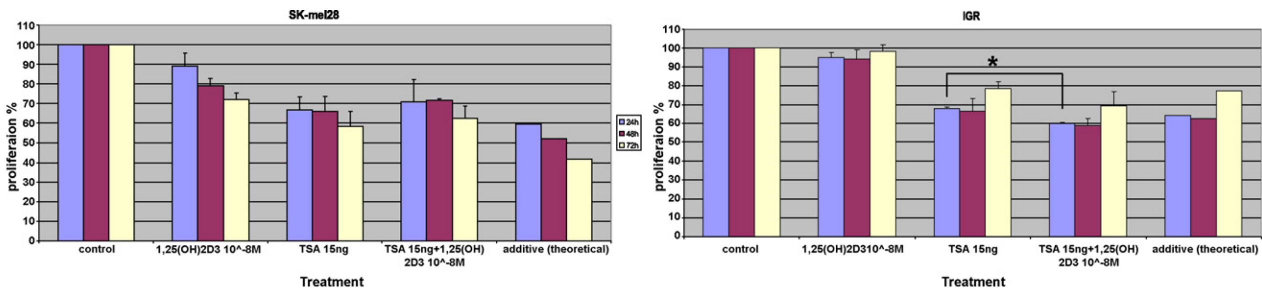

Fig. 2. Proliferation analysis after treatment of melanoma cells with $1,25(OH)_2D_3$ and TSA alone or in combination. Not that HDAC-inhibitor TSA exerts dose- and time-dependent antiproliferative effects on melanoma cell lines. * = Combination therapy with $1,25(OH)_2D_3$ and TSA exerts synergistic antiproliferative effects in a $1,25(OH)_2D_3$-resistant melanoma cell line (IGR) after 24 h ($p < 0.05$). In contrast, the antiproliferative effects of TSA alone in SK-Mel28 are stronger as compared to combination therapy with $1,25(OH)_2D_3$.

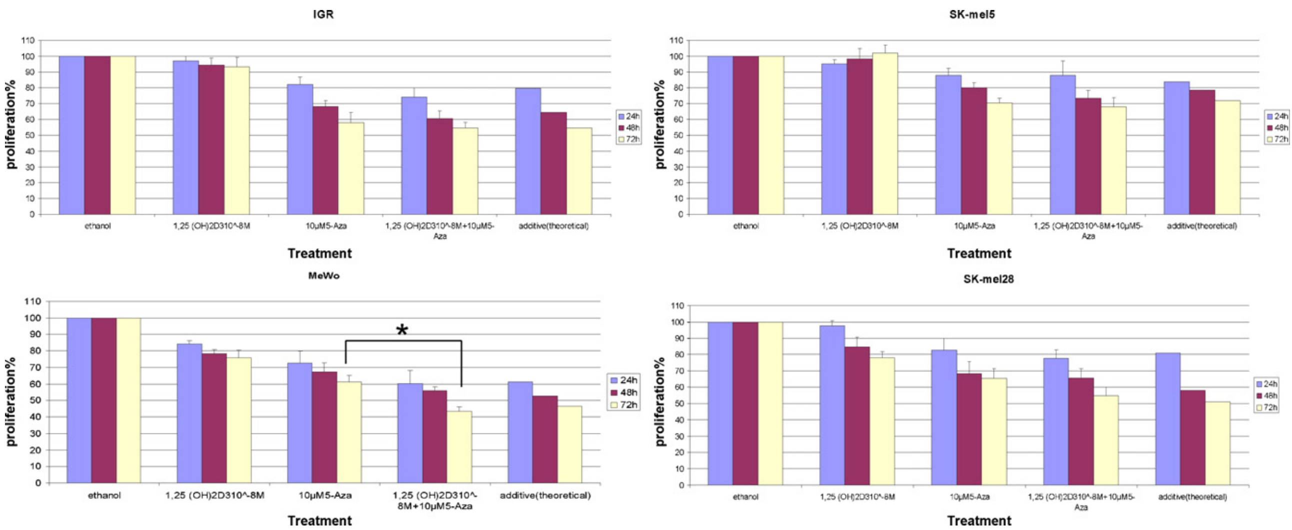

Fig. 3. Proliferation of melanoma cells after treatment with $1,25(OH)_2D_3$ and DNA-methyltransferase inhibitor 5-Aza alone or in combination. Note that 5-Aza exerts dose- and time-dependent antiproliferative effects on melanoma cell lines. * = Combination therapy with $1,25(OH)_2D_3$ and 5-Aza exerts synergistic (MeWo after 72 h, $p < 0.05$) or additive (other melanoma cell lines analyzed) antiproliferative effects.

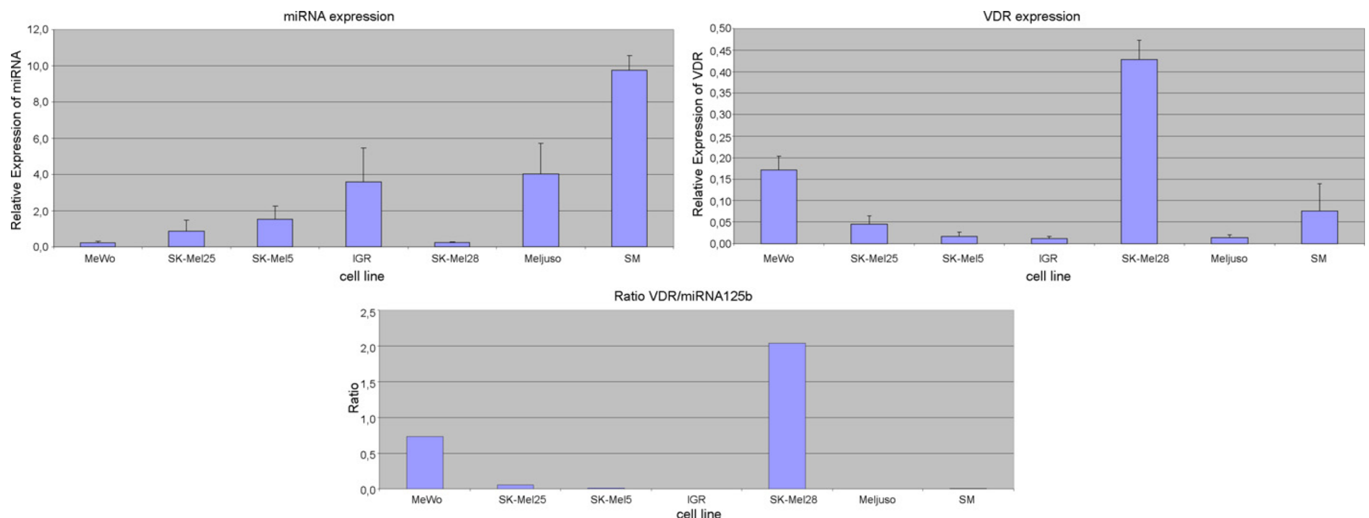

Fig. 4. Expression of miRNA and VDR mRNA in melanoma cell lines, VDR mRNA expression is relatively high in two out of three $1,25(OH)_2D_3$-responsive melanoma cell lines (MeWo, SK-Mel28, but not SM), as compared to resistant cells. Moreover, high VDR expression is associated with relatively low expression of miRNA125b in $1,25(OH)_2D_3$-responsive cells (MeWo and SK-Mel28). We can notice the differences between most $1,25(OH)_2D_3$-responsive and resistant melanoma cells.

VDR expression is associated with low expression of miRNA125b in MeWo and SK-Mel28 cells. Recently, it has been shown that expression of VDR is post-transcriptionally regulated by miR-125b [8]. Our results suggest that the endogenous VDR mRNA level is inversely associated with expression of miRNA125b in melanoma cell lines analyzed in this study. Moreover, miRNA125b may be involved in the regulation of VDR expression and in the resistance against $1,25(OH)_2D_3$ in melanoma cells. It can be speculated whether miRNA125b may be of prognostic importance and/or may represent a therapeutic target for malignant melanoma. Drugs that influence epigenetic mechanisms might be promising therapeutics for the treatment of metastasized malignant melanoma, alone or in combination with antiproliferative or cytotoxic agents such as $1,25(OH)_2D_3$.

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