

Vitamin D3 (1,25(OH)2D3) ile İşlenmiş HL-60 Hücrelerindeki Gen Anlatımının Zamana Karşı Değişiminin Q-RT-PCR ile Analizi

Time Dependent Gene Expression Analysis of Vitamin D3 (1,25 (OH)2 D3) Treated HL-60 Cells by Q-RT-PCR

Aylin KANLI¹, Mehmet Doğan Gülkaç¹, Hakan Savlı²

1Kocaeli Üniversitesi, Tıp Fakültesi, Tıbbi Biyoloji Anabilim Dalı , Kocaeli, Türkiye

2Kocaeli Üniversitesi, Tıp Fakültesi, Tıbbi Genetik Anabilim Dalı, Kocaeli, Türkiye

ÖZ

GİRİŞ ve AMAÇ: Akut myeloid löseminin (AML) 1 α ,25-dihidroxyvitamin D (3) (1,25(OH)2D3) 'ün farklılaştırma etkisiyle tedavisi ön plana çıkan araştırma konularındandır. Bu çalışmanın amacı HL-60 hücrelerinde lösemi hücre döngüsü regülasyonu ve apoptozunda 1,25(OH)2D3 'ün rolünü araştırmaktır.

YÖNTEM ve GEREÇLER: Vitamin D ile indüklenmiş HL-60 hücrelerinde hücre döngüsü ve apoptoz ile ilişkili 13 genin (TNFR1, Bcl-w, Bax, Bak, Caspase-6, Caspase-8, AIF, Survivin, Cdk1 (Cdc2), Cdk2, Cdk4, Siklin D1 ve Siklin E) dört farklı zamanda (18, 36, 48, 72. saatler) gen anlatımlarını gerçek zamanlı kantitatif PZR (polimeraz zincir reaksiyonu) yöntemini kullanarak analiz ettik.

BULGULAR: İncelenen genlerde dramatik düşüş ve yükselişler gözlemlendi. Deneylerimizde, 72. saatte TNFR1, Cdk-4, Siklin D1, Siklin E ve Survivin genlerinin anlatımında azalma, Caspaz-8 ve Bak genlerinin anlatımında artma gözlemledik. Hücre döngüsüne bağlı genlerin eğilimi 18. ila 72. saat arasında azalması, HL60 hücrelerinin farklılaştığını düşündürdü. Anti-apoptotik Bcl-w ve Survivin genlerinin ekspresyonunun azalması ve pro-apoptotik Bak ve Caspase-8 genlerinin ekspresyonunun artması, apoptotik işlem aşamasının 72. saatte başladığını gösterdi.

TARTIŞMA ve SONUÇ: Sonuç olarak, lösemik HL60 hücre hattının D3 vitaminine maruz bırakılması hücreleri farklılaştırmıştır. Klonal gelişim tamamlanmış ve apoptotik aşama başlamıştır.

Anahtar Kelimeler: HL-60 hücreleri, 1, 25(OH)2D3, Hücre döngüsü, Farklılaşma, Apoptozis, Gerçek Zamanlı Kantitatif PZR

ABSTRACT

INTRODUCTION: The treatment of Acute myelogenous leukemia (AML) with differential effect of 1 α ,25-dihydroxyvitamin D(3) (1,25(OH)2D3) has been one of the popular research topics. The aim of this study is to investigate the role of 1.25 (OH) 2D3 in leukemia cell cycle regulation and apoptosis in HL-60 cells.

METHODS: We analyzed 13 genes (TNFR1, Bcl-w, Bax, Bak, Caspase-6, Caspase-8, AIF, Survivin, Cdk1 (Cdc2), Cdk2, Cdk4, Cyclin-D1 and Cyclin-E) for changes in expression associated with the cell cycle and the apoptosis of human promyelocytic leukemia HL-60 cells induced by 1,25(OH)2D3 at 18th, 36th, 48th and 72nd h, using quantitative real-time PCR.

RESULTS: We did not find significantly down or up-regulated expression profiles at mentioned time points. In our experiments, we observed decreased expression of TNFR1, Cdk-4, Cyclin-D1, Cyclin-E and Survivin genes at 72nd hours, and increased expression of Caspase-8 and Bak genes. The tendency of cell cycle related genes decrease from 18th to 72nd hours suggested that HL60 cells differentiated. Decreased expression of anti-apoptotic Bcl-w and Survivin genes and increased expression of pro-apoptotic Bak and Caspase-8 genes suggested that apoptotic process stage starts around 72nd hours.

DISCUSSION AND CONCLUSION: In conclusion, exposure of leukemic HL60 cell line to vitamin D3 derived the cells into differentiation. The clonal development completed and apoptotic stage begun.

Keywords: HL-60 cells, 1, 25 (OH)2 D3, Cell Cycle, Differentiation, Apoptosis, Quantitative Real Time Polymerase Chain Reaction (Q-RT-PCR)

İletişim / Correspondence:

Dr. Aylin Kanlı

Kocaeli Üniversitesi Tıp Fakültesi, Tıbbi Biyoloji Anabilim Dalı , Kocaeli, Türkiye

E-mail: aylinkanli@hotmail.com

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INTRODUCTION

Acute myelogenous leukemia (AML) is described by the accumulation of immature myeloid cells (blasts) and by a differentiation block that appears at different stages of maturation.^{1, 2} This block of the differentiation appears to be reversible as evidenced by previous studies. These findings indicate the potential use of differentiation inducing agents in the therapy of AML.³⁻⁵ The treatment of AML with differential effect of 1 α ,25-dihydroxyvitamin D (3) (1,25(OH)₂D₃) has been one of the popular research topics. The most effective way to determine changes of gene expression related to vitamin D treatment is to determine mechanisms and target genes which play a role in differentiation of myeloid cells.^{6, 7} The HL-60 cell line was derived from peripheral blood leukocytes of a 36-year-old Caucasian female with acute promyelocytic leukemia which provides a unique in vitro system for studying the cellular and molecular events involved in the proliferation and differentiation of normal and leukemic cells.⁸

The physiologically active metabolite of vitamin D₃, 1,25(OH)₂D₃, is a potent inducer of cell differentiation in human myeloid cells.⁹⁻¹² The differentiation effect of vitamin D₃ are mediated by the vitamin D₃ receptor (VDR) which belong to the steroid/hormone nuclear-receptor superfamily.⁹⁻¹¹ VDR binds to specific DNA sequences to regulate the expression of target genes. 1,25(OH)₂D₃ and its analogs have been found to inhibit growth, induce apoptosis in various cell lines.¹³⁻¹⁷ On the other hand, in a study, a contradicting results were reported. The exposure of HL-60 cells to 1,25(OH)₂D₃ resulted in differentiation and made the cells resistant to cell death by apoptosis.¹⁸ This study was not reproduced by the others. In addition to inducing differentiation, 1,25(OH)₂D₃ blocks the G1 phase of the cell cycle and prevents the occurrence of further steps. It was proposed that the G1 block is associated with decreased activity of Cdk2 and reduced levels of cyclin E in the kinase complex.¹⁹⁻²¹ In our previous experiments HL-60 cells were treated with vitamin D for 24 and 72 hours. Different expression levels of 43 genes have been observed using cDNA array technology.²² In another of our previous study, HL-60 cells were treated with 1,25(OH)₂D₃ for 18, 48 and 72 hours. Differential

expression levels of 16 genes have been observed using quantitative real time PCR.²³

There are two different known main mechanisms of apoptosis in the first, apoptosis is triggered by external signals at the surface of the cell, and the extrinsic pathway of apoptosis depends on extracellular stimulation of the death receptors (Fas or TNFR1) to send the signal to caspase-8, which activates caspase-3, -6, and -7 as well as Bid.²⁴⁻²⁷ In the second mechanism, apoptotic signals arise within the cell. The intrinsic mechanism of apoptosis works through mitochondria and is controlled by Bcl-2 family. The balance between pro- and anti-apoptotic Bcl-2 family members is important for protection or initiation of apoptosis. This pathway is activated by hypoxic stress, growth factor withdrawal or irradiation.²⁴ A group of Bcl-2 family proteins protect or initiate apoptosis. Among these proteins Bcl-2, Mcl-1, Bcl2-xL and Bcl-w are anti-apoptotic, while Bax, Bak, Bok, Bad, Bid and Bik are pro-apoptotic.²⁵ However, the pro- and anti-apoptotic Bcl-2 family proteins can make heterodimers, where the ratio determines the sensitivity of leukemic cells to apoptosis.

The aim of this study was to investigate the role of 1,25(OH)₂D₃ in leukemia cell differentiation and apoptosis in HL-60 cells. TNFR1, Bcl-w, Bax, Bak, Caspase-6, Caspase-8, AIF, Survivin, Cdk1 (Cdc2), Cdk2, Cdk4, Cyclin D1 and Cyclin E gene expressions were analyzed in the HL-60 cells treated and non-treated with vitamin D, using quantitative real-time PCR (Q-RT-PCR) method at 18th, 36th, 48th and, 72nd hours.

In here, we demonstrated that exposure of the leukemia cells to 1,25(OH)₂D₃ caused completion of the differentiation process and induced initiation of the apoptosis.

MATERIALS AND METHODS

Cell culture and Vitamin D Incubation: HL-60 cells were treated at 18th, 36th, 48th and, 72nd hours with 1,25(OH)₂D₃ in isopropanol (Leo Pharmaceuticals, Denmark) (4x10⁻⁸M) in Iscove's modified Dulbecco's medium (IMDM; Sigma

Diagnostics, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Biochrome, Wilts, Germany), %1 Penicillin/Streptomycin (Gibco, Los Angeles, CA, USA), %1 L-Glutamin (Gibco, Los

Angeles, CA, USA) at 37°C in a humidified atmosphere 5% CO₂. Non-treated HL-60 cells was also cultured in same conditions.

RNA Isolation and cDNA Synthesis: Cells were harvested and RNA was extracted. Total RNA isolated from specimens by using RNeasy Mini Kit (QIAGEN, CA, USA) according to the manufacturer's instructions. Reverse transcription (RT) and real-time quantitative PCR were performed separately. First strand of cDNA was synthesized starting from 1 µg of total RNA extracted from HL60 and Vitamin D treated HL60 cells using First Strand cDNA Synthesis Kit for RT-PCR (Roche, Indianapolis, IN, USA). Reverse transcription was performed at 25°C for 10 min followed by 42°C for 1 h. The enzyme was inactivated by heating at 95°C for 5 min.

Quantitative Real-Time PCR: Q-RT-PCR analysis was performed by Quantica realtime PCR device (Techne Inc, UK). For Q RT-PCR Light Cycler Faststart SYBR Green I kit (Roche, Indianapolis) was used according to manufacturer's instructions. Real-time PCR profile was 95°C for 10 min (1 cycle), 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min (40 cycles). Primer sequences of investigated genes were shown in Table 1. The levels of housekeeping gene Beta 2 microglobulin gene was used as an internal control for normalization of RNA quantity and quality differences in all samples. Melting curve analysis showed that there were no primer-dimers and non-specific amplifications. These findings confirmed accuracy and efficiency of our data. Gel electrophoresis of the products was validated the reactions. Results were evaluated using REST software.²⁸

Table 1. Primer sequences of investigated genes

Gene	Primer Sequences
Cdk1 (Cdc2)	F: 5'-CCT TGC CAG AGC TTT TGG AAT ACC-3' R: 5'-GAC ATG GGA TGC TAG GCT TCC TGG-3'
Cdk2	F: 5'-GGC CCG GCA AGA TTT TAG TA-3' R: 5'-CTA TCA GAG TCG AAG ATG GG-3'
Cdk4	F: 5'-TGA CAA GTG GTG GAA CAG TC-3' R: 5'-TAA GAG TGC TGC AGA GCT CG-3'
Cyclin D1	F: 5'-GAG ACC ATC CCC CTG ACG GC-3' R: 5'-TCT TCC TCC TCC TCG GCG GC-3'
Cyclin E	F: 5'-ATA CAG ACC CAC AGA GAC AG-3' R: 5'-TGC CAT CCA CAG AAA TAC TT-3'
TNFR1	F: 5'- ACC AAG TGC CAC AAA GGA AC-3' R: 5'- CTG CAA TTG AAG CAC TGG AA-3'
Bcl-w	F: 5'-ATG GTG GCC TAC CTG GAG AC-3' R: 5'-CCC CGT CAG CAC TGT CCT CA-3'
Bax	F: 5'-TGC TTC AGG GTT TCA TCC AG-3' R: 5'-GGC GGC AAT CAT CCT CTG-3'
Bak	F: 5'-GGC CTG CTA AGG CTC CTC-3' R: 5'-GCT GTG CCA ATA GAG AAG GC-3'
AIF	F: 5'-GGA TCC TGG GGC CAG GGT ACT GAT-3' R: 5'-CTC GGG GAA GAG TTG AAT CAC TTC-3'
Survivin	F: 5'-TGC CTG GCA GCC CTT TC-3' R: 5'-CCT CCA AGA AGG GCC AGT TC-3'
Caspase-6	F: 5'-TTA ATC TTC AAT CAT GAG AGG TTC-3' R: 5'-CTC ACA CAA ATC TTG AAT GTA CCA-3'
Caspase-8	F: 5'-AGA GCC TGA GAG AGC GAT G-3' R: 5'-CAC CAT CAA TCA GAA GGG AAG-3'
B2MG (house-keeping gene)	F: 5'-GAT GAG TAT GCC TGC CGT GTG-3' R: 5'-CAA TCC AAA TGC GGC ATC T-3'

RESULTS

Overall results of this study revealed different levels of gene expressions: At 18th h of the study the change was not remarkable, at 36th h expressions of Caspase-6 and Cdk-1 gene were down-regulated (-1.25 and -1.43, respectively), and at 48th h the expressions of AIF, Cdk1, Survivin,

Caspase-6 and Bcl-w gene were down-regulated (-1.02, -1.14, -1.23, -1.35, -1.59, respectively). Q-RT-PCR analysis revealed that 72nd h was critical for the investigated genes. The expression of TNFR1, Cdk4, Cyclin D1, Cyclin E and Survivin genes were decreased (-2.91, -1.45, -1.94, -1.89, respectively),

whereas the expression of Caspase-8 and Bak genes were increased (1.16 and 2.62, respectively) at the end of 72nd h (Figure 1).

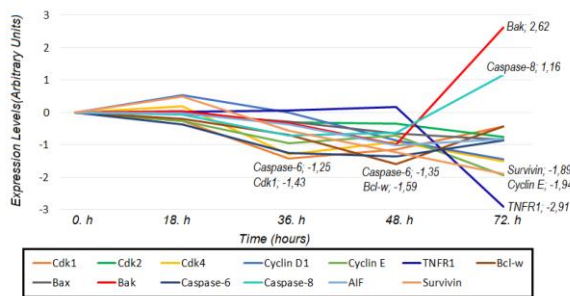


Figure 1. Gene expression levels of 13 cell cycle- and apoptosis-related genes.

DISCUSSION

In this study, we analyzed cell cycle- and apoptosis-related genes expressions in the HL-60 cells treated with vitamin D3. Time dependent reduction of cell cycle related gene expression between 18th h and 72nd h implied that the cells go through proliferation process and pass into the differentiation process. The decreases in anti-apoptotic Bcl-w and Survivin genes expression and the increases in Bak (pro-apoptotic) and Caspase-8 genes expression reveal that apoptosis process was initiated around 72nd h. Moreover, decrease of AIF gene expression towards to 72nd hour could indicate that this gene did not participate in the apoptosis of HL 60 cells.

Horiguchi-Yamada et al (1994) treated HL-60 cells with 12-o-tetradecanoyl 13-acetate (TPA) to and observed differential expressions of various cyclins and Cdk2 gene expression. They found that the expression of cdk2, cyclin A and cyclin E gene dramatically down-regulated, and the expression of cyclin D1 gene was up-regulated at 12th h and 36th h.²⁹ Although we used another differentiation agent, we observed a similar decrease in Cdk2 and cyclin E levels (respectively, -0.75 and -1.95). Harrison et al (1999) investigated cell cycle-related gene expression in 1,25(OH)2D3-treated HL-60 cells at the baseline and 96th h by immunoblotting; they found that Cdk1 expression was decreased ten-fold at 96th h and suggested that decreased level of Cdk1 provided a mechanism for the accumulation of 1,25(OH)2D3-treated HL-60 cells in the G2 cell cycle phase.³⁰ Our Cdk1 gene expression decrease trend at 72nd h suggest consistency with Harrison et al (1999) results. Seol et al (2000)

examined EB1089 (1×10^{-8} M) effect on HL-60 cells for 3 days. They presented down-regulated expressions of Cdk2 and Cdk6, and up-regulated expression of Cdk4 at 24th h. In addition, they found that level of cyclin D1 increased in a time-dependent manner and level of cyclin E did not change. In our study Cdk2 gene expression was consistent with Seol et al study, only. In our previous study, we observed a decrease in expression levels from Cdk2 and cyclin E in vitamin D-treated HL60 cells in a time dependent manner.²³ In our study, this reduction of Cdk1, Cdk2 and cyclin E genes suggest that cell cycle was arrested and cells passed to differentiation phase.

In our study, anti-apoptotic Bcl-w gene expression was down-regulated especially at 48th h. Pro-apoptotic Bak and Bax genes were expressed at basal levels. However, Bak gene expression was distinctly up-regulated at 72nd h. Santos-Beneit and Mollinedo (2000) treated HL-60 cells with DMSO for 4 days and they showed that the expression of Bcl-w and Bak genes gradually increased, and Bcl-2 expression decreased.³¹ Diaz et al (2000) treated colon cancer cell lines with 1 α ,25-dihydroxyvitamin D3 and vitamin D analog EB1089 for 2, 4 and 7 days. As a result of this long incubation period they found reduced level of Bcl-2 expression, and increased Bak expression level. Razzouk and Shapiro (2003) reported that the gene expression of Bax increased 1 to 4-fold at 4th hour and 2.99-fold increase at 8th hour in osteoblast cells.³² Savli et al (2003) analyzed apoptosis associated genes by real time PCR analysis in ATRA treated APL t(15,17) patients and demonstrated the relationship of apoptosis with an increase in bcl2/bax ratio.³³ Kumagai et al (2005) have incubated myeloid leukemia cells (HL-60, NB-4 and U937), myeloma cells (NCI-H929, RPMI8226 and ARH-77), prostate cancer (LNCaP, PC-3 and DU145), breast cancer (MCF-7 and MDA-MB-231), and colon cancer (HT-29) cell lines with various agents. They treated NB-4 APL cells and HL-60 AML cells with non-calcemic vitamin D analog (19-nor-1,25(OH)2D2; paricalcitol) combined with As2O3. They found that this agent reduced Bcl-2 and Bcl-xL anti-apoptotic gene expression, and did not significantly change the level of Bax expression in HL-60 cells.³⁴ Consistent with previous studies, in our experiments the differentiation process was complete, and vitamin D induced differentiation and apoptosis of AML cells.

Razzouk and Shapiro (2003) cultured human bone marrow stromal cells in the Dexamethasone (DMSO) for 28 days and promoted osteogenesis. They induced apoptosis of cells by means of treating the cells with high concentrations of Ca^{2+} and Pi , and then studied the expressions of cell cycle and apoptosis related genes at 4th and 8th hours. They found that TNFR1 gene expression increased 2.66 fold and 1.91-fold at 4th and 8th hours, respectively. Time-dependent decrease in TNFR1 gene expression, they suggested that cell cycle and cell signaling-related genes are expressed in early stages of apoptosis.³² In our study, while TNFR1 gene expression level was at basal level in early stages (between 18th hour and 36th hour), it reduced 2.91 fold at 72nd hour. Although cell lines, differentiation agents, and time periods were different; "time-dependent down-regulation of TNFR1 gene expression" is consistent with our results.

Caspases play a key role in the initiation and signal transduction of apoptosis.^{35, 36} Caspase-8 is one of the initiator caspases and responsible for early apoptotic signal transduction. Caspase-6 is one of the effector caspases and is responsible for execution phases of apoptosis. Caspase-8 is activated by induction of Fas which is a member of TNF receptor family, and activates other caspases and provides apoptosis. Caspase-8 also activated by TNFR1.³⁷ Zhuang and Simon (2000) induced apoptosis in HL 60 cells with peroxynitride (ONOO-) and studied the activity of various caspases. As a result of this study they have determined caspase-8 and caspase-9 as initiator caspases.³⁸ According to our findings, caspase-6 gene expression was down-regulated at 18 h period, and by 72nd h we found an increasing trend. Caspase-8 gene expression was at basal level at 18th, 36th and 48th h, and 1.16-fold increase was found at 72nd hour. Our results imply that apoptotic signal transduction started by the increase of caspase-8 expression by 72nd hour.

AIF has a pro-apoptotic function and cause the formation of apoptotic features such as caspase-independent pathways chromatin condensation and DNA fragmentation.^{39, 40} Several studies have found that different agents increase the expression of AIF in HL 60 cells and induce apoptosis by the caspase-independent pathway.⁴¹⁻⁴⁵ In the literature, there was no change in the expression of AIF in vitamin D treated HL60 cells. In our study, significant AIF gene

expression change in a time-dependent manner was not observed. This result may suggest that the AIF gene is not involved in the apoptotic process in HL-60 cells.

Survivin was first defined as a potential new target for apoptosis-based therapy in cancer and lymphoma by Ambrosini G et al.⁴⁶ Survivin is a member of the inhibitor of apoptosis protein (IAP) family that inhibits caspases and blocks cell death, and is expressed in a large number of malignancies.^{47, 48} Carter et al (2001) examined the effect of ATRA-induced leukemia cell differentiation on survivin expression and they treated HL-60 cells with ATRA for up to 96 hours. They found that survivin gene expression was significantly decreased in the ATRA-treated HL-60 cells.⁴⁹ Wang et al. (2003) have found time-dependent decrease in survivin gene expression in HL-60/ADR at 12th and 48th hours.⁵⁰ In our study, survivin gene expression especially was decreased at 48th and 72nd hours. In this respect, decrease in gene expression of surviving, its relationship with apoptotic process between 48 and 72 hours in HL-60 cells.

As a result, our findings suggest that the leukemic cell line is exposed to the effect of vitamin D3; while clonal development and differentiation process ends, apoptotic stage begins. These results may provide information about the differentiation of these genes, the timing of the cell cycle and the possible genetic pathways in cancer therapy with vitamin D. Furthermore, aside from in vitro studies, in vivo studies should be performed. These studies may shed light on the development of new approaches for treatment of leukemia.

Study Limitation

The findings of this study represent changes occurring in in vitro. These changes should be verified in in vivo models (animal models).

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