In vitro and in vivo inhibition of liver cancer cells by 1,25-dihydroxyvitamin D$_3$

M.H. Pourgholami, J. Akhter, Y. Lu, D.L. Morris*

University of New South Wales, Department of Surgery, St. George Hospital, Kogarah, Sydney, NSW 2217, Australia

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Abstract

Inhibitory effects of 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) on the proliferation of a variety of cancer cell lines have been extensively reported. We have studied the effect of 1,25-(OH)$_2$D$_3$ ($10^{-11}$–$10^{-6}$ M) on the proliferation of a number of human and rat liver cancer cell lines. Additionally, the effect of 1,25-(OH)$_2$D$_3$ (0.02–0.5 μg/kg per day) on the rate of growth of liver cancer cell line xenografts in nude mice was also investigated. In vitro, proliferation of Hep-3B, PLC/PRF/5, and SKHEP-1 cells was significantly inhibited by 1,25-(OH)$_2$D$_3$, while HTC and Novikoff cells were more resistant to the inhibitory effects of the drug. In vivo, treatment of SKHEP-1 tumor bearing nude mice with different doses of 1,25-(OH)$_2$D$_3$ significantly retarded tumor growth without the development of hypercalcemia.

Keywords: Liver cancer; 1,25-Dihydroxyvitamin D$_3$; Proliferation; Nude mice; Xenografts

1. Introduction

Considerable evidence suggests that 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) may play an important role in human cancer. Reduced serum concentrations of 1,25-(OH)$_2$D$_3$ have been associated with an increased risk of breast, prostate, and colon cancer [1], while, high-level vitamin D receptor (VDR) expression has been shown to correlate with a favorable prognosis in colorectal cancer [2]. In vitro, 1,25-(OH)$_2$D$_3$ influences cellular proliferation by promoting differentiation, cell cycle arrest, and apoptosis [3]. Consequently, 1,25-(OH)$_2$D$_3$ has been shown to regulate the proliferation of a number of cancer cell lines in vitro including, breast [4–6], prostate [7,8], colon [9,10], melanoma [11], and pancreatic carcinomas [12]. Studies in vivo have shown that, in immuno-suppressed mice, lung, colon, breast carcinoma, and melanoma xenografts regress when treated with 1,25-(OH)$_2$D$_3$ [13].

We have previously shown that, in vitro, the 1,25-(OH)$_2$D$_3$ analog EB 1089 inhibits the proliferation of prostate [8] and colon [9] cancer cells, and in nude mice, 1,25-(OH)$_2$D$_3$ and its’ analog EB 1089 both significantly inhibit growth of xenografts of human colon cancer cell line LOVO [14]. Furthermore, we have shown that, in vitro, 1,25-(OH)$_2$D$_3$ profoundly inhibits the proliferation of the hepatoblastoma cell line HepG2 [15]. Apart from the preliminary data of Brelvi et al. [16] briefly reporting 1,25-(OH)$_2$D$_3$ induced growth suppression of HepG2 and HTC, this is the first detailed evaluation of the effect of 1,25-(OH)$_2$D$_3$ on liver cancer cell proliferation and xenograft growth.

* Corresponding author. Tel.: +61-2-9350-2070; fax: +61-2-9350-3997.
E-mail address: david.morris@unsw.edu.au (D.L. Morris)
2. Materials and methods

2.1. Cell culture

Hep3-B, PLC/PRF/5, SKHEP-1, HTC, and Novikoff cells were obtained from European Collection of Cell Cultures (UK). Cells were grown in MEM or DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 units/ml streptomycin, and maintained subconfluent at 37°C in humidified incubators containing 5% CO2. 1,25-(OH)2D3 (Calbiochem-Novabiochem, Australian subsidiary) was dissolved in ethanol, the concentration of which in the medium was kept at 0.1%.

2.2. [3H]Thymidine incorporation assay

For the study of [3H]thymidine incorporation, adherent cells (10000) were plated onto 24-well Corning tissue culture dishes and were exposed to culture medium (5% FBS) containing the vehicle or different concentrations of 1,25-(OH)2D3 (10^{-11}–10^{-6} M). For Novikoff cells which is a detached rat cell line, 2000 cells were suspended in 2 ml of DMEM (5% FBS) and kept under the same conditions as for attached cells. Media were replaced with fresh media on alternate days. At the end of the treatment period (5 days), cells were trypsinized and counted with a hemocytometer. In all experiments, cells treated with the medium containing 0.1% ethanol were taken as the control for 1,25-(OH)2D3 treated cells. All counts were obtained in quadruplicate and each experiment was repeated at least twice.

2.3. Cell count

Hep3-B, SKHEP-1, and PLC/PRF/5 cells were plated in 6-well plates at concentrations ranging from 2 × 10^4–5 × 10^4 cells per well. The cell treatment procedure was as described for the [3H]thymidine assay. At the end of the treatment period (5 days), cells were trypsinized and counted with a hemocytometer. In all experiments, cells treated with the medium containing 0.1% ethanol were taken as the control for 1,25-(OH)2D3 treated cells. All counts were obtained in quadruplicate and each experiment was repeated at least twice.

2.4. Xenografts in nude mice

Six- to 10-week-old male BALB/c Nu/Nu mice (Animal Resources Center, Perth, Australia) were inoculated subcutaneously with 10^6 SKHEP-1 cells into the right flank. Twenty-four hours after inoculation, animals were randomly assigned to one of the treatment groups (n = 10), receiving 0.02, 0.1, or 0.5 μg/kg per day of 1,25-(OH)2D3 (i.p., oral on alternate days). The control group was treated with the vehicle (propylene glycol). Using vernier calipers, tumor diameters (mm) were measured every three days for 21 days. Tumor volumes were calculated using the formula 1/6πd^3 [13]. Throughout the experimentation period, food (normal) and water was available to animals ad libitum. For determining serum calcium levels, at the end of the experiment, 0.5 ml of blood was taken under general anesthesia, using cardiac puncture. Calcium levels were determined by the o-cresophthalein complex one method (Boehringer Mannheim Automated Analysis, Hitachi system 717, Boehringer Mannheim, Australia).

2.5. Statistical analysis

Data are presented as mean ± standard error of mean (SEM). For the in vitro work, where applicable, the differences between groups were analyzed by one-way analysis of variance followed by Dunnett’s multiple comparison test. Tumor volume data were analyzed using two-way analysis of variance followed by Tukey’s multiple comparison test. A P-value of <0.05 was considered to represent a significant difference.

3. Results

Results obtained in the [3H]thymidine incorporation study with the three different human liver cancer cell lines are presented in Fig. 1. It is evident from this graph that, amongst the cell lines tested, Hep-3B is clearly the most sensitive of all to 1,25-(OH)2D3 treatment. When treated with 1000 nM 1,25-(OH)2D3, [3H]thymidine incorporation in these cells was reduced to 2.7 ± 0.4 % of the control (P < 0.001). Significant inhibition was present even at the lower concentrations of 0.1 nM (P < 0.05; data not shown) and 1 nM (P < 0.01) 1,25-(OH)2D3. SKHEP-1 and
PlC/PRF/5 were also inhibited by 1,25-(OH)_{2}D_{3}, however, in these two cell lines significant inhibition of [3H]thymidine incorporation was observed only at the higher concentrations of 100 and 1000 nM, respectively. Exposure of SKHEP-1 cells to the highest concentration of 1,25-(OH)_{2}D_{3} employed (1000 nM), led to approximately 50% inhibition of [3H]thymidine incorporation. Compared to human cell lines, the rat hepatoma cell lines displayed lower sensitivity to 1,25-(OH)_{2}D_{3} treatment. When treated with the highest concentration of 1,25-(OH)_{2}D_{3} (1000 nM), compared with control, 47.1 ± 6.7 and 78.4 ± 5.8% [3H]thymidine incorporation was achieved in HTC and Novikoff cells, respectively (Fig. 1).

Results obtained in the cell count study for the human cell lines HEP3-B, SKHEP-1, and PLC/PRF/5 show an almost similar pattern to that seen in the [3H]thymidine assay. Cell proliferation was most effectively inhibited in Hep-3B cells, resulting in dose-dependent ($P < 0.001$) reduction in the number of cells remaining after the 1,25-(OH)_{2}D_{3} treatment. In SKHEP-1 cells compared to control, significant reductions in cell number were observed at 10, 100, and 1000 nM concentrations of 1,25-(OH)_{2}D_{3}, while, for PLC/PRF/5 reduction in cell number was only clearly evident when cells were exposed to the 1000 nM concentration of the drug (Fig. 2).

For the in vivo study in nude mice, SKHEP-1 was chosen, because it has been reported to be the most tumorigenic human liver cancer cell line in nude mice [17]. The response to 1,25-(OH)_{2}D_{3} treatment obtained in nude mice bearing SKHEP-1 tumors was similar to the data obtained in cell culture work. A dose-response inhibition of tumor growth was observed at the three different doses employed (Fig. 3). Amongst the different treated groups, tumor volumes were both dose ($P < 0.001$) and time ($P < 0.001$) dependent. Inhibition of tumor growth was most evident in animals treated with the 0.5 μg/kg per day dose of the drug. Analysis of the serum calcium levels in the different treatment groups indicated that at the doses employed, daily 1,25-(OH)_{2}D_{3} treatment did not induce any significant increase in serum calcium levels.
4. Discussion

In the last 20 years an increasing body of evidence has accumulated to show the anti-proliferative effect of 1,25-(OH)₂D₃ in a variety of malignant cell lines. However, little or no attention has been paid to liver cancers for which there is no effective drug therapy as yet. Following our finding on the profound effect of 1,25-(OH)₂D₃ on the HepG2 cells [15], the present study was undertaken to find out if this steroid hormone does affect the proliferation of liver cancer cell lines. In the human cell lines studied, Hep-3B cells showed extensive sensitivity to 1,25-(OH)₂D₃, which matched that previously seen in HepG2 cells. In this cell line, the significant inhibitory effect of 1,25-(OH)₂D₃ could be seen at concentrations as low as 0.1 nM. PLC/PRF/5 and SkHEP-1 cells were not affected by the lower concentrations of the drug and modestly inhibited at the higher concentrations. The similar pattern of results obtained in the cell count studies, confirmed the [³H]thymidine incorporation data in showing that, while proliferation of Hep-3B was profoundly inhibited in a dose dependent manner, in SKHEP-1 cells, a significant reduction in cell number was obtained only at the higher concentration range of 10–1000 nM 1,25-(OH)₂D₃. Similarly, results obtained in SKHEP-1 tumor bearing mice, indicated that, in this model, without inducing an increase in the serum calcium levels, 1,25-(OH)₂D₃ can modestly inhibit tumor growth in vivo. 1,25-(OH)₂D₃ and its analogs have previously been shown to inhibit tumor growth in breast [13,18,19], prostate [20], colon [14], lung [13] and squamous cell [1,21] animal tumor model systems, but the present study is the first to describe such an effect in a liver cancer derived xenograft.

1,25-(OH)₂D₃ produces biological responses via both genomic and rapid mechanisms. The genomic responses are linked to a nuclear vitamin D receptor [22]. In recent years, an increasing body of evidence has suggested that, 1,25-(OH)₂D₃ is critically involved in cell differentiation and proliferation and more importantly, it can suppress proliferation of a wide array of malignant cell types in which VDR is expressed [13,18,23,24]. Through activation of these receptors, 1,25-(OH)₂D₃ or its analogs, have been shown in culture to inhibit proliferation and also to direct cells towards a more differentiated phenotype. This effect in neoplastic cells may be related to the reported ability of the liganded VDR to arrest cells at the G1 stage by influencing cell cycle regulatory proteins, such as p21 and p27, to control cell growth transcription factors such as c-myc and c-fos, or to elicit apoptosis by down regulating Bcl-2 [24]. The presence of VDR has been demonstrated in both mammalian and avian adult liver [25], and in surgical specimens obtained from patients with hepatocellular carcinoma [26]. In several models, it has been shown that, response to 1,25-(OH)₂D₃ correlates well with the receptor number [27]. The ability of 1,25-(OH)₂D₃ to inhibit the growth of transformed cells appears to be associated with the number of vitamin D receptors per cell [28]. On this basis, the differences observed in the responsiveness of the cell lines examined, to 1,25-(OH)₂D₃ treatment, may be attributable to the different VDR expressions in these cells. However, other mechanisms can not be ruled out as both Hep-3B and HepG2 have been shown to contain high levels of cytochrome P450 enzyme activity [29] which can transform 1,25-(OH)₂D₃ to a number of other metabolites, the biological activity of some of which are still undetermined. On the other hand, SKHEP-1, which was inhibited only modestly by
1,25-(OH)2D3 treatment, apparently lacks these enzymes [30]. Therefore, despite the large number of investigations on the mechanism of action of 1,25-(OH)2D3, the necessity to conduct further studies particularly utilizing liver cancer cell lines still exists. Further studies exploring these issues are warranted.

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References


