Analysis of *ex vivo* Apoptosis of B and T cells from Peripheral Blood and Bone Marrow of Patients with Chronic Lymphocytic Leukemia

Analiza apoptozy *ex vivo* komórek B i T z krwi obwodowej i szpiku kostnego chorych na przewlekłą białaczkę limfocytową

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SUMMARY

In this study we analyzed selected parameters of apoptosis in leukemic cells from peripheral blood and bone marrow of patients with chronic lymphocytic leukemia (CLL). The percentage of apoptotic leukemic B cells ($\Delta\Psi m^{low}/CD19^{+}$) was significantly lower in peripheral blood (median: 0.99%) than in bone marrow (median: 1.41%) ($p<0.0001$). These data can support the idea that these two compartments might have different proliferative statuses. There was an inverse correlation between the *ex vivo* percentage of apoptotic cells and lymphocytosis ($R=-0.34$; $p<0.01$), and a direct correlation between the percentage of PB apoptotic B cells and the Rai stage ($R=0.42$; $p<0.05$). The percentage of $\Delta\Psi m^{low}/CD19^{+}$ cells was significantly higher in ZAP-70-positive patients than from ZAP-70-negative patients ($p<0.01$). There was also a significant difference in the median percentage of apoptotic leukemic B cells between the patients carrying del(11q22.3) or/and del(17p13.1) (2.7%) and the patients without these unfavorable genetic aberrations (1.6%) ($p<0.01$). The B cells from ZAP-70-positive patients with more aggressive disease seem to be more susceptible to spontaneous apoptosis than those from ZAP-70-negative patients. We conclude that the evaluation of ex vivo apoptosis might provide new important information concerning the biology and prognosis of CLL.

Key words: CLL, *ex vivo* apoptosis, CMXRos

STRESZCZENIE

W badaniach przeprowadzono analizę wybranych parametrów apoptozy komórek białaczkowych krwi obwodowej i szpiku chorych na przewlekłą białaczkę limfocytową (PBL). Odsetek białaczkowych limfocytów B ulegających spontanicznej apoptozie *ex vivo* ($\Delta\Psi m^{low}/CD19^{+}$) był większy w szpiku (mediana: 1.41%) w porównaniu z krwią obwodową (mediana: 0.99%) ($p<0.0001$), co może sugerować związek większej apoptozy spontanicznej ze zwiększoną proliferacją komórek białaczkowych w szpiku. Stwierdzono ponadto ujemną korelację pomiędzy odsetkiem komórek apoptotycznych *ex vivo* a limfocytozą chorych na PBL ($R=-0.34$; $p<0.01$) oraz dodatnią korelację pomiędzy odsetkiem komórek apoptotycznych a stadium zaawansowania klinicznego choroby (wg Rai'a) ($R=0.42$; $p<0.05$). Białaczkowe limfocyty B z dodatnią ekspresją ZAP-70 wydają się być bardziej podatne na apoptozę niż limfocyty B pacjentów ZAP-70-negatywnych ($p<0.01$). Na uwagę zasługuje również istotnie wyższy odsetek komórek $\Delta\Psi m^{low}/CD19^{+}$ u pacjentów z del(11q22.3) i/lub del(17p13.1) (2.7%) w porównaniu z chorymi bez tych niekorzystnych zmian cytogenetycznych (1.6%) ($p<0.01$). Obserwacje te sugerują, że ocena apoptozy *ex vivo* może dostarczyć nowych ważnych informacji dotyczących biologii i rokowania PBL

Słowa kluczowe: CLL, apoptoza *ex vivo*, CMXRos

Introduction

The traditional view has been that chronic lymphocytic leukemia (CLL) is a disease deriving from an inherent defect in apoptosis pathways, in which slowly proliferating B lymphocytes accumulate due to this diminished cell death [1]. However, the latest literature data suggest that apart from the long-living peripheral blood cells with deregulated apoptosis pathways, the population of CLL cells may also contain proliferating cells originated in the bone marrow, lymph nodes or spleen [1–3]. Recent studies suggest that CLL is a disease of both proliferation and accumulation [1, 2]. Furthermore, it is known that patients with CLL can have variable degree of T cells dysfunction. Their absolute number have been reported to be elevated, however, the cause for the expansion of various T-cell populations in CLL remains unclear. The increase in T-cell numbers is mainly due to an increased number of $CD8^+$ T cells resulting in an abnormal $CD4^+/CD8^+$ ratio. Moreover, increasing $CD8^+$ numbers are typically paralleled by progression of the disease [4].

We investigated a possible relationship between apoptosis of leukemic B and T cells and clinical and laboratory parameters. We tried to determine whether the percentage of apoptotic cells differs between patients with better prognostic factors and patients with a worse prognosis. Exploring the process of apoptosis is of great importance both for understanding this phenomenon and for improving treatment options.

Materials and methods

Patients and samples

Peripheral blood (PB) and bone marrow (BM) specimens were obtained from 150 newly diagnosed, previously untreated CLL patients (82 men and 68 women). The median age was 64 years (ranging from 32 to 87 years). CLL diagnosis was made on the basis of a clinical examination, morphological and immunological criteria [5]. At the time of diagnosis patients were staged according to the Rai staging system [6] as follows: stage 0 (52 cases), stage 1 (32 cases), stage 2 (34 cases), stage 3 (17 cases) and stage 4 (15 cases). The study was approved by the Local Ethical Committee.

Cell Isolation

All PB and BM samples were collected into heparinized tubes and immediately processed. Mononuclear cells were separated by density gradient centrifugation on Biocol (BIOCHROM) for 25 min at 400×g at room temperature. Interphase cells were removed, washed twice and resuspended in phosphate-buffered saline (PBS).

Determination of apoptosis by Mito Tracker Red CMXRos

The level of apoptosis was measured by chloromethyl-X-rosamine staining (Mito Tracker Red CMXRos; Molecular Probes). CMXRos is a cationic lipophilic fluorochrome that does not accumulate in depolarized mitochondria and can be used to detect disruptions in mitochondrial membrane potential ($\Delta\Psi m$). CMXRos was used in combination with the monoclonal anti-glycophorin A/FITC-conjugated antibody (DAKO) to label glycophorin A on the cell surface of erythrocytes. It was proved to be useful in erythrocyte-contaminated samples with erythrocytes displaying a low $\Delta\Psi m$, similar to apoptotic cells. CMXRos was also used in combination with the monoclonal anti-CD19, anti-CD3, anti-CD4 or anti-CD8/ FITC conjugated antibodies (BD Pharmingen). Cells were incubated with CMXRos for 30 min at 37°C and after 15 min of incubation, anti-glycophorin A, anti-CD19, anti CD3, anti-CD4 or anti-CD8 MoAbs were added. $CD19^+$, $CD3^+$, $CD4^+$ or $CD8^+$ cells considered to be apoptotic displayed a decreased in mitochondrial membrane potential in CMXRos staining ($\Delta\Psi m^{low}$).

Detection of ZAP-70 and CD38 expression

Flow cytometry analysis of ZAP-70 and CD38 expression was performed on fresh PB samples, as described previously [7]. CLL cells were considered ZAP-70-positive or CD38-positive when ≥20% of them expressed the respective antigen.

I-FISH analysis

Peripheral blood mononuclear cells were cultivated for 24 hours in RPMI 1640 medium without mitogen stimulation. After hypotonic treatment and methanol – acetic acid 3:1 fixation cell suspensions were dropped onto microscopic slides and used directly for I-FISH. The following commercially available Vysis probes (Abbott Molecular Europe) were used: LSI ATM SpectrumOrange/CEP 11 SpectrumGreen Probe and LSI TP53 SpectrumOrange/CEP 17 SpectrumGreen Probe. At least 200 nuclei were analyzed for each probe. The cutoff levels for positive values determined for normal controls were 2.5% (mean ± SD).

Statistical analysis

The statistical analysis was performed using Statistica 9.0 PL software. Differences between two groups were assessed using the *U* Mann-Whitney test. The Spearman rank correlation coefficient was used in correlation tests. The Wilcoxon test was used for two dependent variables analyses. Differences were considered statistically with p-value ≤ 0.05.

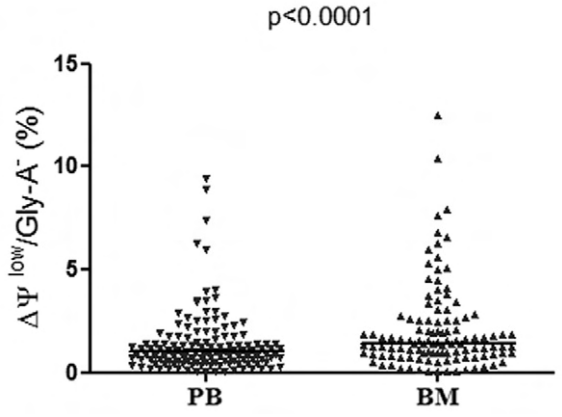

Fig. 1. The percentage of apoptotic cells in peripheral blood (PB) and bone marrow (BM) of untreated CLL patients.
Ryc. 1. Odsetek komórek apoptotycznych we krwi obwodowej oraz szpiku chorych na PBL

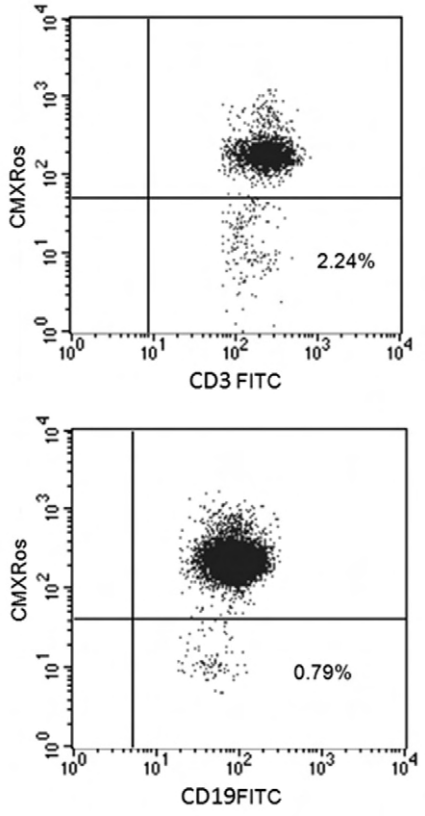

Fig. 2. The flow cytometry dot plots showing the analysis of the percentage of apoptotic cells within T CD3+ and B CD19+ cell populations.
Ryc. 2. Przykładowy obraz z cytometru przepływowego przedstawiający odsetek komórek apoptotycznych pośród limfocytów T CD3+ i B CD19+

Results

We found that the *ex vivo* percentage of PB apoptotic ($\Delta\Psi m^{low}/Gly\text{-}A^-$) cells in CLL patients (median: 0.99%; range: 0.03–9.40%) was significantly lower than that of $\Delta\Psi m^{low}/Gly\text{-}A^-$ apoptotic cells in BM samples (median: 1.41%; range: 0.05–12.5%) p<0.0001) (Figure 1).

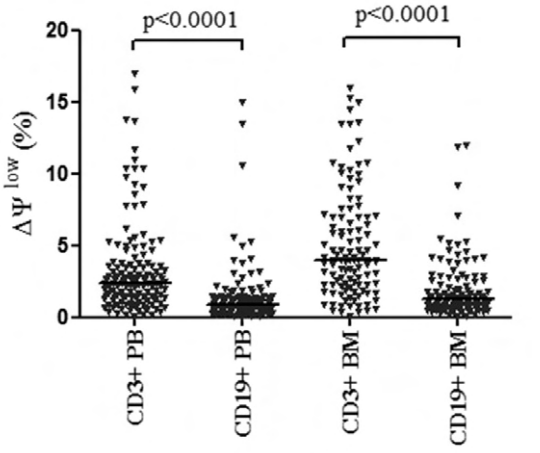

Fig. 3. The percentage of apoptotic cells within T- and B-cell populations ($\Delta\Psi m^{low}$/CD3+and $\Delta\Psi m^{low}$/CD19+) in PB and BM of untreated CLL patients.
Ryc. 3. Odsetek komórek apoptotycznych pośród limfocytów T ($\Delta\Psi m^{low}/CD3^+$) i B ($\Delta\Psi m^{low}/CD19^+$) we krwi obwodowej i szpiku chorych na PBL.

To assess the percentage of apoptotic cells within the T-cell and B-cell populations, we performed CMXRos staining in combination with the monoclonal anti-CD3, anti-CD19, anti-CD8 or anti-CD4/FITC-conjugated antibodies. Figure 2 shows representative plots of $\Delta\Psi m^{low}/CD19^+$ and $\Delta\Psi m^{low}/CD3^+$ cells in PB from CLL patient. In PB, within T-cell population, range of apoptotic cells was 0.11% to 16.93% (median: 2.41%). The apoptotic fraction in the B-cell population was much lower, with ranges of 0.01% to 14.92% (median: 0.94%) (p<0.0001). Likewise, in BM the percentage of $\Delta\Psi m^{low}/CD19^+$ cells was lower (range: 0.05–12.10%; median = 1.25%) than percentage of $\Delta\Psi m^{low}/CD3^+$ seen within the T-cell population (range: 0.09–16.00%; median = 4.00%) (p<0.0001) (Figure 3).

Figure 4 shows the results of CMXRos staining of $CD4^+$ and $CD8^+$ T-cell subsets in a representative CLL patient. Within the $CD8^+$ T-cell subpopulation, the range of apoptotic cells was 0.01% to 9.03% (median = 1.46%) in PB and 0.01% to 14.01% (median = 2.13%) in BM. Within the $CD4^+$ T-cell subpopulation, ranges of apoptotic cells were 0.01% to 11.53% (median = 2.10%) in PB and 0.17% to 17.26% (median = 3.48%) in BM. In PB and BM the apoptotic cell fraction within $CD8^+$ T-cell population was significantly lower than the apoptotic cell fraction within $CD4^+$ T-cell population (p<0.01) (Figure 5).

In this study we confirmed, on a larger group of patients, our previous observations [8] showing inverse correlations between the *ex vivo* percentage of apoptotic cells ($\Delta\Psi m^{low}$/Gly-A) and lymphocytosis (R=-0.34; p<0.01). However, we did not observe significant correlations between the *ex vivo* percentage of apoptotic cells and such prognostic factors as the lac-

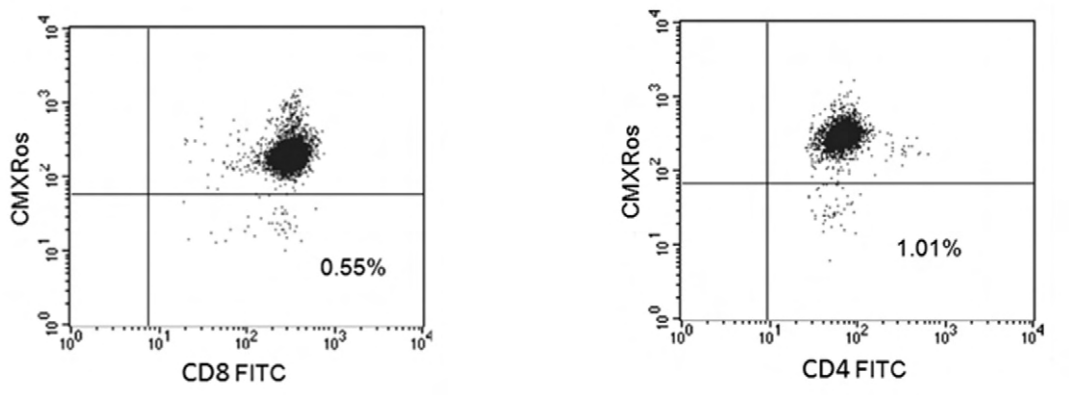

Fig. 4. The flow cytometry dot plots showing the analysis of the percentage of apoptotic cells within CD8+ and CD4+ cell populations.
Ryc. 4. Przykładowy obraz z cytometru przepływowego przedstawiający odsetek komórek apoptotycznych pośród limfocytów CD8+ i CD4+

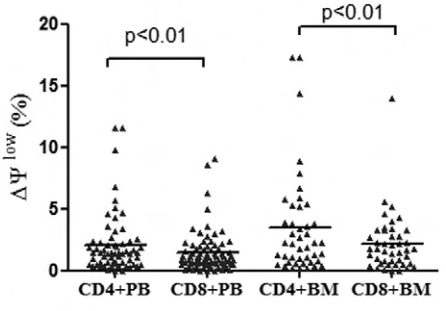

Fig. 5. The percentage of apoptotic cells within $CD8^+$ and $CD4^+$ T cell subpopulations ($\Delta\Psi m^{low}/CD8+$ and $\Delta\Psi m/CD4+$) in PB and BM of untreated CLL patients.
Ryc. 5. Odsetek komórek apoptotycznych pośród limfocytów $CD8^+$ i $CD4^+$ ($\Delta\Psi m^{low}/CD8^+$ i $\Delta\Psi m^{low}/CD4^+$) we krwi obwodowej i szpiku chorych na PBL.

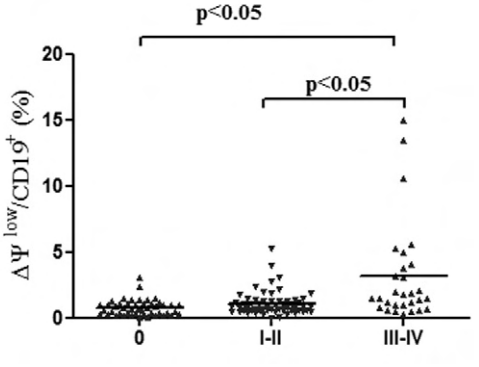

Fig. 6. The percentage of $\Delta\Psi m^{low}/CD19^+$ in CLL patients with different stages of the disease.
Ryc. 6. Odsetek apoptotycznych komórek $\Delta\Psi m^{low}/CD19^+$ u pacjentów w różnych stadiach zaawansowania PBL.

tate dehydrogenase (LDH) or β2-microglobulin levels. We found a direct correlation between the percentage of PB apoptotic B cells ($\Delta\Psi m^{low}/CD19^+$) and the Rai stage (R=0.42; p<0.05). The advanced stage (III-IV) patients showed a significantly higher percentage of $\Delta\Psi m^{low}/CD19^+$ cells (median: 3.13%) than the 0 (median: 0.77%) or I-II Rai stage patients (median: 1.11%) (p<0.05) (Figure 6). Furthermore, we detected a significantly higher percentage of $\Delta\Psi m^{low}/CD19^+$ cells in PB and BM from ZAP-70-positive patients than from ZAP-70-negative patients (p<0.01). We did not show these differences within the T-cell population. Likewise, we found no significant differences between CD38-positive and CD38-negative patients in the analyzed *ex vivo* apoptosis parameters.

We analyzed whether the percentage of apoptotic cells determined at the time of diagnosis correlated with clinical outcome. We found a higher percentage of $\Delta\Psi m^{low}/CD19^+$ cells in the group of patients requiring treatment (median: 2.60%) than in patients who did not (median: 1.72%) (p<0.05). The group of CLL patients with a low (<1%) percentage of $\Delta\Psi m^{low}/CD19^+$ cells had a longer median overall survival (39 months) than the group with high percentage of $\Delta\Psi m^{low}/CD19^+$ cells (32 months), but this difference was not statistically significant. The *ex vivo* percentage of apoptotic B cells before treatment in CLL patients who died due to the progression of the disease was significantly higher (median 3.7%) than the percentage of $\Delta\Psi m^{low}/CD19^+$ cells in patients who survived (median 1.2%) (p<0.01). We did not show these differences within the T-cell population.

Karyotypic analysis at the time of testing was available for 52 out of the 150 patients studied. There was a significant difference in median percentage of $\Delta\Psi m^{low}/CD19^+$ cells between the patients carrying 11q22.3 deletion or/and 17p13.1 deletion (2.7%) and the patients without these genetic aberrations (1.6%) (p<0.01) (Figure 7).

Discussion

Apoptosis is a fundamental biological mechanism involved in embryogenesis, morphogenesis and lympho-

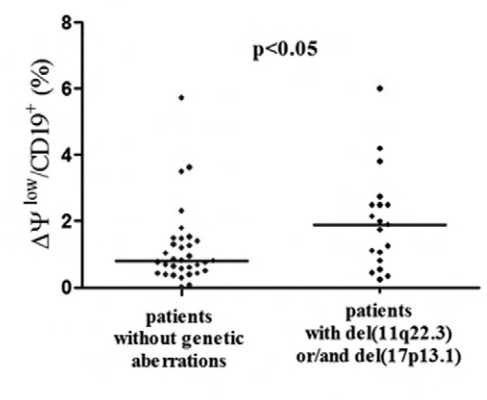

Fig. 7. The percentage of ΔΨm^{low}/CD19^{+} in CLL patients subdivided according to cytogenetic analysis. The first group consisted of the 33 patients having no unfavorable genetic aberrations. The second group (n=19) consisted of 12 patients who had del(11q22.3), 6 patients with del(17p13.1) and 1 patients who had simultaneously both aberrations.
Ryc. 7. Odsetek apoptotycznych komórek ΔΨm^{low}/CD19^{+} u chorych na PBL w zależności od występowania zmian cytogenetycznych: pierwsza grupa (n=33) - pacjenci bez niekorzystnych zmian cytogenetycznych. Druga grupa (n=19): 12 pacjentów z del(11q22.3), 6 pacjentów z del(17p13.1) i 1 pacjent z obiema aberracjami jednocześnie.

id cytotoxicity [9]. The conventional view of CLL has been that it is primarily a disease of failed apoptosis and passive accumulation [10]. In our study, we used CMXRos technique to assess the number of apoptotic cells *ex vivo* in peripheral blood and bone marrow of CLL patients. CMXRos technique detects distributions in the mitochondrial membrane potential (ΔΨm), one of the earliest events in the apoptotic pathway [11,12]. We hypothesised that the assessment of *ex vivo* apoptosis might provide new important information regarding the biology and prognosis of CLL. In our previous study [8], the *ex vivo* spontaneous apoptosis in CLL patients was significantly lower compared to healthy controls. Low spontaneous apoptosis in CLL patients confirmed that defective apoptosis is one of the mechanisms of leukemic lymphocyte accumulation. A new finding of the present study was that *ex vivo* spontaneous apoptosis was higher in bone marrow than in peripheral blood. Moreover, the percentage of apoptotic cells within the T-cell and B-cell populations was evaluated.

Another new finding of our study was that *ex vivo* spontaneous apoptosis of B cells was higher in advanced than in early stage patients. The high percentage of apoptotic B cells in untreated CLL patients with advanced stage is unique and important observation. Furthermore, we confirmed on a larger group of patients our previous data [8] showing inverse correlations between the *ex vivo* percentage of apoptotic cells and lymphocytosis. We can speculate that processes other than the accumulation of undying cells might also be responsible for a high lymphocyte count in the advanced stages of CLL. The previous studies have suggested that proliferation may be an important factor contributing to tumor mass growth in CLL. These data explain that CLL clone proliferate at appreciable rates and cells produced in the bone marrow, lymph nodes or spleen may be slowly released into other compartments [1,13,14]. The earlier studies indicate that there is an ongoing birth and death within CLL clones, and there is an active interaction between these two processes that determines the numbers of leukemic cells [1]. It is likely that proliferating cells show a greater ability to enter spontaneous apoptosis.

In these *ex vivo* studies, we have demonstrated that in CLL, the apoptotic fraction was higher within the T cells population than within the B-cell population. Although the T-cells represent a minority of the circulating lymphocytes in CLL, their absolute number have been reported to be increased. In addition both phenotypic and functional abnormalities have been described including a decreased CD4:CD8 ratio, probably resulting from a relative expansion of CD8^{+} T-cell subset [4,15,16]. In our study, in PB and BM the apoptotic cell fraction within CD4^{+} T cells was significantly higher than the apoptotic cell fraction within CD8^{+} T cells. Apoptosis plays an important role in regulating the size of the mature T lymphocytes, since non-functional and autoreactive T cells are eliminated by this process [15]. The results of Kiaii et al. [4] demonstrated that T cells, both CD4^{+} and CD8^{+}, in CLL patients could prevent apoptosis of leukemic B cells in vitro. Oliveira et al. [17] showed the correlations between the CD8 values and the clinical and laboratory parameters. They concluded that in advanced cases there might be a decreased production of CD8 lymphocytes.

In our study, we tried to assess whether the rate of *ex vivo* apoptosis differs between ZAP-70-positive and ZAP-70-negative patients. Interestingly, in the ZAP-70--positive group, the percentage of apoptotic cells was higher than in the ZAP-70-negative group. One could speculate that the higher rate of apoptosis in ZAP-70-positive patients may be associated with the fact that proliferating cells of more advanced cases are more prone to apoptosis. This could also explain the higher rate of apoptosis in bone marrow than in peripheral blood, as bone marrow cells have a higher proliferation rate than peripheral blood cells. Our result are in line with the observations of Coscia et al. [16]. In their study leukemic B cells obtained from CLL patients with unmutated IgVH showed significantly higher rates of in vitro apoptosis as compared with leukemic B cells isolated from patients with IgVH mutation.

We also analyzed whether the percentage of apoptotic cells determined at the time of diagnosis correlated with clinical outcome. We found a higher percentage of ΔΨm^{low}/CD19^{+} cells in patients requiring

treatment than in patients who did not. Patients with a high percentage of $\Delta\Psi m^{low}/CD19^{+}$ cells had a shorter median overall survival than the patients with a low percentage of $\Delta\Psi m^{low}/CD19^{+}$, but this difference was not statistically significant. Furthermore, there was a higher percentage of apoptotic B cells in patients carrying 11q22.3 deletion or/and 17p13.1 deletion than in patients without these genetic abnormalities. Our findings are in line with the observations of Jahrsdörfer et al [18], who have shown more rapid spontaneous in vitro apoptosis in patients with poor prognosis cytogenetics. They concluded that poor cytogenetics in CLL is not associated with a resistance of the cells to apoptosis.

In conclusion, our results show that bone marrow cells are more sensitive to apoptosis than the cells derived from peripheral blood. The present studies support the idea that these two compartments have different proliferative statuses. The cells from ZAP-70--positive patients with more aggressive disease, seem to enter apoptosis more rapidly than those from ZAP-70-negative patients.

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