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1,25-Dihydroxyvitamin D_3 Regulates the Expression of N-myc, c-myc, Protein Kinase C, and Transforming Growth Factor-β2 in Neuroblastoma Cells[1]

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1α,25-Dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) alters the proliferation of neuroblastoma cells in culture in part via a nerve growth factor (NGF)-mediated pathway. This suggests that factors other than NGF also play a role in the growth arrest induced by 1,25$(OH)_2D_3$. To more fully characterize the effect of 1,25$(OH)_2D_3$ on neuroblastoma cells, we treated the cells with 10^{-8} M 1,25$(OH)_2D_3$ and examined the cells for changes in the expression of N-myc, c-myc, transforming growth factor-β2 (TGF-β2), and protein kinase C (PKC) activity. Our results show that 1,25$(OH)_2D_3$ causes a decrease in the expression of N-myc and c-myc, as well as a two-fold increase in total PKC activity and a dose-dependent increase in TGF-β2 expression. These results show that 1,25$(OH)_2D_3$ regulates the expression of growth-regulatory factors other than NGF in neuroblastoma cells and that 1,25$(OH)_2D_3$ influences the growth of neural cells via multiple growth regulatory pathways. © 1997 Academic Press

1,25-Dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), the active metabolite of vitamin D, is known to regulate epithelial calcium transport, cellular growth, and the transcription of several genes (2-9). 1,25$(OH)_2D_3$ exerts it biological effects primarily through intracellular 1,25$(OH)_2D_3$ receptors (VDR) (6,10). Immunohistochemical studies have located epitopes for the VDR in several tissues of the developing fetus. The VDR has been detected in developing dorsal root ganglia in vivo and in dorsal root ganglion cells maintained in culture (11), suggesting 1,25$(OH)_2D_3$ plays an as yet undetermined role in cellular development within the nervous system.

1,25$(OH)_2D_3$ regulates the growth and differentiation of several cell types maintained in culture. Alterations in the expression of insulin-like growth factors [12-14], insulin-like growth factor binding proteins (13,14), nerve growth factor (NGF) (15,16), and the expression of growth factor receptors in various types of cells (17,18) have been implicated in 1,25$(OH)_2D_3$ regulated growth. Administration of 1,25$(OH)_2D_3$ increases Raf kinase and c-Ki-ras expression (19), and inhibits c-myc expression (20-22) in various cell types. Protein kinase C (PKC) activity is also increased in diverse cell types following 1,25$(OH)_2D_3$ treatment (23-25). Therefore it is possible that 1,25$(OH)_2D_3$ could influence cell growth by regulating the expression and activity of intracellular growth regulatory proteins.

We have previously shown that 1,25$(OH)_2D_3$ causes a decrease in the proliferation and a change in the morphology of neuroblastoma cells, N1E-115 (1). The effect of 1,25$(OH)_2D_3$ was accompanied by an increase in the expression of NGF as well as protein markers of differentiated neuronal cells. Addition of anti-NGF antibody to the medium was able to reverse the effects of 1,25$(OH)_2D_3$ on the cells. In order to further characterize the effects of 1,25$(OH)_2D_3$ on neuronal cells, we examined the expression of various growth factors when cells were treated with 1,25$(OH)_2D_3$. We show that 1,25$(OH)_2D_3$ causes a decrease in the expression of N-myc and c-myc proto-oncogenes, and an increase in TGF-β2 expression and PKC activity, while c-fos expression is not affected by 1,25$(OH)_2D_3$ treatment.

MATERIALS AND METHODS

Cell culture. N1E-115 clones derived from mouse neuroblastoma C1300 cell line (26) were the generous gift of Dr. E. Richelson (Mayo Clinic, Jacksonville, FL). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 7.3 μg/l biotin, 0.002g/l thioctic acid, and 0.00136 g/l vitamin B_{12}.

Isolation of RNA. N1E-115 neuroblastoma cells were grown to confluency in T-75 flasks. At the point of confluency three flasks of cells were treated with 10^{-8} M 1,25$(OH)_2D_3$ while three were treated

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with vehicle (ethanol). Cells were harvested at 1 h, 6 h, and 24 h and total RNA was isolated. Total RNA was isolated using the QIAGEN RNeasy total RNA isolation kit manufactured by QIAGEN Inc. (Chatsworth, CA), following the manufacturer's protocol. The amount of isolated total RNA was quantitated by measuring the absorbance of each sample at 260 nm.

Reverse transcription polymerase chain reaction. N1E-115 neuroblastoma cells were grown to confluency in T-75 flasks. At the point of confluency three flasks of cells were treated with 10^{-8} M $1,25(OH)_2D_3$ while three were treated with vehicle (ethanol). Cells were harvested at 1 h, 6 h, and 24 h and total RNA was isolated. Each sample of total RNA (5 μg) was reversed transcribed into cDNA with oligo$(dT)_{16}$, and the cDNA was amplified by PCR techniques (27) with Taq polymerase and PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1,25 mM $MgCl_2$, pH 8.3) for 25 cycles, using the following synthetic oligonucleotide primer pairs (20 μM);

N-myc (763 bp) 5′ CGAATTGGGCTACGGAGATGCT 3′
5′ TTGTGCTGCTGATGGATGGG 3′
c-myc (501 bp) 5′ TTTGGGGACAGTGTTCTCTGCCTG 3′
5′ GTTCTTGATGAAGGTCTCGTCGTC 3′

The following β-actin primers were obtained from Stratagene (La Jolla, CA).

β-actin (514 bp) 5′ TGTGATGGTGGGAATGGGTCAG 3′
5′ TTTGATGTCACGCACGATTTCC 3′

Expected size of PCR product is given in parentheses. All primers are specific for mouse DNA.

Measurement of total PKC activity. Confluent T-75 flasks of N1E-115 cells were treated with 10^{-8} M $1,25(OH)_2D_3$, vehicle, or serum deprived for 24 hours. Total PKC activity was measured using the total PKC assay kit from Gibco BRL.

TGF-β2 assay. N1E-115 cells were plated out equally into four T-75 flasks and allowed to grow to confluency. Cells were treated with 10^{-6}, 10^{-8}, and 10^{-10} M $1,25(OH)_2D_3$ or vehicle. Supernatant (500 μL) was removed from the cells at 3, 6, and 24 hours. TGF-β2 concentrations in the supernatant were measured using the Quantikine Immunoassay Kits from R and D systems following the manufacturers guidelines.

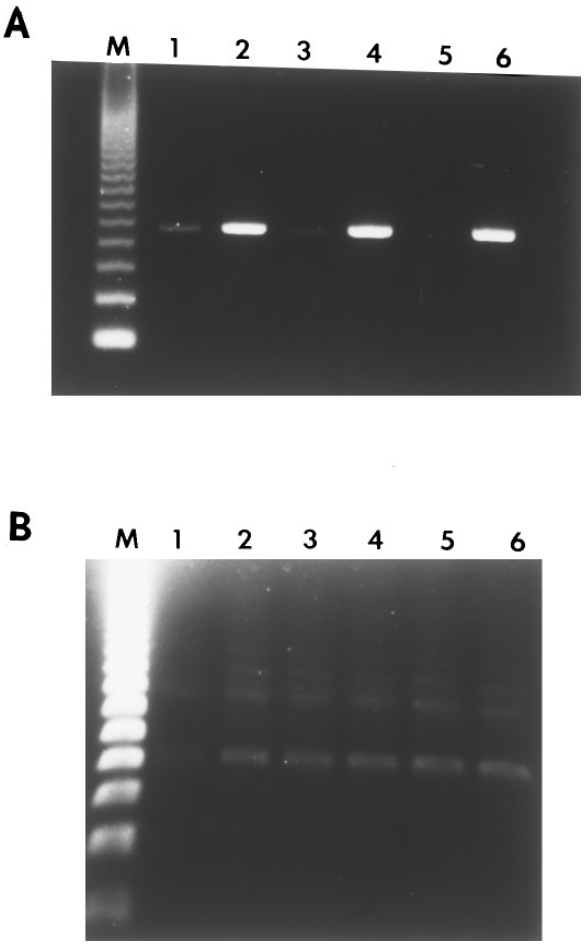

FIG. 1. (A) Effect of $1,25(OH)_2D_3$ on the expression of N-myc mRNA in N1E-115 neuroblastoma cells. Relative levels of expression of N-myc mRNA was determined by RT-PCR of total RNA harvested from 10^{-8} M $1,25(OH)_2D_3$-treated cells at 3, 6, and 24 hours. (B) Expression of β-actin mRNA in neuroblastoma cells. Samples of total RNA from each sample was reverse transcripted and amplified with β-actin specific primers to observe loading of samples. (M) 123 bp DNA marker; (1) 1 h 10^{-8} M $1,25(OH)_2D_3$; (2) 1 h control; (3) 6 h 10^{-8} M $1,25(OH)_2D_3$; (4) 6 h control; (5) 24 h 10^{-8} M $1,25(OH)_2D_3$; (6) 24 h control.

RESULTS

Although $1,25(OH)_2D_3$ is known to play a major role in the control of calcium metabolism and on bone development, there is increasing evidence that the sterol plays a role in tissues that are not related to mineral metabolism. Studies have shown that $1,25(OH)_2D_3$ plays a role in the growth and differentiation of neuronal cells through changes in the expression of various growth related proteins, in particular NGF (1,28,29). However, the mechanisms by which $1,25(OH)_2D_3$ affects neural cell growth or NGF expression are poorly understood. In order to characterize the effect of $1,25(OH)_2D_3$ on neuronal cells, we examined the expression of various growth factors when cells were treated with the sterol.

The expression of N-myc and c-myc was examined in N1E-115 cells treated with 10^{-8} M $1,25(OH)_2D_3$ by RT-PCR. There was a substantial decrease in the expression of N-myc (Fig. 1A) and c-myc (Fig. 2) mRNA in treated neuroblastoma cells compared to control cells.

Results of the assay to measure total PKC activity in $1,25(OH)_2D_3$-treated neuroblastoma cells are shown in Figure 3. Cells treated with 10^{-8} M $1,25(OH)_2D_3$ for 24 hours show a two-fold increase in PKC activity compared to control cells. Serum deprived cells showed little change in total PKC activity compared to the controls.

The effects of $1,25(OH)_2D_3$ on the expression of TGF-β2 expression in N1E-115 cells is shown in Figure 4. $1,25(OH)_2D_3$ caused a significant, dose-dependent increase in TGF-β2 expression in N1E-115 cells, as early as 3 hours after treatment. A five-fold increase in TGF-β2 expression was observed in cells treated with 10^{-6} M 1,25 for 24 hours.

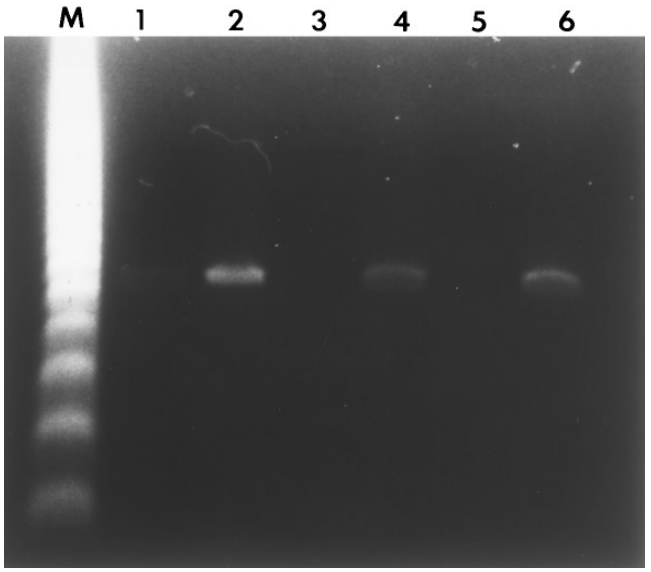

FIG. 2. Effect of $1,25(OH)_2D_3$ on the expression of c-myc mRNA in N1E-115 neuroblastoma cells. Relative levels of expression of c-myc mRNA was determined by RT-PCR of total RNA harvested from 10^{-8} M $1,25(OH)_2D_3$-treated cells at 3, 6, and 24 hours. (M) 123 bp DNA marker; (1) 1h 10^{-8} M $1,25(OH)_2D_3$; (2) 1 h control; (3) 6h 10^{-8} M $1,25(OH)_2D_3$; (4) 6 h control; (5) 24 h 10^{-8} M $1,25(OH)_2D_3$; (6) 24 h control.

DISCUSSION

Previous studies in our laboratory have shown that $1,25(OH)_2D_3$ causes a decrease in the proliferation of neuroblastoma cells as well as increasing the expression of NGF in these cells (1). In order to better characterize the effect of $1,25(OH)_2D_3$ on the proliferation rate of these cells and gain a better understanding of the mechanism of $1,25(OH)_2D_3$-induced NGF expression, we examined $1,25(OH)_2D_3$-treated neuroblastoma cells for changes in the expression of other growth-related proteins.

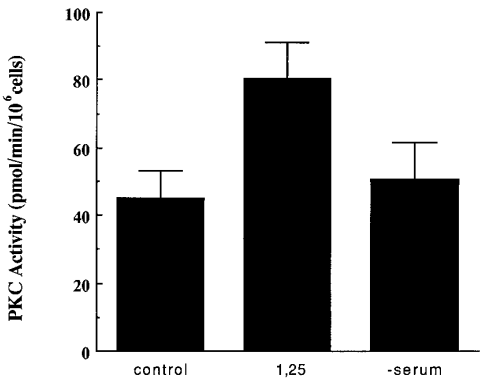

FIG. 3. Effect of $1,25(OH)_2D_3$ on total PKC activity in neuroblastoma cells. N1E-115 cells were treated with 10^{-8} M $1,25(OH)_2D_3$, vehicle, or serum-deprived for 24 hours at which point total PKC activity was measured.

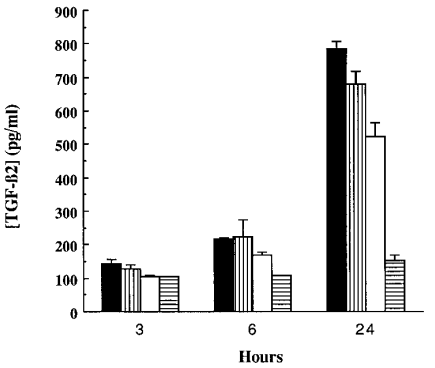

FIG. 4. Effect of $1,25(OH)_2D_3$ on the expression of TGF-β2 in neuroblastoma cells. N1E-115 cells were treated with 10^{-6} M (■), 10^{-8} M (vertical bars), 10^{-10} M (□) $1,25(OH)_2D_3$, or vehicle (horizontal bars) for 3, 6, and 24 hours.

The results presented here show that $1,25(OH)_2D_3$ treatment of N1E-115 neuroblastoma cells causes a decrease in N-myc and c-myc expression. It has been hypothesized that changes in c-myc expression corresponds to cell division blockade and differentiation while changes in N-myc expression are related to an early phase in terminal differentiation (30). Treatment of N1E-115 cells with $1,25(OH)_2D_3$ causes a decrease in cell proliferation as well as an increase in the expression of known markers of differentiated neuronal cells. This observation, along with the observed changes in N-myc and c-myc expression, suggest that $1,25(OH)_2D_3$ is a pro-differentiation factor for this neuroblastoma cell line.

$1,25(OH)_2D_3$ has been shown to increase the expression of PKC activity in several cell lines including chondrocytes, epithelial cells, and bone cells (23-25). However, there are no previous studies showing an effect on PKC activity in neural cells. In our study, as in most others using different cell types, $1,25(OH)_2D_3$ was shown to cause an increase in PKC activity. Increases in PKC activity have been shown to be related to increases in NGF expression in pheochromocytoma cells (31,32). Further investigation is needed to determine if there is a connection between NGF expression and increased PKC activity when N1E-115 neuroblastoma cells are treated with $1,25(OH)_2D_3$.

This study shows that $1,25(OH)_2D_3$ affects the expression of not only NGF (1), but a variety of growth factor-related proteins is neuroblastoma cells, including N-myc, c-myc, PKC, and TGF-β2. It is only recently that the sterol has been shown to play a role in the growth and differentiation of neuronal cells, therefore the mechanisms of $1,25(OH)_2D_3$-regulated growth of these cells are poorly understood. This study provides a basis by which to examine the pathways by which $1,25(OH)_2D_3$ regulates growth and the expression of NGF in neural cells.

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