1,25-Dihydroxyvitamin D₃ Suppresses Gene Expression of Eukaryotic Translation Initiation Factor 2 in Human Promyelocytic Leukemia HL-60 Cells

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ABSTRACT. The physiologically active metabolite of vitamin D₃, 1 α ,25-dihydroxyvitamin D₃ (DVD), is a potent inducer of cell differentiation in human myeloid leukemia cells. In the present study, we examined changes in gene expression during DVD-induced cell differentiation of promyelocytic HL-60 cells employing a DNA microarray technique. The results identified 7 up-regulated and 9 down-regulated genes with a change greater than 1.5-fold after the DVD-treatment for both 2 and 6 days. Seven of these genes were further examined by reverse transcription-polymerase chain reaction (RT-PCR). The results showed that findings obtained from the DNA microarray analysis and RT-PCR are generally comparable with each other. Gene expression of the subunits of eukaryotic translation initiation factor 2 was then examined by methods including RT-PCR and real-time PCR. The results indicated the suppression of these genes, suggesting a linkage to differentiation-associated growth inhibition of these cells.

Key words: vitamin D₃/differentiation/gene expression/initiation factor/HL-60

Introduction

The physiologically active metabolite of vitamin D₃, 1 α ,25-dihydroxyvitamin D₃ (DVD), is a potent inducer of cell differentiation in human myeloid leukemia cells (Bar-Shavit *et al.*, 1983; Murao *et al.*, 1983; Tanaka *et al.*, 1983; Djulbegovic *et al.*, 1986; Amir *et al.*, 1999; James *et al.*, 1999). Differentiation therapy for neoplastic diseases has potential for supplementing existing disease treatments (Koeffler *et al.*, 1985; Nagpal *et al.*, 2001; Song *et al.*, 2003). Nutritional factors DVD and all-trans retinoic acid (ATRA) with differentiation-inducing activity in human promyelocytic leukemia HL-60 cells have been clinically applied as anti-leukemic agents (Nagpal *et al.*, 2001). Although extensive studies have been performed (Wang *et al.*, 1996; James *et al.*, 1999; Song *et al.*, 2003), it is not

fully understood how DVD induces growth inhibition, differentiation, and apoptosis.

In the present study, we examined changes in gene expression during DVD-induced cell differentiation of HL-60 cells employing a DNA microarray technique and compared the results with those obtained by reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR.

Materials and Methods

Cells and cell culture

Human promyelocytic HL-60 cells were obtained from the Riken Cell Bank, Tsukuba, Ibaragi, Japan. HL-60 cells were cultured in 10% fetal bovine serum in RPMI 1640 medium containing 50 U/ml penicillin, 50 μ g/ml streptomycin, and 2.5 μ g/ml amphotericin B at 37°C under a 5% CO₂ atmosphere. HL-60 cells were treated with DVD at 10⁻⁷ M for up to 6 days as described previously (Kuruto-Niwa *et al.*, 1998).

Cell growth

The cell number was determined by the Trypan blue dye exclusion method using a hemocytometer.

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Abbreviations: ATRA, all-trans-retinoic acid; DVD, 1 α ,25-dihydroxyvitamin D₃; eIF, eukaryotic translation initiation factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcription-polymerase chain reaction.

Table I. NUCLEOTIDE SEQUENCES OF PCR PRIMERS

RT-PCR			
Gene name	Forward primer	Reverse primer	Product length (bp)
GAPDH	5'-AAGGTCATCCCTGAGCTGAA-3'	5'-CCCCTCTTCAAGGGGTCTAC-3'	500
Interleukin-8	5'-TTGTGAGGACATGTGGAAGC-3'	5'-ACACAGCTGGCAATGACAAG-3'	395
Lysozyme	5'-GGCAAGGTCTTTGAAAGGTG-3'	5'-TGCTTCTGTCTCCAGCATTG-3'	157
Cathepsin B	5'-GATCTGCATCCACACCAATG-3'	5'-ATAGAGGCCACCAGAAACCA-3'	160
PCNA	5'-GAAGCACCAAACCAGGAGAA-3'	5'-TCACTCCGTCTTTTGACACAG-3'	193
Cyclin D2	5'-TTACCTGGACCGTTTCTTGG-3'	5'-TCCTGAGGCTTGATGGAGTT-3'	159
eIF-2 beta	5'-GATATGGTTGCTGGGGAGAA-3'	5'-GAGATGTTTGGGCTGACGAT-3'	132
NM23B	5'-GGTTGGCAGGAACATCATTC-3'	5'-TGCTGTTGTGTCCACCTCTT-3'	145
eIF-2 alpha	5'-ATTGAAGAAGTCGGCTCTGG-3'	5'-GACATAAGCCCCCATTTTCAG-3'	155
eIF-2 gamma	5'-TGATGGATGCAGCTCTTCTG-3'	5'-AAATGCAAGGATCTGCTCGT-3'	181
Real time PCR			
eIF-2 alpha	5'-ATGACTACGACAACCCTGGA-3'	5'-TCGGCATTCTTCTCTTCAAG-3'	190
eIF-2 beta	5'-GGGCAGGCTCAGAAAGAGAC-3'	5'-AGATGTTTGGGCTGACGATG-3'	210

Fig. 1. Effect of DVD on the cell proliferation of HL-60 cells. The cells were incubated with (black triangle) or without (black square) DVD at 100 nM for the periods indicated and the number of the viable cells was determined using a hemocytometer after the Trypan blue staining.

mRNA preparation

Total RNA was extracted from cells and mRNA was prepared using a QuickPrep™ Micro mRNA Purification Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) according to the manufacturer's directions.

cDNA microarray technique

The microarray used was IntelliGene™ Human Cancer Chip Version 2.0 (TaKaRa Biomedicals, Inc., Tokyo, Japan). Cy3- and Cy5-labeled DNA probes were prepared using mRNA prepared from DVD-treated and untreated cells, respectively, and hybridization was performed according to the manufacturer's directions.

Table II. EFFECTS OF DVD ON GENE EXPRESSION IN HL-60 CELLS^a

Gene name	GenBank Accession No.	2 days	6 days
Up-regulated			
IL-8	M17017	9.75	4.42
Lysozyme	AL157462	1.56	2.84
BCL-2 related protein A1	U29680	1.67	2.20
Protease inhibitor 2	M93056	1.91	1.96
Growth factor receptor-bound protein 2	M96995	1.76	1.86
Prefoldin 5	D89667	1.54	1.80
Cathepsin B	L16510	1.80	1.54
Down-regulated			
PCNA	M15796	0.62	0.40
Cyclin D2	D13639	0.44	0.49
Sema domain, immunoglobulin domain	AB000220	0.55	0.49
Thyroid autoantigen 70 kDa (Ku antigen)	Z83840	0.44	0.50
ATP synthase, H ⁺ transporting, subunit b	X60221	0.66	0.51
eIF-2, subunit beta	M29536	0.66	0.55
Lactate dehydrogenase A	X02152	0.58	0.60
Ku autoantigen, 80 kDa	M30938	0.65	0.60
Non-metastatic cells 2 (NM23B)	M36981	0.38	0.63

^a Each figure represents the ratio of the fluorescence intensity for the DVD-treated cells to that for the untreated cells.

Scanning was performed using a GMS418 Array Scanner (TaKaRa Biomedicals) and data were analyzed using the software ImaGene™ (TaKaRa Biomedicals).

RT-PCR

Total RNA was prepared from 1×10⁶ cells and RT-PCR was per-

Fig. 2.

formed as described previously (Koyama *et al.*, 2004; Ohata *et al.*, 2005). Primers used are listed in Table I. Amplified DNA was subjected to electrophoresis in 2% agarose, stained with SYBR Green I (Molecular Probes Ltd., Eugene, OR, USA), and imaged and calculated using a FluorImager (Molecular Dynamics Tokyo Ltd., Tokyo, Japan) as described previously (Miyoshi *et al.*, 2001; Koyama *et al.*, 2004; Ohata *et al.*, 2005).

Real-time PCR

Quantitative real-time PCR was performed using LightCycler SYBR Green Fluorophore (Roche Diagnostics Ltd., Tokyo, Japan) as described previously (Koyama *et al.*, 2004). Primers used are listed in Table I.

Results

When HL-60 cells were cultured in the presence of 100 nM DVD, the number of cells was reduced to 80% and 26% of that in the culture without DVD after incubation for 2 and 6 days, respectively (Fig. 1). During incubation for 6 days,

most of the cells became attached to the plastic culture flask. The findings are consistent with DVD-mediated growth inhibition reported previously (Djulgovic *et al.*, 1986).

The gene microarray analysis revealed the expression of 7 genes to be 1.5-fold higher in the DVD-treated cells during incubation for 2 and 6 days, and that of 9 genes to be 1.5-fold lower in these cells (Table II).

For confirmation of the results of the microarray analysis, RT-PCR was performed for 7 selected genes (interleukin-8, cathepsin B, lysozyme, PCNA, cyclin D2, eIF-2 beta, and NM23B) in the cells treated and untreated with DVD for 2 days. The results were compatible with those from the microarray analysis (Fig. 2).

We were particularly interested in the reduced gene expression of subunit 2 (beta) of eukaryotic translation initiation factor 2 (eIF-2) (Table II and Fig. 2F), since this may be related to the inhibition of cell growth by DVD. In the next experiment, we examined the gene expression of two other subunits of eIF-2, e.g. alpha and gamma subunits by RT-PCR. The results showed that the expression of these genes was also down-regulated in the DVD-treated cells

Fig. 2. Effects of DVD on the gene expression in HL-60 cells. A–G, Effects on the gene expression of interleukin-8, cathepsin B, lysozyme, PCNA, cyclin D2, eIF-2 beta, and NM23B. The cells were incubated with DVD at 100 nM for the periods indicated and extracted RNA was analyzed by RT-PCR (23–26 cycles). Amplified cDNA separated by agarose gel electrophoresis was detected by staining with CYBR Green and imaged using FluorImager (a). The results in (a) are normalized using data for GAPDH and are expressed in (b) as the value relative to that for control (100%).

(Fig. 3).

To obtain quantitative information, we performed real-time PCR for alpha and beta subunits of eIF-2 in the cells treated with DVD for 2 days. The results indicated that these genes were down-regulated consistent with the results of the RT-PCR experiments (Fig. 4).

Discussion

Of the 425 genes examined, the microarray analysis identified 7 that were up-regulated and 9 that were down-regulated in DVD-treated HL-60 cells. Seven of these genes were examined further by RT-PCR, and the results were compatible with those from the DNA chip analysis. The mRNA levels of interleukin-8, lysozyme, and cathepsin B were found to increase with the DVD treatment time-dependently and the findings were consistent with the monocytic differentiation of HL-60 cells (Murao *et al.*, 1983; Meier *et al.*, 1995; Berquin *et al.*, 1999). The down-regulation of cyclin D2 and proliferating cell nuclear antigen (PCNA) expression can be correlated to the reported cell cycle arrest

of these cells treated with DVD (Wang *et al.*, 1996). The biological significance of the decreased expression of NM23B is not known at present.

We were specifically interested in the down-regulated expression of eIF-2 beta and examined further the expression of other subunits of eIF-2 by RT-PCR. The results showed that the alpha and gamma subunits were also down-regulated. The results of quantitative real-time PCR confirmed that the alpha and beta subunits were down-regulated in the DVD-treated cells.

eIF-2 is one of the key initiation factors controlling translation for the synthesis of new protein. eIF-2 is composed of three subunits, alpha, beta and gamma, and binds GTP and Met-tRNAⁱ leading to transference of the Met-tRNA to the 40S subunit, resulting in the formation of the 43S pre-initiation complex (Dever, 2002). eIF-2 is involved in the regulation of protein synthesis in response to conditions such as growth activation, differentiation, and metabolic changes (Efioke *et al.*, 1994). An increase in c-myc function by transformation leads to elevated expression of eIF-2 and increases in protein synthesis and cell proliferation (Rosenwald *et al.*, 1993; Rosenwald, 1996). The activation

Fig. 3. Effects of DVD on the gene expression in HL-60 cells. A, Effects on eIF-2 alpha expression. Cells were treated with DVD and the data were processed as in Fig. 2. B, Effects on eIF-2 gamma expression. The cells were incubated with DVD at 100 nM for 2 days and extracted RNA was analyzed by RT-PCR (25 cycles). The data were processed as in Fig. 2. The results in (a) are normalized using data for GAPDH and are expressed in (b) as the value relative to that for the control (100%) from three experiments. *Different from the control with statistical significance at $p < 0.01$ (Student's *t*-test).

Fig. 4. Effects of DVD on the gene expression of eIF-2 alpha (a) and beta (b) in HL-60 cells. The cells were incubated with DVD at 100 nM for the periods indicated and extracted RNA was analyzed by real time PCR. The results are normalized using data for GAPDH and are expressed as the value relative to that for control (100%) from three experiments. *Different from the control with statistical significance at $p < 0.01$ (Student's *t*-test).

by a mitogen causes increased expression of eIF-2 α in primary T lymphocytes (Cohen *et al.*, 1990). These observations indicate the intimate association of eIF-2 with cell proliferation.

In the present study, the results of RT-PCR demonstrated that DVD caused a decrease in the gene expression of the three subunits of eIF-2. The results from the quantitative real-time PCR for alpha and beta subunits confirmed the down-regulation revealed by RT-PCR. These findings suggest that the expression of the three subunits of eIF-2 was down-regulated in a synchronized manner and that the down-regulation can be correlated to the growth inhibition of DVD-treated HL-60 cells.

Recently, Song *et al.* (2003) reported the gene expression profiles of monocytic and granulocytic lineages of HL-60

cell differentiation using microarray gene chips containing 827 genes. However, there was no description for any eIFs including eIF-2. Therefore, to the best of our knowledge, this is the first paper to describe the DVD-mediated down-regulation of the three subunits of eIF-2. We believe that the down-regulation of eIF-2 is pivotal in determining the physiological outcome of HL-60 cells after the DVD-treatment.

On comparing the gene expression during the retinoic acid-induced differentiation of two types of acute promyelocytic leukemia cells, Lee *et al.* (2002) found no common gene expression in NB4 and HL-60 cells. They suggested that ATRA-induced differentiation of acute promyelocytic leukemia cells may be different and cell type-specific. Therefore, it would be important to examine the cell type-dependent and inducer-dependent changes in differentiation.

Proteomic analysis of ATRA-treated NB4 cells has revealed a significant down-regulation of the protein expression of eukaryotic initiation factors such as IF-2, eIF-4A1, eIF-4G, eIF-5, and eIF-6 (Harris *et al.*, 2004). In these experiments, RT-PCR demonstrated that there was essentially no change in the expression of the eIF-4A1 gene as a function of the ATRA treatment. Although the authors did not examine the changes in the gene expression of IF-2, eIF-4G, eIF-5, and eIF-6, they cited previous data and claimed that a previous microarray analysis indicated that the mRNA expression of these genes was unchanged. Since data with a 2-fold or greater difference between two hybridizations has usually been utilized in microarray analyses, one cannot exclude the possibility that a moderate down-regulation escaped detection in previous studies. Therefore, it would be interesting to clarify whether the gene expression of eIF-2 is down-regulated moderately during the ATRA-mediated differentiation of HL-60 by conducting a more precise analysis.

Preliminary experiments using RT-PCR have shown that expression of eIF-2 alpha is also down-regulated in the HL-60 cells treated with phorbol 12-myristate-13-acetate, suggesting that this down-regulation is not unique to DVD-mediated differentiation of these cells. A study is now in progress to reveal how DVD affects the gene expression of eIFs other than eIF-2.

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