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The assessment of spontaneous apoptosis of peripheral blood and bone marrow malignant cells in B-cell chronic lympocytic leukemia

B-cell chronic lymphocytic leukemia (B-CLL) is the most frequent kind of adult leukemia in western countries. It is characterized by the expansion of monoclonal neoplastic CD5+ B cells, which appear to be non-dividing and arrested in G0 phase of cell cycle (3, 5). These malignant cells accumulate in the blood, bone marrow and lymphoid organs. In this process the dysregulation of programmed cell death rather than increased proliferation rate seems to play the crucial role (7). Apoptosis, being a mode of cell death is a physiological process playing an important role both during embryonic development and in mature organism. A sequence of changes occurs in cells during this process like lowering of mitochondrial membrane potential, loss of plasma membrane asymmetry, activation of caspase cascade and DNA cleavage, initiating the irreversible steps of cell demise. Many proteins that act as inducers or inhibitors can control apoptosis. The high level of antiapoptotic Bcl-2 protein is detected in B cells of B-CLL. Interestingly, it was reported that long-living malignant B lymphocytes of B-CLL die rapidly cultured *in vitro* (4). Thus some external factors, e.g. cytokines may suppress apoptosis of these cells (8,11). In this study we investigated the apoptosis of B-CLL cells cultured in vitro. The experiments were done in peripheral blood (PB) and bone marrow (BM) samples obtained from 5 B-CLL newly diagnosed, previously untreated patients. We used recently described method based on detection of activated caspase with the use of FAM-VAD-FMK that is the fluorochrome labelled inhibitors of caspases and is considered as a pan-caspase marker (1, 10).

MATERIAL AND METHODS

Patients. The patients were selected at the Hematology Department, Medical University of Lublin. The diagnosis of B-CLL was set up according to defined clinical, morphological and immunological criteria (2). At the time of the study all patients were untreated. Venous blood and bone marrow samples were collected after informed consent and tested immediately. All procedures were approved by the Ethical Committee of the Medical University of Lublin.

Cell separation and culture. PB and BM mononuclear cells were isolated by density gradient centrifugation (Lymphoprep, Nycomed Pharma, Norway), then washed twice with phosphate buffer saline (PBS) and finally resuspended at a concentration of $2x10^6$ cells/ml in culture medium consisting of RPMI 1640 (Gibco Laboratories, USA) with 1% 2 mM 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 the atmosphere of 5% CO_2 in the air.

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. Prior to use the stock solution was diluted 1:5 in PBS to obtain 30 x working solution. The B-CLL cells in the concentration of 10^6/ml were added to 10 ml of 30 x working dilution of FAM-VAD-FMK, cells were then incubated for 1 hour at 37°C under

5% CO_2 protecting the tubes from the light. Following incubation time the cells were washed twice with 1 x working Dilution Wash Buffer according to manufacture's instruction. For bicolor analysis 2 ml 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.

RESULTS

During samples analysis we detected the presence of four distinct cell subpopulations according to differences in binding of VAD and PI. Non-apoptotic cells showed neither VAD nor PI fluorescence (VAD-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.

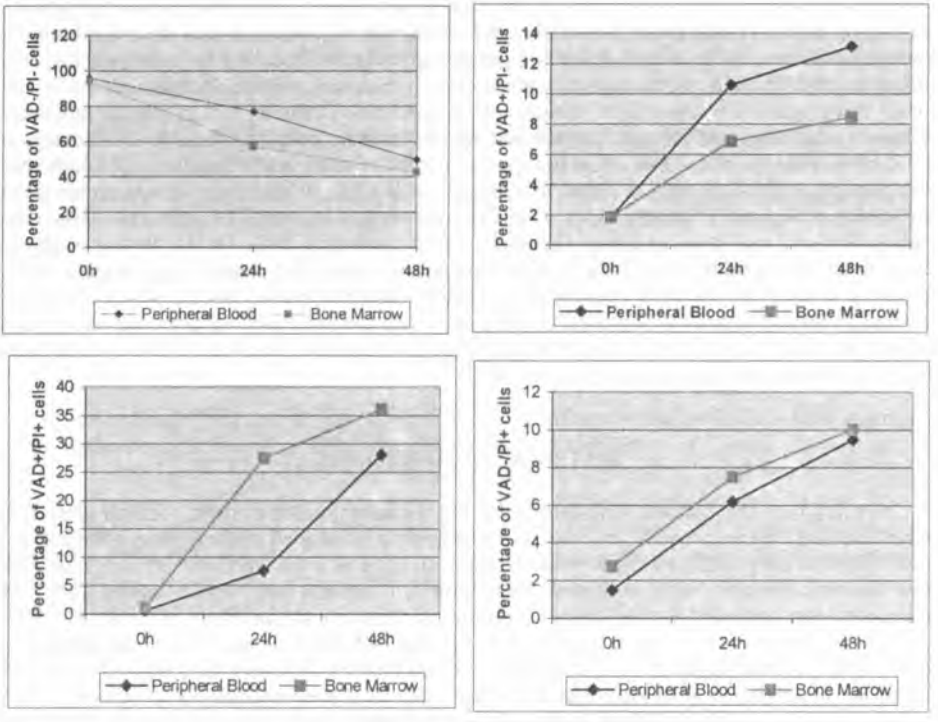

Fig. 1. The time-dependent changes in percentage of VAD-/PI-, VAD+/PI-, VAD+/PI+, VAD-/PI+ cells of peripheral blood and bone marrow in B-cell chronic lymphocytic leukemia

The spontaneous apoptosis was detected in both PB and BM samples. The mean percentage of living PB cells was lower at 24 and 48 h of cell culture in comparison to 0 h culture. Similarly the significant decrease in percentage of living cells in time-dependent analysis was observed in BM samples, however the percentage of living cells in BM was significantly lower in comparison to PB both at 24 and 48 h.We detected an increase in the percentage of VAD+ cells both with and without PI internalization in PB and BM samples. Interestingly, there was the higher percentage of early apoptotic VAD+/PI- cells in PB than BM, while the percentage of late apoptotic cells

(VAD+/PI+ and VAD-/PI+) was lower in PB than BM both at 24 and 48 h. The results presenting as mean standard deviation values are shown in Table 1 and in Figure 1.

Tab.1. The mean percentage ± standard deviation of living (VAD-/PI-), early apoptotic (VAD+/PI-) late apoptotic (VAD+/PI+) and necrotic (VAD-/PI+) cells of peripheral blood and bone marrow in 24 and 48 h cell culture in B-CLL patients

Cell culture	Peripheral blood				Bone marrow			
	VAD-PI-	VAD+PI-	VAD+PI+	VAD-PI+	VAD-PI-	VAD+PI-	VAD+PI+	VAD-PI+
0 h	95.84±0.47	1.83±0.75	0.63±0.20	1.48±0.71	94.18±0.29	1.87±0.93	1.18±0.44	2.75±0.31
24 h	76.90±11.01	10.58±8.79	7.56±2.59	6.19±1.54	57.02±17.87	6.87±4.79	27.42±18.07	7.51±5.69
48 h	49.72±24.25	13.13±7.06	28.01±21.64	9.47±9.02	42.57±21.16	8.46±5.25	36.10±20.11	10.02±6.74

DISCUSSION AND CONCLUSIONS

The obtained results show the time-dependent activation of apoptosis during culture of B-CLL cells, indicating that these cells have the shorter life-span *in vitro* in comparison to prolonged life-span *in vivo*. Collins et al. (4) for the first time reported that B-CLL cells derived from peripheral blood died spontaneously by apoptosis when cultured *in vitro*. By 30 h of culture approximately 20% of the leukemic cells were affected. There was no significant difference in the incidence of apoptosis in T-cell depleted and undepleted cultures or when either autologous or normal human serums were used. Cell density was unlikely to be of importance in producing apoptosis, either.

There are some reports regarding the mechanism of spontaneous apoptosis of B-CLL cells. Sanz et al. (9) showed the important role of members of Bcl-2 family in regulation of this process. mRNA expression for Bcl-2, Bcl-w, Bfl-l and Mcl-1 significantly decreased at the onset of B-CLL spontaneous apoptosis, suggesting that all these anti-apoptotic proteins play a role in maintaining B-CLL cell survival *in vivo*. mRNA for pro-apoptotic Bak and Bax were also reduced as cells become apoptotic. However the Bcl-2/Bax, which correlates with cell survival, was significantly reduced as cells undergo spontaneous apoptosis *in vitro*.

In our study we used the method based on detection of activated caspases. Thus our results may further explain the mechanism of B-CLL cells spontaneous apoptosis indicating this process as caspase-dependent. Such a caspase activation in spontaneous apoptosis of leucemic cells was also reported by King et al. (6). They showed activation of caspase-3 and -7 and the processing of caspase-8 both in spontaneous and drug-induced B-CLL apoptosis.

According to our knowledge it was for the first time that the analysis of the process of spontaneous apoptosis in B-CLL cells derived from bone marrow was done. It seems that the rate of apoptosis is higher in BM than in PB, because of the higher decrease of living cells in BM in comparison to PB. However, the higher percentage of late apoptotic cells in BM than in PB with lower percentage of early apoptotic cells may indicate that apoptosis process undergoes more rapidly in BM than in PB.

In summary, our study shows that *in vitro* spontaneous apoptosis of B-CLL occurs both in PB and BM cells, however it is more rapid in BM. It seems that caspase activation play the important role in spontaneous apoptosis of B-CLL cells, however further investigation should elucidate their role in this process more precisely.

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SUMMARY

The aim of this study was the assessment of apoptosis of B-CLL cells cultured *in vitro*. The experiments were done in peripheral blood and bone marrow samples obtained from newly diagnosed, previously untreated B-CLL patients. We used the method based on detection of activated caspases and flow cytometry technique. The obtained results indicate that time-dependent spontaneous apoptosis and caspase activation occur in both peripheral blood and bone marrow samples. It seems that the apoptosis process undergoes more rapidly in bone marrow than in peripheral blood, because of the higher decrease of living cells in bone marrow in comparison to peripheral blood.

Ocena procesu spontanicznej apoptozy komórek białaczkowych krwi obwodowej i szpiku w przewlekłej białaczce limfocytowej B-komórkowej

Celem pracy była ocena procesu spontanicznej apoptozy komórek przewlekłej białaczki limfocytowej B-komórkowej w warunkach hodowli *in vitro*. Analizie poddano komórki wyizolowane z krwi obwodowej i szpiku chorych na przewlekłą białaczkę limfocytową. Zastosowano metodę opartą na ocenie aktywowanych kaspaz oraz technikę cytometrii przepływowej. Uzyskane wyniki wskazują na postępującą w czasie i wyrażającą się aktywacją kaspaz indukcję procesu apoptozy, zarówno w komórkach krwi, jak i szpiku. Z uwagi na szybsze obniżania się odsetka żywych komórek oraz szybszy wzrost odsetka komórek późnoapoptotycznych w szpiku w porównaniu z krwią można sądzić, że proces apotozy komórek białaczkowych szpiku postępuje szybciej.