Gene expression during camptothecin-induced apoptosis in human myeloid leukemia cell line ML-2*

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Malignant cell proliferation and accumulation depends on the balance between the rates of cell production and cell death. Recent evidence indicates that apoptosis is important in the development of cancer. Apoptosis is strictly controlled by various regulators, which can take part in the apoptotic process, proliferation and differentiation alike.

Apoptosis was induced in myeloid cell line ML-2 by camptothecin, an inhibitor of topoisomerase I. After 18 hours of induction by camptothecin 50% of cells were apoptotic. The apoptotic effect of CAM was reversible in the cells studied. The induction of apoptosis influenced the expression of apoptosis and cell cycle regulators as detected by cDNA arrays, RT-PCR or Western blotting. According to cDNA arrays e.g. bax, bfl1, bak, pRb2, c-jun, jun-B were upregulated, and cdk4, cyclin B1, wee1, CRAF1, DP1 were downregulated. A number of other regulators like p21 and cdc25A, as well as some other genes linked with apoptosis, as p53 and the bcl-2 family, were up- or down-regulated as determined by real-time PCR. Changes in gene expression were found not only in the group of regulators of apoptosis and the cell cycle, but also among regulators of differentiation.

Key words: apoptosis, camptothecin, ML-2 cell line, p53, wee1, jun family, MAPKK

Apoptosis is a complex, strictly regulated physiological process. Any failure in its control results in an up- or down-regulation of cell death and in dysfunction of the organism. For instance, an upregulated apoptosis in neurons results e.g. in Alzheimer or Parkinson diseases [3]. On the other hand, downregulated or missed apoptosis, e.g. after DNA damage, leads to the uncontrolled cell proliferation and cancer development [3]. Apoptosis can be induced by various extra- and intra-cellular signals, as has been described elsewhere [3, 22]. The executive aparatus of apoptosis after induction signal is caspase-mediated cleavage of specific substrates [21].

Apoptotic process is also involved in cancer therapy and many chemotherapeutic agens act by induction of apoptosis. On the other hand, the inhibition of apoptosis may confer resistance to chemotherapy [8]. For example camptothecin (CAM), which was used in our study, is an inhibitor of topoisomerase I [4]. Topoisomerase I and II inhibitors are anticancer drugs that stabilize a transient intermediate of topoisomerase reactions in which enzymes are linked to the 3'(top1) or 5'(top2) terminus of a DNA duplex producing DNA single- or double-strand breaks. These topoisomerase-linked DNA strand breaks are reversible prelethal lesions that inhibit DNA metabolism such as replication and transcription [17]. Topoisomerase-linked DNA breaks results in G1 or G2 cell cycle arrest in some cells, while rapidly induce apoptosis in other cells (with caspase activation) [18]. Caspase activities drive proteolysis of specific substrates, which leads cells irreversible to apoptosis [2]. Cells deficient in the caspase pathway are protected from apoptosis induced e.g. by anticancer drugs [12]. Blocking caspase activities in myeloid cells provoked a switch from apoptosis to necrosis [9].

The aim of present study was to detect changes in expression of the regulators of apoptosis and processes such as differentiation or proliferation in connection with CAM-mediated apoptosis in the human myeloid cell line ML-2.

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Material and methods

Cells and reagents. Human acute myeloid leukemia (AML) cell line ML2 (Dr. Minowada, Fujisaki Cell Centre, Okayama, Japan). Cells were maintained in RPMI 1640 medium (Sevac, Prague, Czech Republic) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO_2.

Camptothecin (CAM) and ethidium bromide (Sigma, St. Louis, USA). Phenol, chloroform and ethanol (Merck, Darmstadt, Germany). Syber Green I and II (Boehringer, Mannheim, Germany). All the primers used and ultra pure agarose (Gibco BRL, Life Technologies, New York, USA). RNasin, dithiothreitol (DTT), moloney murine leukemia virus reverse transcriptase (M-MLV), random hexamers and dNTP (Promega, Madison, USA). FastStart Taq DNA Polymerase (Roche, New York, USA) and cyclin D1(R-123), p21 (F-5), p53 (Pab 122), Bcl-2 (Bcl-2-100) antibodies (Santa Cruz, USA).

Determination of cell viability and proliferation. ML-2 cells were seeded and allowed to reach exponential growth for 24 hrs. Cells were plated at an initial density of $5x10^5$ cells/ml and CAM (5 μg/ml) was added to induce apoptosis. Following the induction, adherent and non-adherent cells were harvested and counted by the Trypan blue dye-exclusion method (at least 200 cells were counted for each assay). Cell viability was calculated as the percentage of cells that excluded the Trypan blue dye.

Proliferation was estimated by measuring ^{3}H-thymidine incorporation (cells were cultivated in the presence of ^{3}H-thymidine for 4 hrs at the end of the induction period, harvested on a glass filter and the radioactivity incorporated was counted).

Apoptosis assays. The magnitude of the apoptotic cell pool was estimated from the flow cytometric profile of the DNA content. Harvested cells were washed in PBS, fixed in cool 75% ethanol, incubated with propidium iodide and analyzed by flow cytometry (Becton Dickinson). A prominent peak in the sub-G1 area appeared in CAM-induced cells, being negliable in normal cells. Parallel measurement of caspase-3 activity was performed according to the manufacturers protocol (ApoAlert Caspase-3 Fluorescent Assay Kit, Clontech, Palo Alto, USA).

Semiquantitative and quantitative PCR analysis. Harvested cells ($2x10^6$ cells for one isolation) were washed in PBS and total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method [5] as modified by HAŠKOVEC et al [7] and stored at –80 °C. The quality of the RNA was checked by electrophoresis on 2% agarose gels stained with Syber Green II. cDNA (an aliquot of $2x10^6$ cells) was synthesized from the RNA isolated using random hexanucleotides (25 mM) and M-MLV reverse transcriptase (120 U per reaction). Semiquantitative RT-PCR (reverse transcription-polymerase chain reaction) was performed using 100 mmol of each dNTP, 1.5 mM $MgCl_2$, appropriate concentration of primers (Tab. 1) and 0.6 U of AmpliTaq Gold polymerase in total volume 25 μl. The cycling conditions in terms of the number of cycles and the annealing temperatures were optimised for each pair of primers (Tab. 1). β2-microglobulin gene was used as an internal control of the amplification. The PCR conditions were as follows: at 95 °C for 10 min, 25–35 cycles: at 94 °C for 1 min, for 1 minute at suitable annealing temperature (Tab. 1), 1 minute at 72 °C, and at the end of the amplification at 72 °C for 10 min.

The preparation of cDNA for RQ-PCR (quantitative real-time reverse transcription-polymerase chain reaction) was the same as in the semiquantitative RT-PCR analysis. RQ-PCR (real-time quantitative PCR) of β2-microglobulin, p53 and p21 were performed with 100 mM dNTP, 1U FastStart Taq DNA polymerase (Roche, New York, USA), 3.5 mM $MgCl_2$, 0.5 μM of each primer and 0.2 μM FAM probe in total volume 20 μl. There were no non-specific products in optimalized amplification after 45 cycles. Fluorogenic probe
6FAM-CCTCCATGATGCTGCTTACATGTCTCp,
6FAM-CCCCTCCTCAGCATCTTATCC-GAGTGGTXTp and
6FAM-ACGGCGGCAGACCAGCATGAXTp were used for the quantification of β2-microglobulin, p53 and p21, respectively. The thermal cycle parameters for real-time amplifications were: 1 cycle 95 °C for 5 min followed by 40 cycles at 94 °C for 20 sec, 60 °C for 45 sec. The Rotor-Gene apparatus (Corbett Research, Australia) was used for amplifications, measurements and quantifications.

pENTR 1A carrying inserted cDNA of β2-microglobulin was used as a standard for the construction of the calibration curve. The amounts of the genes tested were normalized to the same amount of β2-microglobulin.

cDNA expression arrays. Harvested cells ($5x10^7$ cells for each isolation) were washed and total RNA was isolated as described above. The isolation was followed by treatement with DNAse I in 37 °C for 30 min and total RNA was re-isolated. This pure RNA was quantified by agarose electrophoresis and 2–5 μg of total RNA was used for radioactive probe labelling (^{32}P) and hybridization acording to manufacturer's protocol (Atlas Human Apoptosis cDNA array, Clontech, Palo Alto, USA).

Western blot analysis. Cells ($3x10^7$) were washed and incubated in lysis buffer (0.15 M NaCl, 10% Triton X-100, 100 mM phenylmethylsulphonyl fluoride), centrifuged at 13 000 x g for 30 min and the supernatant was frozen at –75 °C. The amounts of proteins was estimated from a calibration curve for bovine serum albumin by spectrophotometry at 280nm. 160 μg of total protein in each lysate was loaded onto gel. The cell lysate was blotted onto a nitrocellulose membrane (Hybond-ECL, Amersham, Vienna, Austria) after separa-

Table 1. PCR conditions and primers used for comparative RT-PCR

Name	Sequence of primers	Final primer concentration	Reference	Annealing temperature	Numbre of cycles
β2-microglobulin	5'GAGTATGCCTGCCGTGTG 3'	20 μM	*	55 °C	34
	5'AATCCAAATGCGGCATCT 3'	20 μM			
Ki-67	5'GGAGGCAATATTACATAATTTCA 3'	11 μM	*	60 °C	30
	5'CAGGGTCAGAAGAGAAGCTA 3'	8 μM			
cyclin D1 AS	5'GTCACACTTGATCACTCTGG 3'	11 M	23	50 °C	33
cyclin D2 AS	5'CATGGCAAACTTAAAGTCGG 3'	2 μM			
cyclin D3 AS	5'CCAGGAAATCATGTGCAATC 3'	2 μM			
cyclin D1,D2,D3 multiplex sense	5'CTGGCCATGAACTACCTGGA 3'	11 μM			
$p21^{Waf1/Cip1}$ AS	5'CCGTTTTCGACCCTGAGAG 3'	10 μM	20	58 °C	30
	5'CCTCTTCGGCCCGGTGGAC 3'	10 μM			
cdc25A	5'CTCCTCCGAGTCAACAGATT 3'	12 μM	*	60 °C	30
	5'CAGAGTTCTGCCTCTGTGTG 3'	12 μM			
E2F-1	5'CAGGAGGTCACTTCTGA 3'	22 μM	11	52 °C	33
	5'CCCTGTCAGAAATCCAG 3'	22 μM			
mdm-2	5'TGATGAAAGCCTGGCTCTGT 3'	11 μM	*	58 °C	30
	5'CTCTTCAGCTTGTGTTGAGT 3'	13 μM			
bcl-2	5'TGCACCTGACGCCCTTCAC 3'	22 μM	1	66 °C	33
	5'AGACAGCCAGGAGAAATCAAACAG 3'	22 μM			
bax	5'ACCAAGAAGCTGAGCGAGTGTC 3'	22 μM	1	68 °C	33
	5'ACAAAGATGGTCACGGTCTGCC 3'	22 μM			
p53	5'AAGTCTGTGACTTGCACG 3'	80 μM	10	50 °C	34
	5'CTGGAGTCTTCCAGTGTG 3'	80 μM			
p73	5'CGGGACGGACGCCGATG 3'	22 μM	6	62 °C	33
	5'GAAGGTCGAAGTAGGTGCTGTCTGG 3'	22 μM			

*primers were designed in our laboratory by the Primer3 Input software.

tion by SDS-PAGE [14]. The blots were blocked in non-fat milk for 2 hrs and incubated with primary antibody overnight in 4 °C. The blots were then incubated with appropriate secondary antibody labelled with horse-radish peroxidase and proteins were detected by enhanced chemiluminescence according to the manufacturers protocol (ECL Western Blotting System, Amersham, Vienna, Austria).

Table 2. Distribution of cell cycle phases determined by DNA content analysis using flow cytometry

	G1 phase (% of the cells)	S phase (% of the cells)	G2/M phase (% of the cells)
Non-induced control cells	32%	53%	15%
CAM-induced cells	70%	18%	12%

Non-induced control cells and cells induced with CAM were measured for their DNA content 18 hours after incubation.

Results

Induction of apoptosis in the studied myeloid cell line ML-2 was accompanied by cell cycle arrest, which was indicated by a decrease in DNA synthesis within 24 hrs (measured by ^{3}H thymidine incorporation – data not shown), retardation of cell growth and a diminished amount of cells in the S phase as compared with G1 phase (G1 arrest) (Tab. 2). This decrease in proliferation correlated with a decreased expression of proliferating antigen Ki-67 as measured by RT-PCR (data not shown).

According to DNA content analysis, highest levels of apoptosis were detected after 24 hours (100% of apoptotic cells). Maximum caspase-3 activity was detected after 8 hours of the induction and then it was decreasing (Fig. 1). There were no marks of RNA degradation on the agarose electrophoresis. The greatest changes were found in the expression of about 20 genes, linked with apoptosis or cell proliferation, using cDNA expression arrays and relevant software (Atlas Human Apoptosis cDNA Array listing 200 genes and Atlas Image 1.0 software) (Tab. 3). Among these genes are the apoptotic regulators of bcl-2 family such as anti-apoptotic Bfl-1 or pro-apoptotic bax and bak which were upregulated. CRAF-1 and CD27BP are membrane receptors of TNF super-family, whose activation leads to growth activation and survival. Their expression was down-regulated. A regulator of the cell cycle like E2F dimerisation partner (DP1) was down-regulated, whereas the expression of e.g. Rb-like protein 2 (130-kDa) was up-regulated. The expression of the mitogen-activated kinase

Table 3. Expression profile of CAM-induced ML-2 cell line determined by Atlas Human Apoptosis cDNA Array

Protein/gene	Adj. intensity		Difference
	control cells	CAM-induced cells	
Downregulated			
(CDK4); PSK-J3	41684	28667	–13017
serine/threonine-protein kinase PCTAIRE 1 (PCTK1)	38282	24406	–13876
G2/mitotic-specific cyclin B1 (CCNB1)	19216	4528	–14688
wee1Hu CDK tyrosine 15-kinase	26344	6400	–19944
NEDD5 protein homolog; DIFF6; KIAA0158	22276	8539	–13737
MAPKK 1; ERK activator kinase 1	39504	13848	–25656
CD40 receptor-associated factor 1 (CRAF1)	17482	5232	–12250
CD27BP (Siva)	25534	14070	–11464
E2F dimerization partner 1; (DP1)	38700	19768	–18932
GRB3-3;	42786	32417	–10369
MCL-1	36974	25985	–10989
brain-specific tubulin alpha 1 subunit (TUBA1)	43394	14574	–28820
(HLAC)	31660	18524	–13136
40S ribosomal protein S9	24362	12919	–11443
Upregulated			
apoptosis regulator bax	4144	20672	16528
retinoblastoma-like protein 2 (RBL2; RB2); 130-kDa	6930	17431	10501
BCL-2-related protein A1 (BCL2A1); BFL1 protein;	4392	23162	18770
bcl2 homologous antagonist/killer (BAK)	3164	20978	17814
IL-1 beta converting enzyme (ICE); p45; caspase-1	7766	22980	15214
caspase-4 precursor (CASP4); ICH-2 protease; TX protease;	20744	35738	14994
c-jun proto-oncogene; transcription factor AP-1	3066	20732	17666
jun-B	1	12973	12973
(GADD153);	28030	38747	10717
(GADD45);	20794	34704	13910
p58/HHR23B	11338	24698	13360

Software estimation of Adjustened Intensity (the intensity of autoradiographic spots of control cells and cells incubated with CAM for 18 hrs) was normalized to the background and nine house-keeping genes. Difference = cells + CAM – control cells. Values ‹0 marks downregulation and values ›0 marks upregulation of the gene expression. Gene and protein nomenclature in this table is noted according to Clontech database.

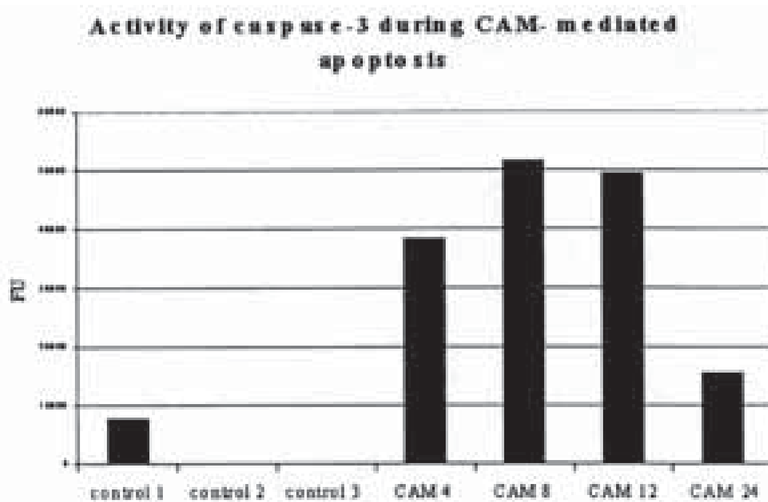

Figure 1. Detection of caspase-3 activity in ML-2 cells after induction by CAM (4–24 hours as indicated in the figure). FU – fluorescence units, μM of substrate cleaved by cas-3 in 1 hour. Negative controls: control 1 – non-induced control cells, after 12 hours of cultivation; control 2 – ML-2 cells incubated with CAM for 12 hours, no addition of cas-3 substrate; control 3 – ML-2 cells incubated with CAM for 12 hours, addition of cas-3 inhibitor (DEVD-CHO).

MAPKK1 was down-regulated while that of the genes of an early response, as c-jun and jun-B, were up-regulated. GADD45 is involved in DNA repairs and its expression was upregulated after the induction of apoptosis as well as the expression of caspases precursors (cas-1 and cas-4). The expression of several house-keeping genes like brain-specific tubulin alpha 1 subunit (TUBA 1), HLAC or 40S ribosomal protein S9 was also influenced (Tab. 3).

The results of cDNA arrays were normalized to nine house-keeping genes (whose expression was not changed). Only those genes whose expression were changed by a difference greater than 10000 AU are included in Table 3. We did not detect any remarkable changes in several genes, as p21 or p53, which are supposed to be important in apoptosis. Therefore they were studied individually by RQ-PCR. The changes in gene expression de-

Figure 2. Western blot analysis of p53 expression in ML-2 cell line. Lane 1 – positive control cell line RAJI. Lane 2–5 – ML-2 cells incubated with camptothecin for 4, 8, 12 and 24 hours, respectively. Lane 6 – non-induced ML-2 control cells.

Figure 3. Western blot analysis of p21 and bcl-2 in ML-2 cell line. Lane 1 – non-induced control cells. Lane 2 – cells incubated with camptothecin for 18 hours. Lane 3 – RAJI cell line used as a positive control of bcl-2 expression.

Table 4. Gene expression during CAM-mediated apoptosis (18 hours after induction) detected by comparative RT-PCR or real-time RT- PCR

Gene	Expression
cyclin D1, D2, D3	=
p21	+ +
p53	=
p73	–
mdm-2	=
E2F-1	– –
bcl-2	– – –
bax	+ + +
cdc25A	– –
Ki-67	– – –

The semiquantitative estimation of upregulation is indicated with + and downregulation with –; = indicates no considerable changes in the gene expression.

tected by PCR only (in p21, p73, E2F-1, bcl-2 and cdc25A) were due to a higher sensitivity of the PCR method (to compare with the cDNA arrays). Where only slight differences (<10.000 AU) were detected by cDNA arrays, these genes were not included in the Table 3.

Good correlation was found between the peak of caspase-3 activity (8 hours of the induction) and maximum expression of p53 protein (4–8 hours after induction). The increased level of p53 was not stable, it decreased in 12–24 hrs (Fig. 2). In contrast to the protein level, the mRNA level of p53 remained unchanged (during the whole studied period, data show only those after 18 hours). (Tab. 4). As shown in Table 4 CAM-mediated apoptosis did not influenced the expression of D cyclins at both the mRNA level and the protein level (data not shown). Another member of cyclin family – cyclin B1 was downregulated and so was kinase wee1, which inhibits this cyclin/cdk complex. Cdk4, its relative PCTAIRE 1 and activating transduction kinase MAPKK1 were also downregulated by CAM as shown in Table 3. The expression of Cdc25A was down-regulated during both apoptosis and differentiation. No changes of mdm-2 were detected at the mRNA level. The mRNA expression of p73, E2F1, Ki-67 and cdc25A was downregulated (Tab. 4). Bcl-2 mRNA was decreased (Tab. 4) in contrast to bcl-2 protein whose expression remained unchanged (Fig. 3). An increase of p21 mRNA correlated well with that of p21 protein (Tab. 4, Fig. 3). In contrast to bcl-2, bax mRNA was strongly up-regulated (Tab. 4). The results presented show how many genes are involved both in the apoptotic and differentiation processes.

Discussion

The present study of mRNA expression was focused on apoptosis and members of apoptotic, proliferation and differentiation pathways which could be involved in CAM-mediated cell death. Some of the regulators of apoptosis, differentiation and proliferation are involved in more than one of these processes, i.e. transcription factor E2F-1 plays a dual role, both in apoptosis and proliferation [13, 15]. In our study, a decrease of its expression was detected by RT-PCR and we also found a lowered expression of its dimerisation partner, DP-1. Active E2F-1 has to dimerise with DP-1 to acquire its regulatory function.

It is not yet clear whether the binding of p73 to mdm-2 targets p73 to its degradation, similarly as in the case of p53. Decrease of p73 and E2F-1 mRNA expression correlated with cell cycle arrest and apoptosis in our experiments. It seems that the changes in mRNA expression of p53, p73, E2F-1 and mdm-2 were not necessary events in this apoptotic pathway, because the expression of some genes is regulated at the transcriptional (mRNA) level, of others at protein level, i.e. by regulation of translation or degradation of the proteins. For example, p53 was not changed at mRNA level during the apoptotic process, but only at the protein level. Therefore, some results obtained at mRNA level require checking at the protein one.

The increased expression of p53 protein was evidence of its involvement in CAM-mediated apoptosis. Nevertheless its expression was not stable and after an initial increase it came down. This may have been due to a reversible effect of CAM [19]. The p53 protein could also regulate the mRNA levels of important regulators of apoptosis, such as bcl-2 family members. The increase in bax and bak mRNA levels correlated well with a decrease of bcl-2 mRNA level and with the induction of apoptosis. The decreasing Bcl-2 mRNA level contrasted with the unchanged expression of its protein. An upregulation of Bfl-1 could have been caused by the molecular pattern of this cell line. It is known that p53

can also activate p21 expression [16]. While p21 upregulation correlated well with the upregulation of cyclin D1 during differentiation in ML-2 cells [24], there was no such correlation during apoptosis. The down-regulation of Cdc25A during both apoptosis and differentiation could be a linking point, as in the case of E2F-1.

Our results showed a complexity of apoptosis and its connection with other cell processes such as proliferation.

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