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SHORT COMMUNICATIONS

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DIFFERENTIAL EXPRESSION OF 16 GENES CODING FOR CELL CYCLE- AND APOPTOSIS-RELATED PROTEINS IN VITAMIN D-INDUCED DIFFERENTIATION OF HL-60 CELLS

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Objective: **The aim of the study was to analyze expression of several genes related to cell cycle regulation and apoptosis in realization of 1alpha,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)-induced differentiation of HL-60 cells.** ***Methods:*** **The cultured HL-60 cells were treated with $1,25(OH)_2D_3$. Quantitative real-time PCR was used for analyzing the changes in expression of 16 genes (Bcl-2, Bcl-xL, Mcl-1, Bik, caspase 6, caspase 7, cytochrome c, TNFR1, Myc, TGF-beta, JNK1, p38MAPK, p21, p27, Cdk2, cyclin E) at early phases of cell differentiation of HL-60 cells induced by $1,25(OH)_2D_3$.** ***Results:*** **Among investigated genes,** ***Bik*** **and** ***Myc*** **gene expression was down-regulated at 48 h time points.** ***JNK1*** **gene was markedly up-regulated and caspase-6 and cyclin E genes were down-regulated at 18 h time point.** ***Conclusion:*** **These findings suggest that there are no distinct apoptotic signals at early phases of cell differentiation. It is speculated that changes in the expression of the genes involved in vitamin D-induced apoptosis of HL-60 cells could be better visualized after the terminal stages of cell differentiation.**
Key Words: **HL-60, $1,25\ (OH)_2\ D_3$, cell cycle, differentiation, apoptosis, quantitative real time PCR.**

Acute myelogenous leukemia (AML) is a disease resulting from neoplastic proliferation of myeloid precursor cells [1]. There is a series of genetic alterations rather than a single event leading to leukemic transformation. AML is characterized by a defect in differentiation leading to an imbalance between proliferation and maturation. Differentiation induction has become the treatment of choice in a subset of AML.

The differentiation effect of vitamin D_3 is initiated by its binding to a nuclear vitamin D receptor receptor (VDR) [2]. In addition to inducing differentiation, $1,25(OH)_2D_3$ blocks the cell cycle during the G1 phase. It was proposed that the G_1 block is associated with decreased activity of Cdk2 and reduced levels of cyclin E in the kinase complex [3]. Exposure of HL-60 cells to $1,25\ (OH)_2D_3$ results in differentiation and makes these cells resistant to cell death by apoptosis [4]. In our previous experiments HL-60 cells were treated with vitamin D for 24 and 72 h and gene expression was analyzed performed using cDNA array technology. Different expression levels of 43 genes have been observed [5].

The extrinsic pathway of apoptosis depends on extracellular stimulation of the death receptors (Fas or TNFR1) to send the signal downstream to caspase-8, which activates caspase-3, -6, and -7 as well as Bid [6, 7]. It has been shown that MYC induces apoptosis via this pathway [8]. The important mediators of signal transduction, mitogen-activated protein (MAP) kinases play a key role in the regulation of many cellular processes, such as cell growth, cell proliferation, differentiation, and apoptosis. In mammalian cells, MAP kinases have been identified as three major groups: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase. It is well documented that ERK is typically stimulated by growth-related signals, whereas the JNK and p38 MAP kinase cascades are activated by various stress stimuli [9].

TGF- β is a negative growth factor of main interest linked to apoptosis and cell cycle inhibitors [10]. TGF-β can directly inhibit cdk4/cyclin D complex and cdk2/cyclin E complex, blocking Rb phosphorylation. In addition, the block of cdk2/cyclin E and cdk4/cyclin D complexes can be also indirectly performed by TGF- β, inducing the cell cycle-inhibitors p27Kip1 and p15INK4b [11].

The intrinsic mechanism of apoptosis works through mitochondria and is controlled by Bcl-2 family. This pathway is activated by hypoxic stress, growth factor withdrawal or irradiation, which can shift the balance between pro- and anti-apoptotic Bcl-2 family members [12]. 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 [13]. However, the pro- and anti-apoptotic Bcl2-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 $1,25(OH)_2D_3$ in leukemia cell differentiation and apoptosis in HL-60 cells. In three different time periods (18, 48, 72 h) Bcl-2, Bcl-xL, Mcl-1, Bik, Caspase 6, Caspase 7, Cytochrome-c, TNFR1, Myc, TGF-beta, JNK1, p38MAPK, p21, p27, Cdk2 and Cyclin E gene expressions were analyzed in the HL-60 cells treated and non-treated with vitamin D using Quantitative Real Time PCR method.

MATERIALS AND METHODS

Cell culture and vitamin D treatment. HL-60 cells were treated for 18, 48, and 72 h with $1,25\ (OH)_2D_3$ in isopropanol (Leo Pharmaceuticals, Denmark) (4×10^{-8} M) in Iscove's Modified Dulbecco's medium (IMDM; Sigma Diagnostics, USA) supplemented with 10% fetal calf serum (FCS; Biochrome, Germany), 1% Penicillin/Streptomycin

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Abbreviations used: AML – acute myelogenous leukemia; β2MG – beta 2 microglobuline; ERK – extracellular signal-regulated kinase; JNK – c-Jun N-terminal kinase; PCR – polymerase chain reaction; p38MAPK – p38 mitogen-activated protein kinase; TGF-β1 – transforming growth factor β1; VDR – vitamin D receptor.

(Gibco, USA), 1% L-glutamin (Gibco, USA) at 37 °C in a humidified atmosphere 5% CO_2. Non-treated HL-60 cells were cultured at the same conditions.

RNA isolation and cDNA synthesis. Cells were harvested and total RNA was isolated from specimens using RNeasy Mini Kit (QIAGEN, USA) according to the manufacturer's instructions. Reverse transcription (RT) and Quantitative Real Time 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. Quantitative real time PCR analysis was performed in Light Cycler (Roche, USA) using Light Cycler Faststart SYBR Green I kit (Roche, Indianapolis) according to manufacturer's instructions. Real–time PCR profile was 95 °C for 10 min (1 cycle), 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min (40 cycles). Primer sequences of investigated genes are shown in the Table.

Table. Primer sequences of investigated genes

Gene	Primer sequences
Bcl-2	F: 5'-AGG AAG TGA ACA TTT CGG TGA C-3'
	R: 5'-GCT CAG TTC CAG GAC CAG GC-3'
Bcl-xL	F: 5'-GTA AAC TGG GGT CGC ATT GT-3'
	R: 5'-TGG ATC CAA GGC TCT AGG TG-3'
Mcl-1	F: 5'-GAT GAT CCA TGT TTT CAG CGA C-3'
	R: 5'-5'-CTC CAC AAA CCC ATC CCA G-3'
Bik	F: 5'-TCT GCA ATT GTC ACC GGT TA-3'
	R: 5'-5'-TTG AGC ACA CCT GCT CCT C-3'
Caspase-6	F : 5'-CTA ATC TTC AAT CAC GAG AGG TTC-3'
	R: 5'-CTC ACA CAA ATC TTG AAT GTA CCA-3'
Caspase-7	F: 5'-AGC CTG GGT TTT GAC GTG-3'
	R: 5'-ACC GTG GAA TAG GCG AAG-3'
Cytochrome-c	F: 5'-AGG CCC CTG GAT ACT CTT ACA CAG-3'
	R: 5'-TCA GTG TAT CCT CTC CCC AGA TG-3'
TNFR1	F: 5'- ACC AAG TGC CAC AAA GGA AC-3'
	R: 5'- CTG CAA TTG AAG CAC TGG AA-3'
Myc	F: 5'-GGC AAA AGG TCA GAG TCT GG-3'
	R: 5'-GTG CAT TTT CGG TTG TTG C-3'
TGF-beta	F: 5'-TGA ACC GGC CTT TCC TGC TTC TCA TG-3'
	R: 5'-GCG GAA GTC AAT GTA CAG CTG CCG C-3'
JNK1	F: 5'-CAA TGG CTC TCA GCA TCC ATC ATC-3'
	R: 5'-CAA TGA CTA ACC GAC TCC CCA TCC-3'
p38 MAPK	F: 5'-GCC CAA GCC CTT GCA CAT-3'
	R: 5'-TGG TGG CAC AAA GCT GAT GAC-3'
p21	F: 5'-TGA GCG ATG GAA CTT CGA CT-3'
	R: 5'-GAC AGT GAC AGG TCC ACA TGG-3'
p27	F: 5'-TCA GAC GGT TCC CCA AAT GC-3'
	R: 5'-TGC TAC ATC CAA CGC TTT TAG AGG-3'
Cdk2	F: 5'-GGC CCG GCA AGA TTT TAG TA-3'
	R: 5'-CTA TCA GAG TCG AAG ATG GG-3'
Cyclin E	F: 5'-ATA CAG ACC CAC AGA GAC AG-3'
	R: 5'-TGC CAT CCA CAG AAA TAC TT-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'

The level of housekeeping 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 confirming accuracy and efficiency of the data. Gel electrophoresis of the products validated the reactions. Results were evaluated using REST software [14].

RESULTS AND DISCUSSION

We observed that upon the treatment of HL-60 cells with vitamin D expression of *Bik* and *Myc* genes was down-regulated (–1.84 and –1.56 respectively) in 48 h period, while *JNK1* gene expression was markedly up-regulated in 18 hr period (+4.52). Caspase-6 and Cyclin E genes were in down regulated state at 18 h period (–2.49 and –1.38 respectively). However, the expression levels of these genes tended to increase at 72 h period (–1.14 and –0.57 respectively) (Figure).

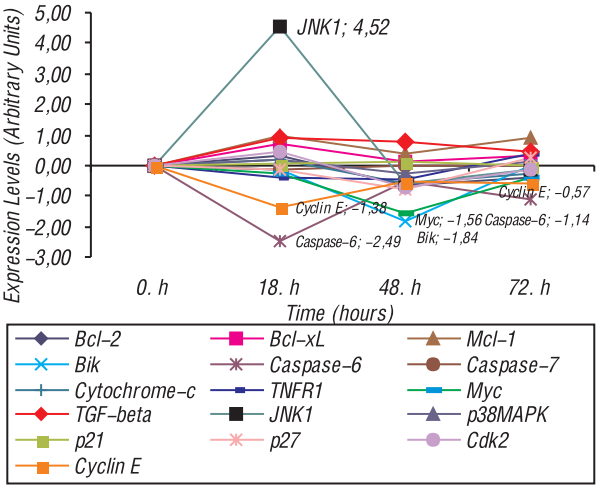

Figure. Gene expression levels of 16 cell cycle- and apoptosis-related genes.

Watanabe et al (1985) demonstrated that induction of HL-60 differentiation was associated with decrease in *c-Myc* RNA as an early event in HL-60 differentiation. Thus, the appearance of the mature phenotype and loss of proliferative capacity are associated with the decline in *c-Myc* RNA [15]. Consistent with previous studies, our findings suggest that down-regulation of *c-Myc* gene expression closely correlated to cell growth arrest, normal cell maturation and HL60 cell differentiation.

Recent studies revealed that the MEK/ERK module of the mitogen-activated protein kinase (MAPK) signaling cascades is up-regulated in the early stages of $1,25(OH)_2D_3$-induced monocytic differentiation of human leukemia cells HL60 [16]. In aggrement with previous studies, the increase in JNK1 activity in 18 h period suggests that it play a role in the early stages of monocytic differentiation of HL60 cells.

Seol et al. (2000) treated HL-60 cells with EB1089 ($1x10^{-8}$ M) for 3 days and found that Cyclin E gene expression was not changed, while cdk2 gene expression was down-regulated in 24 hours [17]. Our findings related to Cdk2 and Cyclin E genes were not consistent with this study. Horiguchi-Yamada et al. (1994) treated HL-60 cells with 12-o-tetradecanoyl 13-acetate (TPA) and studied changes of cyclins and cdk 2 gene expressions. They found that the expressions of Cdk 2 and cyclin E gene were markedly down-regulated between 12 and 36 h periods [18]. Although, different differentiation agent was used in this study, the expression of cyclin E gene is consistent with their results. Cdk2 gene expression was at basal level in three time periods, but it was slightly reduced in 48 hours period. Cyclin E gene was in down regulated state in 18 hr period (–1.38), while the

expression of Cyclin E gene was tend to increased to 72 hr period (–0.57). This reduction of Cyclin E and Cdk2 gene expression suggests that cell cycle was arrested and cells passed to differentiation phase.

In our study, pro-apoptotic Bik gene expression was down-regulated especially in 48 h and the expression of Bik gene tended to increased in 72 h. Anti-apoptotic genes Bcl-2, Bcl-xL and Mcl-1 were expressed at basal levels in these time periods.

Caspase-6 and Caspase-7 are the effectors caspases responsible for execution phases of apoptosis [19]. According to our study, caspase-6 gene was in down regulated state in 18 h period. However the expression of caspase-6 gene tended to increase in 72 h. Caspase-7 gene expression was at basal level in 18, 36 and 48 h periods. Our results imply that caspase-6 gene expression tended to increase in 72 h, while the caspase cascade was not promoted and caspase-7 did not take role in this process.

In conclusion, our results suggest that there are no distinct apoptotic signals in early phases of differentiation. The genes involved in vitamin D-induced apoptosis of HL-60 cells seem to be more clearly visible after the terminal differentiation process.

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РАЗЛИЧНАЯ ЭКСПРЕССИЯ 16 ГЕНОВ, КОДИРУЮЩИХ БЕЛКИ КЛЕТОЧНОГО ЦИКЛА И АПОПТОЗА, ПРИ ДИФФЕРЕНЦИРОВКЕ КЛЕТОК HL-60 ПОД ДЕЙСТВИЕМ ВИТАМИНА D

Цель: **проанализировать экспрессию ряда генов, участвующих в регуляции клеточного цикла и апоптоза в дифференцировке клеток HL-60 промиелоцитарного лейкоза человека, индуцированной 1альфа,25-дигидроксивитамином D_3 ($1,25(OH)_2D_3$).** ***Методы:*** **культивируемые клетки HL-60 инкубировали с $1,25(OH)_2D_3$. Количественную ПЦР в режиме реального времени использовали для анализа изменений экспрессии 16 генов (** ***Bcl-2, Bcl-xL, Mcl-1, Bik,*** **каспаза 6, каспаза 7, цитохром с,** ***TNFR1, Myc, TGF-beta, JNK1, p38MAPK, p21, p27, Cdk2, ц*****иклин E) при индукции апоптоза и дифференцировки в клетках HL-60 под действием $1,25(OH)_2D_3$.** ***Результаты:*** **среди исследованных генов отмечено снижение экспресси** ***Bik*** **и** ***Myc*** **через 48 ч. Экспрессия гена** ***JNK1*** **повышена, а генов каспазы-6 и циклина E снижена в точке 18 ч.** ***Выводы:*** **эти данные свидетельствуют об отсутствии четких сигналов экспрессии апоптоз-ассоциированных генов на ранних фазах клеточной дифференцировки. Предполагается, что изменения в экспрессии генов, участвующих в реализации апоптоза клеток HL-60, индуцированного витамином D, могут быть выражены лишь на терминальных стадиях дифференцировки этих клеток.**

Ключевые слова: **HL-60, $1,25(OH)_2D_3$, клеточный цикл, дифференциация, апоптоз, количественная ПЦР в режиме реального времени.**