

QUANTITATIVE ANALYSIS OF EXPRESSION LEVEL OF BCL2 AND BAX GENES IN HEP-2 AND HL-60 CELLS AFTER TREATMENT WITH ETOPOSIDE

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Summary: Apoptotic cell death is a highly regulated process, which plays a crucial role in many biological events. The aim of the present research was to investigate the expression of the apoptosis related genes BCL2 and BAX in Hep-2 and HL-60 cells. Apoptosis was induced in these cell lines during treatment with etoposide. The expression levels of BCL2 and BAX genes were measured after 6 h and 12 h of treatment by quantitative real-time RT-PCR. In Hep-2 cells the expression level of BCL2 significantly increased both 6 h and 12 h of treatment, whereas expression level of BAX didn't change. In HL-60 cells the expression level of BCL2 decreased after 6 h of treatment and expression of BAX increased both 6 h and 12 h of treatment with etoposide. Those findings show distinct reactions of Hep-2 and HL-60 cells to etoposide treatment and different upregulation or downregulation of apoptosis-related genes BCL2 and BAX.

Key words: Apoptosis; Etoposide; BCL2, BAX; Real-time RT-PCR

Introduction

Many cytostatic drugs have been reported to induce apoptosis in tumor cells both in vitro and in vivo (1, 27, 30). Apoptosis is a complex, strictly regulated physiological process which is characterized by several molecular and biochemical features such as upregulation of proapoptotic genes, activation of specific enzymes, degradation of sub-cellular organelles, cell contraction and rounding, formation of spikes and blebs, DNA fragmentation and cell fragmentation into apoptotic bodies (19).

Apoptosis is also involved in cancer therapy and many chemotherapeutic agents act by induction of apoptosis. For example etoposide, which was used in our study, belongs to the most commonly used classes of anticancer drugs and it has a broad anti-tumor spectrum. The drug increases level of topoisomerase II-mediated DNA breaks and it acts by inhibiting the ability of topoisomerase II to ligate cleaved DNA molecules (3).

Etoposide can induce apoptosis in human tumor cell lines Hep-2 and HL-60 and this process is accompanied by typical morphological changes as a cell blebbing, DNA fragmentation and changes in mitochondrial potential (5, 25). These morphological changes in apoptotic cells are accompanied with changes in expression of apoptosis-related genes BCL2 and BAX (12, 8). During apoptosis is

BAX transported to the mitochondria and induces the release of cytochrome c from the mitochondria. Released cytochrome c binds APAF1 and CASP9 and induces apoptosis in the cells (10, 20). On the other hand, BCL2 binds to BAX and can form heterodimers, thus inhibiting BAX activity. The ratio of BCL2 to BAX protein has been reported to be correlated with apoptosis in cancer cells (9, 22). Expression level of these apoptosis-related genes is various and depends on the type of cell lines. The analysis of changes of expression levels of BCL2 and BAX genes may provide new information about the chemoresistance or chemo-sensitivity (16). Expression level could also differ between tumor and noncancerous samples (15, 29).

In the present study we investigated the occurrence of changes in the expression levels of apoptosis-related genes (BCL2 and BAX) in cancer cell lines Hep-2 and HL-60 during etoposide treatment.

Materials and Methods

Cell lines and cytostatic

Etoposide (Vepesid inj., Bristol-Myers Squibb, USA) was diluted from the original ampoules supplied by manufacturer in a serum-free DMEM to the tested concentration of the 10 µg/ml.

Human epitheloid cell line Hep-2 (ECACC, No. - 86030501, Porton Down, England) was cultivated as stationary monolayer in plastic tissue-culture dishes (Nunc, Denmark). Cells were grown in Dulbecco's modified Eagle's medium (Gibco, Germany) supplemented with 10 % fetal serum (Gibco, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin.

Human promyelocytic leukemia HL-60 cells (ECACC, No. - 85011431, Porton Down, England) were grown in RPMI 1640 medium (Gibco, Germany) supplemented with 10 % fetal calf serum (Gibco, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin.

Cells were grown under standard laboratory conditions (37 °C, 5 % CO₂). After 24 hours of cultivation cells in a control group were harvested and washed with PBS. In groups where the effect of etoposide on Hep-2 and HL-60 cells was investigated, the standard medium was replaced with a medium containing 10 µg/ml of etoposide. After incubation for 6 and 12 hours, cells were harvested and washed with PBS.

Quantitative PCR analysis

Total RNA was extracted using NucleoSpin RNA II kit (Macherey -Nagel, Germany), according to the manufacturer's protocol. Total RNA was eluted with 50 µl of RNase free water and stored at -70 °C. The RNA concentration was determined using spectrophotometry. Complementary DNA (cDNA) was synthesized with 1 µg total RNA and 0,8 µg oligo (dT)12-18 primer (Generi-Biotech, Czech Republic), 200 U M-MuLV Reverse Transcriptase (Finnzymes, Finland) and 40 U of RNasin Ribonuclease Inhibitor (Promega, USA) per reaction.

Primers and the TaqMan probes for detecting gene expression of BAX and PBGD by real-time PCR were designed and synthesized by Generi-Biotech (Czech Republic), sequences of primers for BCL2 were taken from Ikeguchi et al. (16) and synthesized by Generi-Biotech (Czech Republic) (Tab. 1).

Quantification of gene expression was performed with RotorGene 3000 detection system (Corbett Research, Australia). PCR solution (20 µl) was composed of 1 µl cDNA, 5 mM MgCl₂, 0.2 mM of each dNTP, 1.25 U of Thermo-Start DNA Polymerase (AB gene, UK) forward primer 300 nM, reverse primer 900 nM and FAM probe

100 nM (PBGD), forward primer 900 nM, reverse primer 900 nM and FAM probe 100 nM (BCL2), forward primer 300 nM, reverse primer 300 nM and FAM probe 100 nM (BAX). The thermal cycle parameters were: 1. cycle 95 °C for 15 min followed by 45 cycles at 95 °C for 20 sec, 60 °C for 40 sec.

Standard curves for BAX, BCL2 and PBGD were generated using serial dilution of cDNA derived from the cell lines. PBGD was monitored as a reference gene and BCL2 and BAX expression levels were normalized with respect to PBGD transcript and calculated by 2^{-ΔΔCt} method (21).

All experiments were repeated three times and statistical analysis was done with the program QC. Expert 3.0 (TriloByte, Czech Republic). A two-sided P-value, lower than 0.05 was considered as statistically significant difference.

Results

In Hep-2 cells, the relative expression level of anti-apoptotic BCL2 gene was significantly higher after 6 h of treatment than the untreated control (p=0.048). The relative expression level of BCL2 was significantly higher also after 12 h of treatment (p=0.001) and the expression level also significantly increased between 6 h and 12 h of treatment (p=0.03) with etoposide (Fig. 1). Whereas, the relative expression level of proapoptotic BAX did not significantly change neither after 6 h nor 12 h of treatment with etoposide (Fig. 2).

In HL-60 cells, the relative expression level of anti-apoptotic BCL2 gene was significantly lower after 6 h of treatment than the untreated control (p=0.013), but the expression level after 12 h of treatment was significantly higher (p=0.012) compared to untreated control. The significant distinction was also between 6 and 12 h (p=0.0003) of treatment with etoposide (Fig. 3). The relative expression level of proapoptotic BAX gene was significantly higher after 6 h (p=0.007) and 12 h (p=0.0009) of treatment with etoposide compare to untreated control (Fig. 4).

Discussion

Etoposide induces apoptosis in various cancer cell lines (14, 17, 18, 23). To investigate the mechanism of apoptosis

Tab. 1: Sequences of the oligonucleotide primers and probes used in real-time RT-PCR assays.

Target gene	Function	Sequence	PCR product size (bp)
BCL2	Forward primer	5'-TTGGCCCCCGTTGCTT-3'	65
	Reverse primer	5'-CGGTTATCGTACCCCGTTCTC- 3'	
	Probe	5'-AGCGTGCGCCATCCTTCCCAG-3'	
BAX	Forward primer	5'-GTCGCCCTTTTCTACTTTGCCAG-3'	124
	Reverse primer	5'-TCCAGCCCAACAGCCGCTCC- 3'	
	Probe	5'-CCAATGTCCAGCCCATGATGGTTCTGA-3'	
PBGD	Forward primer	5'-ACCATCGGAGCCATCTGCAAG-3'	106
	Reverse primer	5'-CCCACCACACTCTTCTCTGGCA-3'	
	Probe	5'-CCCTCATGATGCTGTTGTCTTTCACCCAA-3'	

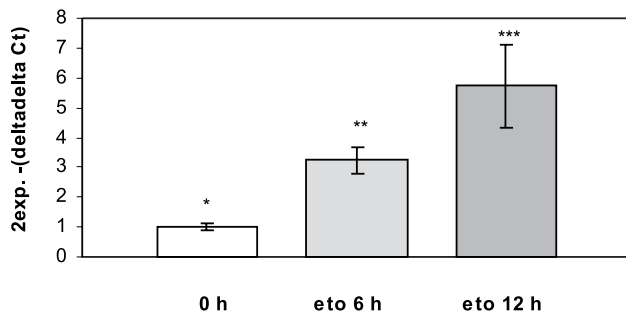


Fig. 1: Hep-2, BCL2/PBGD ratio.

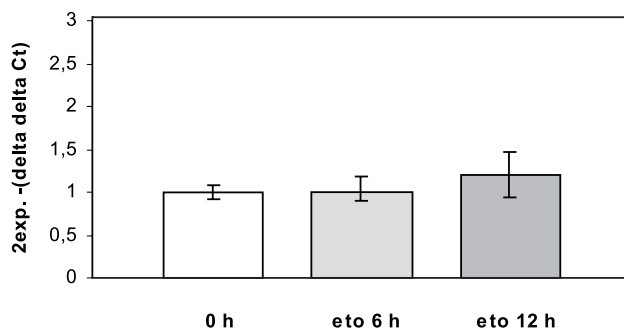


Fig. 2: Hep-2, BAX/PBGD ratio.

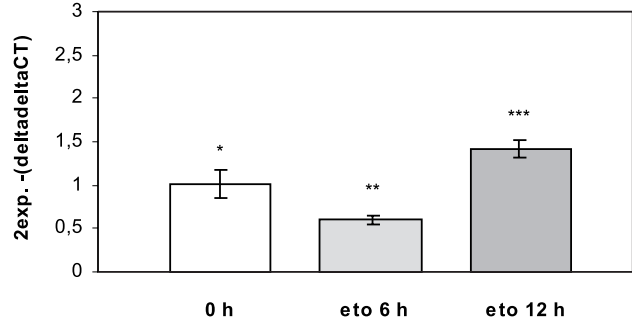


Fig. 3: HL-60, BCL2/PBGD ratio.

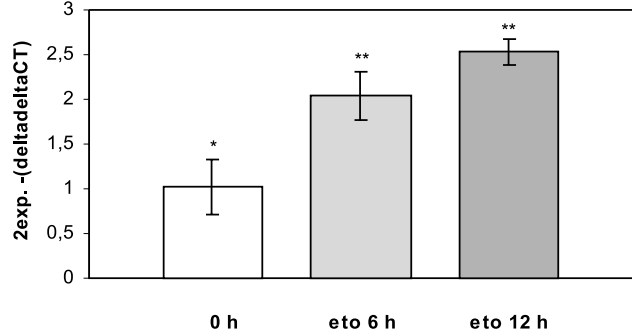


Fig. 4: HL-60, BAX/PBGD ratio.

Fig. 1-4: Relative expression of apoptosis-related genes during treatment with etoposide. A significant difference ($p < 0.05$) was detected between *, ** and ***.

the expression levels of apoptosis-related genes (BCL2 and BAX) were analyzed in cancer cells lines Hep-2 and HL-60 during etoposide treatment.

Etoposide is one of the most widely prescribed anti-cancer drugs in the world. It is used to treat a variety of cancers, including small cell lung cancer, sarcomas, leukemias and lymphomas. Etoposide is derived from podophylotoxin, the natural product from the plant *Podophyllum peltatum*. The primary cellular target for etoposide is topoisomerase II. This essential enzyme removes knots and tangles from the genome by introducing transient double-stranded breaks in the DNA strand. The effect of etoposide is in a stabilization of covalent enzyme-cleaved DNA complex (known as the *cleavage complex*) which is an intermediate in the catalytic cycle of topoisomerase II. The accumulation of cleavage complexes in treated cells leads to the generation of permanent breaks in the genetic material, which finally trigger cell death (3).

Induction of apoptosis in Hep-2 cells was described in previous work of Červinka et al. (5). Cells were treated with etoposide at 10 $\mu\text{l/ml}$ concentration. In the period of 4–8 h after beginning of the treatment the cell becomes rounded and plasma membrane formed numerous of pseudopodia. Typical formation of DNA ladders due to an internucleosomal hydrolysis of the DNA was also observed. In the following works they described other typical hallmarks of apoptosis such as membrane blebbing and activa-

tion of caspase 3 in Hep-2 cells after treatment with etoposide (6, 25, 26). So these data show, that etoposide of concentration 10 $\mu\text{l/ml}$ can induce typical apoptotic changes in Hep-2 cells. Duran et al. (7) also observed typical morphological changes such as granulation, nuclear enlargement and rounding of Hep-2 cells after treatment with etoposide at 5 $\mu\text{l/ml}$ and 50 $\mu\text{l/ml}$ concentration.

Quantitative real-time RT-PCR analysis showed that expression level of BCL2 gene significantly increased both 6 h and 12 h after treatment with etoposide in Hep-2 cells. Whereas the expression level of BAX did not significantly changes neither after 6 h or 12 h.

These results do not correspond with ours expectations about induction of apoptosis in Hep-2 cells. However, apoptosis is a complex process and can be induced by other genes of BCL2 family which were not analyzed.

References about induction of apoptosis in Hep-2 cells with etoposide or about expression of BCL2 and BAX genes are very rare. Some authors report about downregulation of BCL2 protein after induction of apoptosis in Hep-2 cells with venom from lionfish (2) or after induction of apoptosis with carboplatin and 5-fluorouracil (28). The similar results, but in PANC-1 cells, has been reported by Ikeguchi et al. (16). The expression levels of BCL2 increased and BAX expression level did not changes during cisplatin treatment, so overexpressing of BCL2 gene may play an important role in the chemo-resistance of PANC-1 cells.

Induction of apoptosis in HL-60 cells was described in previous work of Rudolf et Červinka (25). Cells were treated with etoposide at 10 µl/ml concentration which induced typical cell blebbing and subsequent cell death. Induction of apoptosis in HL-60 cells after treatment with etoposide was also described by many others authors. Higginbottom et al. (14) induced apoptosis in HL-60 cells after incubation with etoposide at concentrations 1 µl/ml and 10 µl/ml for 6 h and the cleavage of caspase 9 was indicative of apoptotic cell death. Kravtsov et al. (19) treated HL-60 cells with 1, 2.5, 5, 10 and 20 µmol/L concentration of etoposide and the most effective in apoptosis induction was 10 µmol/L. Typical morphological changes such as membrane blebbing were also observed. Eliseev et al. (8) described morphological changes such as membrane blebbing, condensation of chromatin and fragmentation of DNA in HL-60 cells during treatment with 50 µM etoposide. Zuryn et al. (30) induced apoptosis in HL-60 with etoposide at concentrations 20 and 200 µM and Bjorling-Poulsen et Issinger (4) induced apoptosis in HL-60 cells by incubation with 30 µM etoposide for 5 h.

Quantitative real-time RT-PCR analysis showed that expression level of BCL2 gene significantly decreased after 6 h of etoposide treatment in HL-60 cells. Whereas expression level of BAX significantly increased after both 6 h and 12 h of treatment.

These results indicate that etoposide induces apoptosis in HL-60 cells and morphological changes are accompanied by changes in expression of apoptosis-related genes BCL2 and BAX. Similar results as upregulation of BAX gene in HL-60 cells after treatment with etoposide and downregulation of BCL2 gene after treatment with topotecan and methotrexate reported Floros et al. (12, 11). Rózsalski et al. (24) noted downregulation of BCL2 and upregulation of BAX in HL-60 cells after treatment with doxorubicin and amifostine. Floros et al. (13) reported about downregulation of BCL2 in HL-60 cells after cisplatin treatment.

Conclusions

Our findings show that etoposide, the topoisomerase II inhibitor, induce apoptosis in HL-60 cells. The expression level of proapoptotic gene BAX significantly increased after 6 and 12 h during etoposide treatment, whereas expression of antiapoptotic BCL2 decreased. On the contrary in Hep-2 cells the expression of BCL2 significantly increased both 6 h and 12 h after etoposide treatment, whereas expression of BAX did not change. Those findings show the distinct reactions of Hep-2 and HL-60 cells to etoposide treatment and different upregulation or downregulation of apoptosis-related genes BCL2 and BAX.

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