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Microarray analysis of 1α,25-dihydroxyvitamin D_3-treated MC3T3-E1 cells☆

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Abstract

The active form of Vitamin D, 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], demonstrates potent antiproliferative actions on normal as well as on malignant cell types by blocking the transition from the G1- to the S-phase of the cell cycle. Key target genes for 1,25-$(OH)_2D_3$ in this non-classic effect remain largely unknown. Therefore, this study aims to identify genes that, through changes in expression after 1,25-$(OH)_2D_3$ treatment, contribute to the observed antiproliferative effect.

cDNA microarrays containing 4600 genes were used to investigate changes in gene expression in MC3T3-E1 mouse osteoblasts at 6 and at 12 h after treatment with 1,25-$(OH)_2D_3$ (10^{-8} M), preceding (6 h) or coinciding with (12 h) the G1/S block in these cells. Approximately one fifth of the genes that were significantly down-regulated after a 12 h incubation period with 1,25-$(OH)_2D_3$ were genes involved in the DNA replication process, a basic process for cell growth that starts at the end of G1-phase and continues in S-phase. Down-regulation of these genes by 1,25-$(OH)_2D_3$ was confirmed by quantitative RT-PCR in MC3T3-E1.

In conclusion, cDNA microarrays revealed that treatment of MC3T3-E1 cells with 1,25-$(OH)_2D_3$ resulted in the down-regulation of DNA replication genes in parallel with the observed G1/S-arrest.

Keywords: Vitamin D; Microarray; Antiproliferative effect; DNA replication; Osteoblasts

1. Introduction

In addition to the well known action on bone metabolism and on calcium and phosphate homeostasis, 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] has a potent antiproliferative and prodifferentiating effect on a wide variety of cells including malignant cell types [1]. Treatment of these cells with 1,25-$(OH)_2D_3$ induces a growth reduction characterized by a blocked transition from the G1- to the S-phase of the cell cycle [2,3]. A possible way for 1,25-$(OH)_2D_3$ to establish this antiproliferative effect is by acting on the DNA replication process. This process starts in G1-phase and ultimately leads to accurate DNA synthesis during S-phase (reviewed in [4]). Key players in this process are, among others, a cell division cycle 6 homolog (Cdc6), DNA polymerases α, δ and ε (Polα, δ, ε), proliferating cell nuclear antigen (PCNA) and a Flap-structure specific endonuclease (FEN1). At the origin of DNA replication Cdc6 attracts different Minichromosome Maintenance (Mcm) proteins needed to unwind the duplex DNA. Once the strands are separated, the Polα-primase complex synthesizes an RNA-DNA primer which is used by Polδ and Polε as a starting point for DNA synthesis. PCNA is needed to load Polδ and Polε onto the DNA and FEN1 serves to remove the remainder of the RNA primer.

To further investigate the antiproliferative effect of 1,25-$(OH)_2D_3$, cDNA microarrays were used to study the expression of 4600 genes in MC3T3-E1 mouse osteoblasts treated with 1,25-$(OH)_2D_3$ (10^{-8} M) for 6 or for 12 h. Remarkably, 20% of the genes that were down-regulated after 12 h treatment with 1,25-$(OH)_2D_3$, were genes involved in DNA replication. Since the down-regulation of these genes paralleled the 1,25-$(OH)_2D_3$-induced G1/S-block, it might contribute to the antiproliferative effect of 1,25-$(OH)_2D_3$.

2. Materials and methods

2.1. Cell culture

MC3T3-E1 cells (Riken Cell Bank, Tsukuba, Japan) were maintained in α-MEM with 2 mM glutaMAX™-I

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(Invitrogen, Merelbeke, Belgium) containing 10% fetal bovine serum (Biochrom KG, Berlin, Germany), 100 units/ml penicillin and 100 μg/ml streptomycin. Approximately 1×10^4 cells/cm^2 were seeded for microarray analysis as well as for quantitative RT-PCR.

Cells were treated with 1,25-$(OH)_2D_3$ (10^{-8} M), a gift of JP van de Velde (Solvay, Weesp, The Netherlands) or solvent (ethanol) 24 h after seeding.

2.2. RNA isolation

Total RNA from MC3T3-E1 cells used for microarray analysis was extracted using TRizol LS reagent (Invitrogen). Total RNA from MC3T3-E1 used for quantitative RT-PCR analysis was prepared using the RNeasy kit (Qiagen, Hilden, Germany). Both RNA extraction methods were performed as specified by the manufacturer.

2.3. Microarray analysis

Microarray analyses were performed at the Microarray Facility of the Flanders Interuniversity Institute for Biotechnology (VIB). Information about construction of the arrays can be found on their website (http://www.microarrays.be).

2.4. Quantitative RT-PCR analyses

1.5 μg RNA was reverse transcribed at 42 °C for 80 min using 150 ng random primers and 200 U SuperScript II (Invitrogen). PCR reactions contained 1× TaqMan buffer A, 200 μM dNTPs, 2 or 5 mM $MgCl_2$, 0.65 U AmpliTaq Gold (Applied Biosystems, Foster City, CA), 300 nM forward primer, 300 nM reverse primer, 200 nM of a dual-labelled detection probe (Eurogentec) and 0.5 μl cDNA or 0.5 μl of a corresponding RNA sample that had not been reverse transcribed and served as a negative control. Amplification reactions were performed in triplicate in an ABI-prism 7700 sequence detector (Applied Biosystems) at previously described conditions [5]. For all samples a detection probe for β-actin was used in order to normalize the obtained data. For each detection probe used, plasmid clones containing partial target cDNA sequences were made. These plasmid clones represent known amounts of target cDNA and serial dilutions of the plasmid clones served as standard curves. Quantification of the amount of target cDNA in the samples was done using these standard curves [5].

2.5. Cell cycle analysis

At 6, 12 and 48 h after treatment with 10^{-8} M 1,25-$(OH)_2D_3$ or vehicle, approximately 1×10^6 MC3T3-E1 cells were washed with PBS twice and fixed in ice-cold 70% ethanol for 30 min. After fixation, cells were washed twice with PBS containing 0.05% Tween-20 and resuspended in PBS containing 0.05% Tween-20, 0.5 mg/ml propidium iodide and 1 mg/ml RNase A (Sigma). Analysis of samples was done using the CellQuest and Modfit program on a FACSort flow cytometer (Becton Dickinson, Erembodegem, Belgium).

3. Results

To quantify the 1,25-$(OH)_2D_3$-induced G1/S-block in MC3T3-E1 cells, FACS-based cell cycle analysis was used to determine the number of cells in S-phase at 6, 12 and 24 h after treatment. At 6 h after treatment there was no significant drop in S-phase cells whereas at 12 and 48 h after treatment with 1,25-$(OH)_2D_3$ samples contained 26% and 40% less S-phase cells than vehicle-treated samples (Table 1).

According to the cDNA microarray analysis, 11 out of 61 genes that were significantly down-regulated after 12 h treatment with 1,25-$(OH)_2D_3$ (10^{-8} M) were genes involved in DNA replication. A three-fold down-regulation was observed for Cdc6 and FEN1, whereas Polα2 (the 68 kDa subunit of the polymerase α-primase complex), Polδ1 and Polδ2 (125 kDa catalytic subunit and 50 kDa regulatory subunit of DNA polymerase δ, respectively) showed a more than two-fold decrease. PCNA showed a 1.92-fold down-regulation (data not shown). Quantitative RT-PCR analyses were performed in MC3T3-E1 cells at 6, 12 and 48 h after treatment with 1,25-$(OH)_2D_3$ to confirm the down-regulation of the abovementioned six DNA replication genes. Already at 6 h after treatment QRT-PCR data showed a clear down-regulation of these genes albeit less pronounced for Polδ2 and Polα2. Down-regulation at 12 h after treatment was indeed at least two-fold for all the genes except for Polδ2 (1.8-fold). After 48 h the decrease in expression was even more pronounced (Table 1).

Table 1
Cell cycle analysis and QRT-PCR analyses on MC3T3-E1 cells treated with 1,25-$(OH)_2D_3$ (10^{-8} M)

	Number of S-phase cells	Cdc6	Polα2	Polδ1	Polδ2	PCNA	FEN1
6 h	92 ± 11	58 ± 9.8	85 ± 3.3	62 ± 2.5	83 ± 2.2	65 ± 2.5	64 ± 1.3
12 h	74 ± 5[a]	33 ± 11.8	40 ± 6.6	41 ± 1.6	56 ± 7.7	46 ± 8.9	38 ± 7.0
48 h	60 ± 4[a]	15 ± 3.5	24 ± 2.8	22 ± 1.0	35 ± 0.6	24 ± 0.1	21 ± 0.2

MC3T3-E1 cells were treated with 1,25-$(OH)_2D_3$ (10^{-8} M) or vehicle for 6, 12 and 48 h. All data are expressed as percentages of corresponding vehicle treated cells. The data from the FACS-based cell cycle analysis are the mean ± S.E.M. of at least three independent experiments. QRT-PCR data are the mean ± S.E.M. of two independent experiments performed in triplicate. Overall down-regulation by 1,25-$(OH)_2D_3$ was significant for each of the six genes according to Fisher's Least Significant Differences (LSD)-multiple comparison test ($P < 0.05$).

[a] Indicates $P < 0.05$, 1,25-$(OH)_2D_3$-treated vs. vehicle treated (Student's *t*-test).

4. Discussion

The non-classic antiproliferative effect of 1,25-$(OH)_2D_3$ is undoubtedly an intriguing research topic. This study used cDNA microarrays to search for 1,25-$(OH)_2D_3$-induced changes in gene expression in MC3T3-E1 cells that might contribute to this antiproliferative action. At 12 h after treatment, in parallel with the observed G1-arrest, we found the significant down-regulation of at least eleven DNA replication genes. The decrease in expression of six of these genes, namely Cdc6, FEN1, Polα2, Polδ1, Polδ2 and PCNA was confirmed by QRT-PCR analyses and was shown to actually start at 6 h after treatment. The early time point at which 1,25-$(OH)_2D_3$ represses the expression of DNA replication genes leads to the suggestion that down-regulation of these genes contributes to the antiproliferative effect of 1,25-$(OH)_2D_3$.

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