The assessment of caspase activation during apoptosis of malignant cells in B-cell chronic lymphocytic leukaemia

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Abstract

In this study we analysed caspase activation in peripheral blood and bone marrow cells of B-cell chronic lymphocytic leukaemia (B-CLL) exposed to apoptosis both spontaneous and induced via external and internal pathway. We used the method based on detection of activated caspases with FAM-VAD-FMK that is the fluorochrome labelled inhibitor of caspases and flow cytometry technique. We detected the higher rate of apoptosis induced by mitochondrial way in comparison to receptor one that may suggest dominance of this mechanism in B-CLL. We also observed the higher rate of spontaneous and mitochondrial-induced apoptosis in peripheral blood in comparison to bone marrow indicating the differences in apoptosis process depending on cell environment.

Key words: *B-CLL, apoptosis, caspases.*

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Introduction

B-cell chronic lymphocytic leukaemia (B-CLL) is characterised by the accumulation of malignant CD5+ B cells, in which. dysregulation of apoptosis rather than increased proliferation rate seems to play the crucial role [1, 2]. During apoptosis the activation of cysteine-aspartic acid-specific proteases (caspases) is the critical event, initiating the irreversible steps of cell demise. Even though there are some reports concerning caspase activation in B-CLL apoptosis the importance of this process in spontaneous and drug-induced cell death of malignant cells should be elucidated yet. The differentiation of the main ways of caspase activation – external (via death receptors) and internal (mitochondrial) seems to be of great importance.

In this study we investigated the activation of caspases in B-CLL cells exposed to apoptosis both spontaneous and induced via external and internal pathway. The experiments were done in peripheral blood (PB) and bone marrow (BM) samples obtained from newly diagnosed, previously untreated B-CLL patients. We used the method based on detection of activated caspase with use of FAM-VAD-FMK that is the fluorochrome labelled inhibitors of caspases and is considered as a pan-caspase marker [3, 4].

Material and methods

Cell separation

PB and BM mononuclear cells obtained from 5 B-CLL patients were isolated by density gradient centrifugation (Lymphoprep, Nycomed Pharma, Norway) and resuspended at a concentration of $2x10^6$ cells/ml in culture medium consisted of RPMI 1640 with 1% 2mM L-glutamine, 1% antibiotics (penicillin and streptomycin), 10% heat-inactivated fetal calf serum (FCS). All reagents used were purchased from Sigma, Germany. The cultures were maintained at 37˚C in an atmosphere of 5% CO_2 in air.

Induction of apoptosis

The B-CLL cells were cultured without apoptosis inductors to assess the spontaneous apoptosis. To induce apoptosis the cells were treated either with 0.15 μM of DNA topoisomerase I inhibitor camptothecin (CPT) or with

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a combination of 0.3 nM tumour necrosis factor-α (TNF-α) and 5 μM cycloheximide (CHX), all from Sigma, Germany. Cells from all samples were harvested after 24 and 48 h of incubation and submitted to further procedures.

Cell staining

The fluorochrome labelled caspase inhibitor FAM-VAD-FMK that is considered as a pan-caspase marker was obtained as a component of CaspaTag Caspase Activity Kit (Intergen Company, USA). Before cell staining FAM-VAD-FMK was dissolved in dimethyl sulfoxide (DMSO, Sigma) to obtain 150 x concentrated (stock) solution. The aliquots of the stock solution were stored at -20°C in the dark. Before using the stock solution was diluted 1:5 in PBS to obtain 30 x working solution. The B-CLL cells in concentration of 10^6/ml were added to 10 μl of 30x working dilution of FAM-VAD-FMK and then incubated for 1 hour at 37°C under 5% CO_2. After incubation time the cells were washed twice with 1x working Dilution Wash Buffer according to manufacture's instruction. 2 μl of propidium iodide (PI) solution was added to cell suspension prior to flow cytometry acquisition.

Flow cytometry analysis

The bicolor flow cytometry technique by FACSCalibur (Becton Dickinson, USA) instrument and Cell Quest Software for data analysis were used. The green fluorescence on FL1 channel and red fluorescence (PI) on the FL3 channel were measured. Then a log FL1 (X-axis) versus log FL3 (Y-axis) dot plot was generated and the analysis in the quadrant cursors was performed.

Statistical analysis

The statistical analysis was done with use of Wilcoxon test (STATISTICA 6.0).

Results

We analysed the samples both of PB and BM in which the mean percentage of CD19+/CD5+ cells was comparable (75.07% and 73.60%, respectively).

Table 1. The mean percentage of apoptotic FAM-VAD-FMK+/PI-cells of peripheral blood and bone marrow in B-CLL patients

	Time of cell culture [hours]	Peripheral blood	Bone marrow
		VAD+PI-(%)	VAD+PI-(%)
Spontaneous apoptosis	0	1.83	1.87
	24	10.58	6.87*
	48	13.3	8.46*
Apoptosis induced by TNF-α + CHX	0	1.83	1.87
	24	9.91	8.71
	48	10.87	10.57
Apoptosis induced by CPT	0	1.83	1.87
	24	8.79	5.86*
	48	14.88**	12.27*, **

** statistically significant lower in comparison to peripheral blond (p<0.05);*
*** statistically significant higher in comparison to apoptosis induced by TNF-α + CHX (p<0.05)*

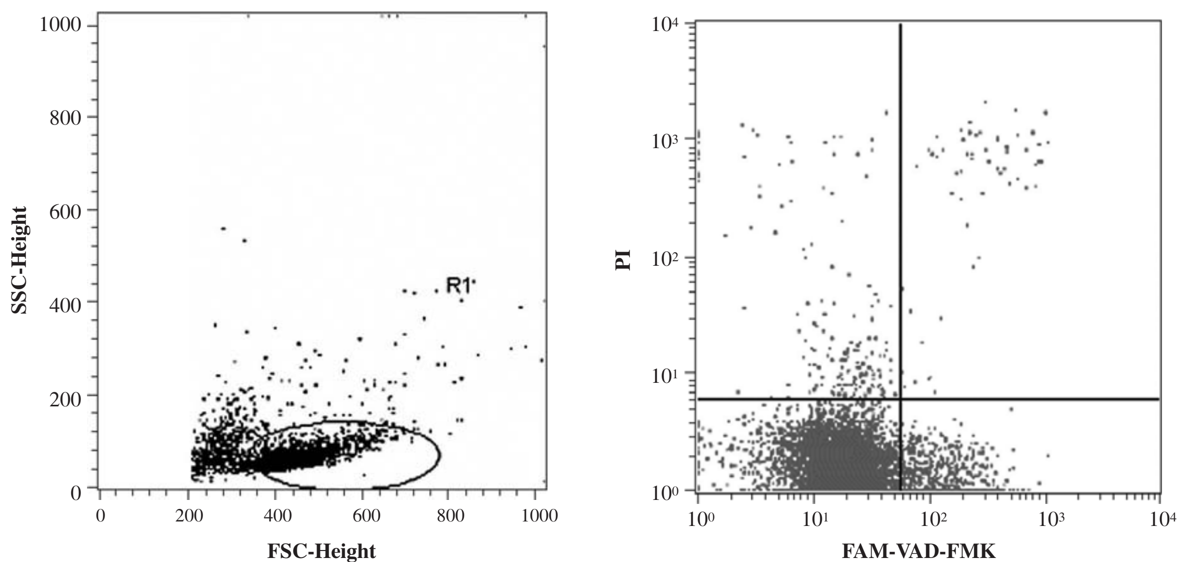

Fig. 1. The flow cytometry presentation of the distribution of peripheral blood cells staining by FAM-VAD-FMK and PI. The cells are gated according to forward scatter versus side scatter dot plot (R1 gate)

All samples tested in the study revealed the presence of four distinct cell subpopulations, which differed in binding of VAD and PI (figure 1). Non-apoptotic cells showed neither VAD nor PI fluorescence (FLICA-PI-). The cells in early phase of apoptosis were VAD+PI-. VAD+PI+ and VAD-PI+ cells represented two consecutive phases of the "necrotic stage" of apoptosis. In further analysis we concerned the group of VAD+/PI- points as the best indicator of apoptotic cells. The results presenting as mean values are shown in table 1.

The spontaneous apoptosis was detected in both PB and BM samples in time-dependent analysis. There was the significantly higher percentage of VAD+/PI- cells in PB than BM samples both at 24 and 48 h. We detected an increase of apoptotic cells number in PB and BM after induction of apoptosis by both TNF-α and CPT. In PB and BM culture with CPT we observed significantly higher number of VAD+/PI- cells at 48 h in comparison to TNF-α-culture. The percentage of apoptotic cells in PB culture with CPT was higher than in BM, while there was no difference between PB and BM in TNF-α-culture.

Discussion

The obtained results indicate the time-dependent caspase activation during spontaneous and induced apoptosis of B-CLL cell. Comparing the receptor and mitochondrial way of apoptosis induction we can presume that although both ways cause caspase activation, the level of caspase activation is higher after mitochondrial induction. The rate of mitochondrial-induced apoptosis in PB cells seems to be higher than in BM. The differences between the percentage of CD19+/CD5+ of PB and BM were not statistically significant, thus differences of apoptosis rate between these two cell environments seem not to be connected with percentage of malignant cells.

There are some reports concerning caspase activation during spontaneous or induced apoptosis of B-CLL PB cells based mainly on Western blot analysis. The activation of caspase-9 involving in mitochondrial way of apotosis followed by actvation of effector caspases-3 and -7, as well as caspases-8 activation involving in receptor way in B-CLL cells was reported by Almond et al. [5]. Perez-Galan et al. [6] detected caspase-3,-7-,8, and -9 activation induced by cladribine. King et al. [7] reported activation of caspase-3 and -7 and the processing of caspase-8 in spontaneous and drug-induced B-CLL apoptosis. Jones et al. [8] detected procaspase-8 processing induced by chlorambucil, fludarabine or γ radiation. High level of caspase-8 in B-CLL in conjunction with resistance to Fas/FasL signaling system is interesting and require elucidation [9]. On the contrary MacFarlane et al. [10] reported resistance of B-CLL cells to TRAIL induced-apotosis connected with little or no caspase-8 processing.

Our results, similarly to those presented above, indicated on involvement of caspases in apoptosis process of B-CLL cells obtained from PB. Additionally, for the first time the analysis of B-CLL cells obtained from BM was presented. We detected the higher rate of caspase activation during spontaneous and mitochondrial induced apoptosis in PB than in BM, that may indicate the differences in apoptosis process depending on cell environment. In view of controversial results reported by other authors, the differentiation between receptor and mitochondrial way of apoptosis seems to be difficult. We detected caspase activation both in receptor and mitochondrial induction of apoptosis, but with higher rate in mitochondrial one, that may suggest dominance of this mechanism in B-CLL, however, activation of caspases connected with receptor way cannot be excluded.

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