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Vitamin D-dependent suppression of endothelin-induced vascular smooth muscle cell proliferation through inhibition of CDK2 activity

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ABSTRACT

1,25 dihydroxyvitamin D_3 (1,25 (OH)2 D) and its less hypercalcemic analogues have been shown to inhibit the proliferation of vascular smooth muscle cells (VSMC) in culture. However, the mechanism(s) underlying this suppression is not well understood. Here we have shown that 1,25 (OH)2 D and its analogues (RO-25-6760 and RO-23-7553) inhibit endothelin (ET)-dependent DNA synthesis and cell proliferation in neonatal rat aortic VSMC. While ET stimulation of mitogenic activity requires activation of the MEK/ERK signal transduction cascade, 1,25 (OH)2 D neither affected the ET-dependent activation of ERK nor synergized with the MEK inhibitor PD98059 in reducing DNA synthesis in these cultures, implying that the locus of 1,25 (OH)2 D actions lies between ERK and the cell cycle machinery. 1,25 (OH)2 D suppressed ET-induced activation of cyclin-dependent kinase 2 (Cdk2), a key cell cycle kinase, but had no effect on the expression of this protein. Collectively, the data identify Cdk2 as the target of 1,25 (OH)2 D in the cell cycle machinery and imply a potential role for 1,25 (OH)2 D, or its less hypercalcemic analogues, in the treatment of disorders of VSMC proliferation involving the vascular wall.

1. Introduction

Vitamin D is a secosteroid hormone precursor whose levels are determined by dietary intake or *de novo* synthesis following interaction of ultraviolet light with cholesterol precursors in the epidermis [1]. The most polar metabolite of vitamin D, 1,25 dihydroxyvitamin D_3 (1,25 (OH)2 D), is believed to be the bioactive form of the hormone that binds to the vitamin D receptor (VDR) in the nuclei of target cells and, along with its heterodimeric partner the retinoid X receptor (RXR), triggers the cascade of downstream events that lead to predictable phenotypic changes in these cells.

While most early investigation dealing with vitamin D and its metabolites focused on the capacity of this hormone to mobilize calcium across the intestinal mucosa, thereby providing substrate for mineralization of bone, a growing number of studies have demonstrated both the presence of vitamin D receptors and 1,25 (OH)2 D-dependent biological activity in a variety of tissues and cell types that might be regarded as atypical targets. These include breast [2,3], colon [3,4], pancreatic [5,6] and prostate [2,3,7] cancer cells, pancreatic islets [8,9], cells of the immune system [10], parathyroid cells [11,12], cardiac myocytes [13,14] and VSMC [15].

While 1,25 (OH)2 D, as well as it less hypercalcemic analogues [3,16], have been shown to exert a wide spectrum of effects in these atypical targets, it is, perhaps, its effects on the growth and differentiation of these cells that have attracted the most attention. While exceptions have been noted [15,17], the preponderance of published data support a growth suppressant role for 1,25 (OH)2 D, as well as other VDR ligands. These ligands inhibit proliferation of breast [2,3], colon [3,4], pancreatic [5,6] and prostate [2,3,7] cancer cells. They suppress growth of parathyroid cells [11,12], prevent hypertrophy of cardiac myocytes [18] and inhibit proliferation of VSMC [15,19,20], The latter effect is of particular interest in that it suggests potential utility of these agents in the management of atherosclerosis, post-transplant vasculopathy, re-stenosis post-angioplasty and other disorders characterized by growth and remodeling in the vascular wall.

While the growth suppressant effect of vitamin D in the VSMC is clear, very little information has been published describing the underlying mechanism(s). Parenthetically, in other cell types 1,25 (OH)2 D effects on the cell cycle machinery have been heterogeneous with a number of proteins involved in cell cycle regulation shown to be targets of 1,25 (OH)2 D action [16,21].

In the present study, we have investigated the effects of 1,25 (OH)2 D and less hypercalcemic analogues of 1,25 (OH)2 D (RO-25-6760 and RO-23-7553) on DNA synthesis and cell proliferation in cultured neonatal rat aortic VSMC. We demonstrate that the VDR agonists uniformly suppress endothelin (ET)-stimulated, but not basal, DNA synthesis and mitogenesis, and that the suppression is

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linked to a reduction in the activity, but not the levels, of cyclin-dependent kinase 2 (Cdk2), a key cell cycle kinase that operates in G1 and at the G1/S transition.

2. Materials and methods

2.1. Materials

ET was purchased from Peninsula Laboratories Inc. (Belmont, CA). Anti-Cdk2, anti-cyclin A, anti-cyclin E, anti-Cdk4, anti-Cdk6, anti-cyclin D1, anti-ERK2, anti-JNK1, anti-p27, anti-p21, anti-p16, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-VDR antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [^{3}H]Thymidine, [α-^{32}P]dCTP, and [γ-^{32}P]ATP were purchased from PerkinElmer Life Sciences (Boston, MA). The Cell-Titer 96 Aqueous nonradioactive cell proliferation assay kit was purchased from Promega (Madison, WI). PD098059 was from Research Biochemicals International (Natick, MA). Bovine myelin basic protein was from Upstate Biotechnology Inc. (Lake Placid, NY). RO-23-7553 and RO-25-6760 were kindly provided by M. Uskokovic of Bioxell Inc. (Nutley, NJ).

2.2. Cell culture

Neonatal VSMC were obtained from Jones [22]. Cells were cultured at 37 °C in a 5% CO_2-humidified incubator in DME H-21 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 mg/ml streptomycin, and 2% (v/v) broth, tryptose phosphate (growth media). Cells were used after reaching 75–80% confluence.

2.3. [^{3}H]Thymidine incorporation

Cells were cultured in media containing 10% FBS in 24-well plates for 24 h and then changed to serum-substitute media [23] for the ensuing 24-h period. At that point, all cells were treated with different concentrations of 1,25 (OH)2 D, one of two non-hypercalcemic 1,25 (OH)2 D analogues, or vehicle for the next 48 h. Where indicated, ET was included for the final 24 h of culture. Four hours before collection, cells were pulsed with [^{3}H]thymidine (1 mCi/well) in modified Eagle's medium (MEM) with Earle's balanced salt solution. [^{3}H]Thymidine incorporation was determined as described previously [24].

2.4. Cell proliferation assay

VSMC were cultured in 96-well plates and growth-arrested in serum-substitute/DME for 24 h. Quiescent cells were treated with different concentrations of 1,25 (OH)2 D or its analogues for 48 h in the presence or absence of ET for the final 24 h of culture. To terminate the experiment, media was evacuated and MTS solution was added to each well for the final 4 h. Cell numbers were determined by the Cell-Titer 96 Aqueous nonradioactive cell proliferation assay kit according to the instructions provided by the manufacturer.

2.5. Expression and purification of recombinant glutathione S-transferase (GST)-c-JUN

GST-c-JUN$^{1-79}$ was transformed into *Escherichia coli* strain HB101. Synthesis of the recombinant protein was induced with 100 μM isopropyl-β-D-galactoside for 6 h. Bacteria were collected by centrifugation and sonicated in buffer containing 50 mM Tris–HCl (pH 7.5), 2 mM EDTA, 2 mM DTT, and protease inhibitors (1 Complete^{TM} tablet/50 ml buffer, Roche Applied Science). The supernatant was incubated with GSH-Sepharose (Amersham Biosciences) for 2 h at 4 °C, and then washed with 3 volumes of phosphate-buffered saline containing 2 mM DTT and 0.1% Triton X-100. GST-c-JUN was eluted with 5 mM glutathione in 50 mM Tris–HCl, pH 8.0, and 2 mM DTT.

2.6. Immunoprecipitation and immune complex kinase assay

Cells were incubated with the agents indicated for different time intervals and lysed with lysis buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM glycerophosphate) containing protease inhibitors (1 Complete^{TM} tablet/50 ml buffer). 200 μg of soluble protein was incubated with 1 μg of anti-ERK2, anti-JNK1, anti-Cdk2, anti-cyclin A or anti-cyclin E antibodies and 10 μl of protein G-Sepharose for 1–2 h at 4 °C. Immunoprecipitation and immune complex kinase assay were carried out as described previously [24] with appropriate substrate [2 μg of histone 1 for measurement of Cdk2, cyclin A-, and cyclin E-associated kinase; 5 μg of myelin basic protein (MBP) for measurement of ERK; 5 μg of GST-c-JUN for c-Jun N-terminal kinase (JNK) [24]]. Reaction products were electrophoresed on SDS-polyacrylamide gels which were then dried and exposed to X-ray film.

2.7. Immunoblot analysis

Cells were treated with 10^{-8} M 1,25 (OH)2 D for different time intervals. Cellular lysates were generated, subjected to 12.5% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Chemical Corp., Amersham, IL). Membranes were blocked with 5% nonfat milk in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20 (TBST), and probed with anti-cyclin E, anti-cyclin A, anti-cyclin D1, anti-Cdk4, anti-Cdk6, anti-Cdk2, anti-p27, anti-p21, anti-p16, anti-GAPDH and anti-VDR antibodies, each diluted 1: 50–100 in TBST. A horseradish peroxidase-conjugated secondary antibody (diluted 1:1000–2000 in TBST) was employed to detect immunoreactive bands using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Chemical Corp.).

2.8. Statistical analysis

Data were evaluated using one-way analysis of variance and the Newman–Keuls post-hoc test to assess significance.

3. Results

Treatment of neonatal VSMC with 1,25 (OH)2 D resulted in a dose-dependent reduction in ET-dependent ^{3}H-thymidine incorporation in these cultures. As shown in Fig. 1A, ET treatment led to a 5–6-fold increment in ^{3}H-thymidine incorporation while treatment with 1,25 (OH)2 D led to a stepwise reduction. At 10^{-8} M 1,25 (OH)2 D, ET treatment resulted in only a 2-fold increase in DNA synthesis. 1,25 (OH)2 D had no effect on basal incorporation of ^{3}H-thymidine in the absence of ET. The ability to suppress DNA synthesis was shared by two synthetic analogues of 1,25 (OH)2 D with reduced hypercalcemic activity, RO-23-7553 and RO-25-6760 (Fig. 1B and C, respectively). Each of these agents effected a similar dose-dependent reduction in ET-dependent DNA synthesis. RO-25-6760, based on its dose response, appeared to be somewhat more effective than either 1,25 (OH)2 D or RO-23-7553 in promoting this inhibition.

The reductions in DNA synthesis were matched by qualitatively similar effects on cell number, implying that the aforementioned inhibition of ^{3}H-thymidine incorporation is linked to a bonafide reduction in cell division (versus repair synthesis) in these cultures. As shown in Fig. 2A, ET treatment led to a 2-fold increment in cell number which was, again, reduced to levels only slightly above