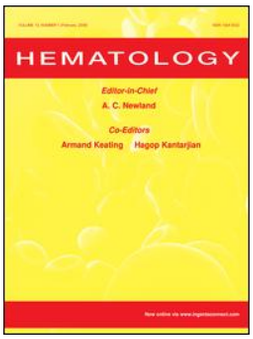

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Hematological Malignancies

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Hematological Malignancies

Aberrant Morphology, Proliferation, and Apoptosis of B-cell Chronic Lymphocytic Leukemia Cells

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B-cell chronic lymphocytic leukemia (CLL) has been traditionally described as a disease characterized by an accumulation of quiescent small lymphocytes with decreased susceptibility to apoptotic cell death. However, small numbers of "atypical" lymphocytes and prolymphocytes (PL) are frequently observed in the bone marrow (BM) of patients with CLL. In this study, we examined BM biopsy and aspirate specimens obtained from seven patients with atypical CLL. Using a double labeling (Ki-67$^+$/CD20$^+$) immunohistochemical method, we found that an appreciable number of the atypical CLL cells expressed the proliferation-associated protein Ki-67. Because CLL is characterized by a slow change in the peripheral blood (PB) lymphocyte count, we reasoned that a subpopulation of CLL cells probably undergoes spontaneous apoptosis. Using Western blot analysis, we observed expression of procaspase-9, procaspase-10, and poly(ADP-ribose) polymerase by the neoplastic cells in all seven cases of CLL, and procaspase-3 and procaspase-8 expression in six neoplasms. We also detected cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase in four and five CLL cases, respectively. To determine whether CLL cells undergo spontaneous apoptosis, we performed the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using BM biopsy specimens. We found TUNEL-positive lymphocytes in areas infiltrated by CLL. In summary, our data show that subpopulations of B-lymphocytes are proliferating or undergoing spontaneous apoptotic cell death in patients with atypical CLL.

Keywords: Chronic lymphocytic leukemia (CLL); Apoptosis; Proliferation; Atypical morphology; Cytogenetics

INTRODUCTION

B-cell chronic lymphocytic leukemia (CLL) is the most common type of adult leukemia in the United States and Western Europe [1]. It is thought to be caused primarily by defects in programmed cell death (apoptosis) and is characterized by a gradual accumulation of small, long-lived, immunologically dysfunctional mature B-lymphocytes, most of which are in G0/G1 phase of the cell cycle [2–4]. During diagnosis or during the course of the disease, 15% of patients with CLL show atypical morphologic features characterized by an increased (>10%) number of circulating prolymphocytes (PL), designated as CLL/PL, or an increased (>15%) number of circulating lymphoplasmacytic and cleaved cells, designated as "atypical" CLL [1,5]. Patients with atypical CLL more commonly have an accelerated clinical course and trisomy 12 [1,6,7]. In addition, atypical CLL cases have increased expression of the nuclear proliferation protein Ki-67 [8], and ultrastructural features suggestive of apoptosis [9].

Expression of the human protein Ki-67 is strictly associated with cell proliferation, being expressed during all active phases of the cell cycle (G1, S, G2, and mitosis) but absent in resting cells (G0 phase) [10]. The predominant proliferative component in CLL is localized in proliferation centers that are

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usually identified in the lymph nodes but less commonly are present in bone marrow (BM) [11,12]. There have been no published reports of simultaneous assessment of proliferation and apoptosis in BM biopsy specimens obtained from patients with CLL with atypical morphology.

In our experience, small percentages of atypical lymphocytes (i.e. lymphoplasmacytic and cleaved cells) and PL can be identified in BM aspirate smears in most cases of CLL, in patients at any clinical stage. Thus, we hypothesized that CLL cells are more heterogeneous than is appreciated in the literature. Thus, in this study group we assessed both proliferation and apoptosis. We show that cases of CLL with atypical morphologic features, regardless of stage, have subpopulations of cells that are proliferating or undergoing spontaneous apoptosis, in addition to quiescent cells, *in vivo*.

MATERIALS AND METHODS

Patient Specimens

Bone marrow aspirate and biopsy specimens were obtained from seven patients with B-cell CLL. Bone marrow aspirate and peripheral blood (PB) specimens were also obtained from two hematologically normal individuals (BM transplant donors) at the time of diagnostic evaluation at The University of Texas M. D. Anderson Cancer Center. All subjects gave their informed consent, but this retrospective study did not require approval by the Institutional Review Board when it was initiated.

The clinical characteristics of the seven CLL patients are summarized in Table I. All previously treated patients had not received treatment for several months at the time that the specimens were obtained. The diagnosis of CLL was based on a combination of clinical, morphologic, laboratory, and immunophenotypic criteria as defined previously [4]. Specifically, the criteria for CLL included involvement by a B-cell neoplasm composed predominantly of small lymphocytes that expressed monotypic immunoglobulin light chain, pan-B-cell antigens (CD19, CD20), CD5 and CD23 and negative for CD3, CD10 and cyclin D1.

Morphologic analysis was performed using Wright-Giemsa stained PB and BM aspirate smears and hematoxylin-eosin stained BM biopsy specimens. Flow cytometric immunophenotyping was performed using BM aspirates with a panel of antibodies specific for immunoglobulin kappa and lambda light chains, CD3, CD5, CD10, CD19, CD20, and CD23 according to well-established methods [13]. Other antibodies were used in a subset of these cases. Conventional G-band karyotype analysis of all samples was performed according to a previously reported method [13].

Immunohistochemical Methods

Immunohistochemical studies were performed using formalin-fixed, paraffin-embedded tissue sections (4 μm thick) and an avidin–biotin peroxidase technique [14]. The primary antibodies were specific for Ki-67 (1:1000; DAKO Corporation, Carpinteria, CA, USA) and cleaved caspase-3 (1:5000; R & D Systems, Minneapolis, MN, USA). Peroxidase staining was performed using the anti-mouse, anti-rabbit link antibody (DAKO LSAB2-HRP, 15 min; DAKO Corporation). Sequential immunoenzymatic staining combining the immunoperoxidase and immunoalkaline phosphatase techniques was used for simultaneous detection of Ki-67 and CD20 (DAKO). Negative and positive controls were run.

At low power magnification, the entire tissue section was scanned and microscopic fields were selected based on areas with the greatest number of Ki-67-positive and cleaved caspase-3-positive cells. In each case, at least 500 CLL cells were evaluated for nuclear (Ki-67, cleaved caspase-3) and membranous (CD20) positivity.

TABLE I Patient characteristics

Patient	Age in years/sex	Rai stage	WBC ($\times 10^3$/ml)	Hb level (g/dl)	Plt count ($\times 10^3$/ml)	β_2M level (mg/l)	Prior treatment	LDT (months)
1	57/M	II	33.4	12.8	167	3.4	Chb, CTX-P	6
2	57/M	I	96.3	13.5	132	3.4	None	12
3	42/F	I	22.2	13.1	132	2.8	None	4
4	55/M	IV	13.1	8.6	130	2.0	CHOP/CTX, O, VP-16, P, F, DHAP, thiotepa; bleomycin	6
5	73/M	II	104.5	13.1	118	5.6	None	N/A
6	46/F	I	28.2	12.8	178	N/A	None	N/A
7	76M	IV	175	11.2	75	11.4	Chb, F, 2CdA	6

WBC, white blood count; Hb, hemoglobin; Plt, platelet; β_2M, β_2-microglobulin; LDT, lymphocyte doubling time; Chb, chlorambucil; CTX, cyclophosphamide; CHOP, cyclophosphamide, adriamycin, vincristine, and prednisone; O, vincristine; VP-16, etoposide; P, prednisone; F, fludarabine; DHAP, dexamethasone, cytarabine, and cisplatin; 2CdA, 2-cholorodeoxyadenosine.

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-end Labeling (TUNEL) Assay for Detection of Apoptosis

Cells undergoing apoptosis were detected with a TUNEL method as described previously [14]. Apoptotic figures, including positively labeled intact nuclei and nuclear fragments derived from a single cell, were counted per high-power field (HPF) in areas with 100% cellularity with the use of an Olympus BX40 microscope with a 40× objective and 10× ocular (Olympus America Inc., Melville, NY, USA). In all cases, there were sufficient areas with adequate morphology to cover four or more HPFs. Using this approach, it was found that 0.23 mm^2 area within a HPF contained approximately 2950 cells when the cellularity was 100%. The number of apoptotic cells derived from a field with lower cellularity was normalized proportionally to 100% cellularity (e.g. when two TUNEL-positive cells were identified in a HPF with a cellularity of 50%, a score of 4 was recorded). The median score in each case was used for the final analysis.

Western Blot Analysis

Western blot analysis was performed using cell lysates of PB and low-density aspirate BM cells of seven CLL patients as described previously [15]. Cell lysates from 5×10^5 BM cells were assayed to determine the protein concentration using the BCA Protein Assay Reagent kit (Pierce Chemical Co., Rockford, IL, USA). Each set of paired samples was then adjusted so that they would have the same protein concentration. The following antibodies were used for their respective proteins: mouse anti-human CPP32 (procaspase-3), mouse anti-human FLICE (procaspase-8), rabbit anti-human procaspase-10, mouse anti-human poly(ADP-ribose) polymerase (PARP; Pharmingen, San Diego, CA, USA), and rabbit anti-human cleaved caspase-3 (New England BioLabs, Beverly, MA, USA). Normal mouse IgG and rabbit IgG (Sigma Chemical Co., St. Louis, MO, USA) were used as controls. Lysates of Jurkat, HL-60, and K-562 cells were used to confirm the detection of these proteins. After initial probing, blots were re-probed with an antibody specific for actin (1:1000, mouse monoclonal, Sigma Chemical Co.) to assess protein loading.

Statistical Analysis

Statistical analysis was performed using the chi-square test. p values <0.05 were considered statistically significant.

RESULTS

Clinical Features

The clinical features for the seven patients are summarized in Table I. We studied five male and two female patients whose ages ranged from 42 to 76 years (median, 57 years). Four patients were not previously treated, and three had been treated at other institutions. All previously treated patients had been off therapy for several months at the time the samples were obtained. Most patients received fludarabine-based chemotherapy in combination with rituximab after diagnostic evaluation at The University of Texas M. D. Anderson Cancer Center. Three patients (1, 4 and 7) received alemtuzumab alone or in combination with other agents. Four patients achieved complete remission and three patients had partial response.

Expression of Ki-67 Protein by CLL Cells

In all CLL patient specimens, we detected a small number of binucleated and trinucleated and/or large plasmacytoid cells in BM aspirate smears and expansion of the medullary space in BM biopsy specimens by CLL (Fig. 1A–C). Because the presence of these cells has been associated with an increased proliferation rate [6], we performed immunohistochemical studies to detect Ki-67 protein in BM biopsy specimens. As shown in Table II, we found that the immunoreactivity for this protein was relatively high (>5%) in BM biopsy specimens of all seven patients in typical areas of CLL infiltration (Fig. 1D–E). The CLL infiltrate distorted and displaced the normal Ki-67^+ erythroid and myeloid islets compared with that in the surrounding uninvolved BM (Fig. 1F).

To establish with certainty that CLL cells rather than normal hematopoietic cells were proliferating, we performed double labeling immunohistochemical staining with Ki-67 and the B-cell marker, anti-CD20. As shown in Fig. 2, a subset of CLL cells co-expressed Ki-67 and CD20.

Expression of Procaspase and Caspase Proteins and Detection of Apoptosis in CLL Cells

In previous studies, we found that patients with untreated acute leukemias had high levels of procaspase and caspase proteins and that PARP, a DNA repair enzyme known to protect cell integrity, was spontaneously cleaved in acute leukemia cells [15–17]. Using a similar method, we used Western immunoblotting to detect these proteins in BM aspirates from seven cases of CLL. Procaspase-9 and procaspase-10 were expressed in all neoplasms, with procaspase-8 and procaspase-3 were expressed in

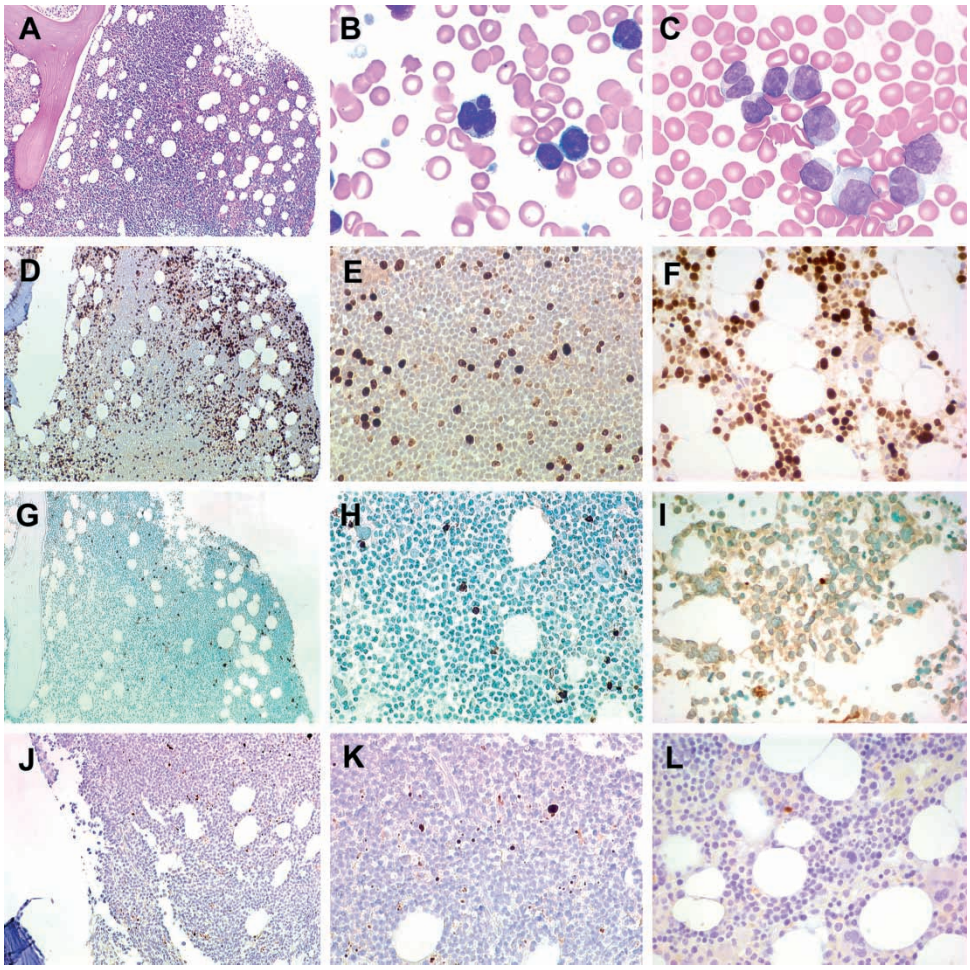

FIGURE 1 Histologic, immunohistologic, and cytologic stains of representative BM biopsy and aspirate specimens from patient 3. (A) Extensive infiltration by small lymphocytes in the BM biopsy specimen (hematoxylin and eosin stain, ×400). (B and C) Cleaved, multinucleated lymphocytes, prolymphocytes and small round lymphocytes in the BM aspirate smears (Wright-Giemsa stain, ×1000). (D–F) Immunohistochemical stain for Ki-67 shows Ki-67-positive CLL cells (D and E, CLL BM biopsy specimens [×400 and ×600, respectively]; F, normal BM biopsy specimen [×600]). (G–I) TUNEL staining of BM biopsy specimens (G and H, BM specimens showing several TUNEL-positive cells [×400 and ×600, respectively]; I, normal BM biopsy specimen showing rare positive cells [×600]). (J–L) Immunohistochemical staining for cleaved caspase-3. Activated caspase-3 was found often in CLL cells (J and K [×400 and ×600, respectively]) but rarely in normal BM specimens (L [×600]).

TABLE II Cytogenetic and BM findings in CLL cases

Patient	Cytogenetic abnormality	Histologic pattern in BM biopsy specimen	Lymphocytes (%)	Cleaved caspase-3 (%)	TUNEL (per HPF)	Ki-67 (%)
1	Diploid	N/I	95	15	8	12
2	Diploid	D	88	9	12	12
3	47,XX,+12[3] 47,XX,t(6;14)(p13;q32),+12[1]	D	80	5	29	50
4	46,XY,inv(9)(p11q12),del(11)(q22)[5]	N/I	80	3	9	10
5	46,XY,del(14)(q23)[13] 46,XY[7]	D	93	3	8	8
6	90–91,XXXX,−4,−6,der(7;?)(p10;?),−10, add(14)(q32)x2,der(19)del(19) (p13.1)del(19)(q13.1)x2, +0–4mar[cp5]	D	95	2	1	45
7	46,XY,ins(12;?)(q13;?),del(13) (q12q22)[2] 46,XY[6]	D	82	18	6	40

BM, bone marrow; N/I, nodular/interstitial; D, diffuse; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling; HPF, high power field.

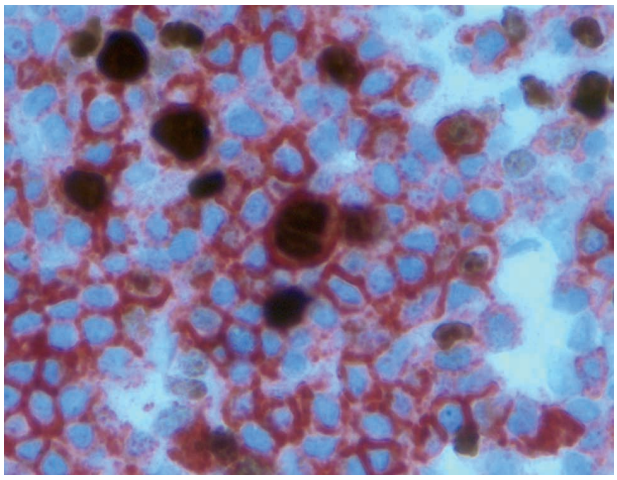

FIGURE 2 Double immunostaining for nuclear Ki-67 and membranous CD20. Many of the CLL cells were $CD20^{+}/Ki\text{-}67^{+}$; the remaining CLL cells were $CD20^{+}/Ki\text{-}67^{-}$.

six, cleaved PARP in five (Fig. 3), and cleaved caspase-3 in four neoplasms.

To determine whether these proteins were activated in CLL cells, we performed immunohistochemical staining for the detection of cleaved caspase-3 in BM biopsy sections. We found that CLL cells expressed cleaved caspase-3 protein (Fig. 1J–K, Table II) whereas normal BM cells did not (Fig. 1E). Furthermore, to determine whether spontaneous cleavage of PARP results in apoptosis of CLL cells, we performed TUNEL staining of BM sections. We found TUNEL-positive CLL cells (Fig. 1G–H and Table I) in all seven neoplasms (Fig. 1G–H, Table II), indicating that a subset of the CLL cells underwent spontaneous apoptosis. TUNEL-positive cells were rarely found in normal BM sections (Fig. 1I).

Cytogenetic Results

Conventional cytogenetic studies were performed on BM aspirates of all seven patients. Five patients (71%) had chromosomal anomalies and two patients (29%) were diploid (Table II). The chromosomal abnormalities included three chromosome 14 abnormalities (one del(14)(q23), one add(14)(q23), and one t(6;14)(p13;q32)), two chromosome 12 abnormalities (one trisomy, one insertion), one del(13)(q12q22), and one del(11)(q22). Additional cytogenetic abnormalities were seen in three patients.

DISCUSSION

CLL is a disease thought to be characterized by monoclonal expansion of circulating, mature B-lymphocytes that are not in cell cycle [2–4]. However, "atypical" lymphocytes (i.e. plasmacytoid, cleaved, and/or binucleated lymphocytes) and PL

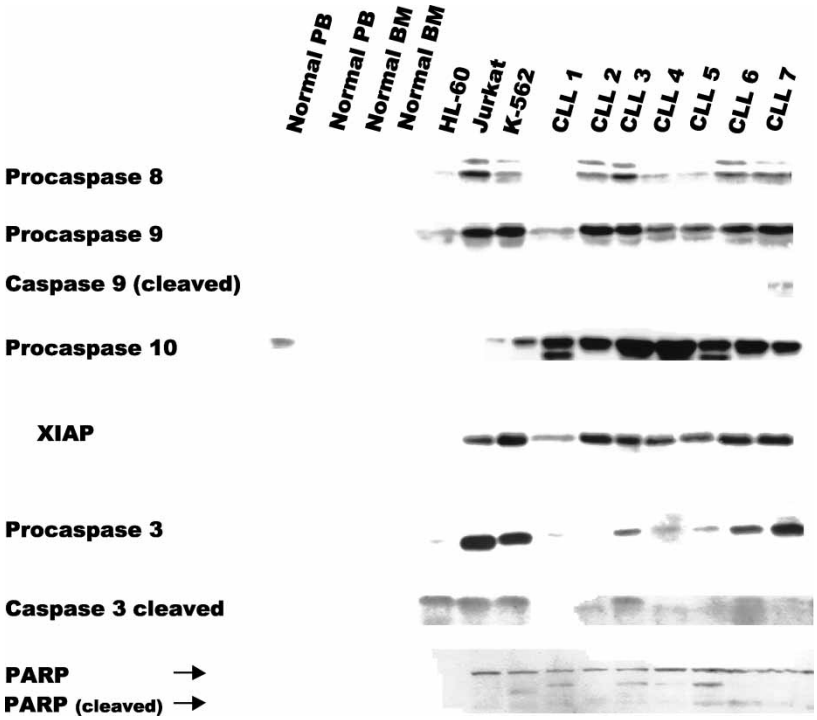

FIGURE 3 Detection of procaspase-8, procaspase-9, procaspase-10, and procaspase-3 and cleaved caspase-9, procaspase-3, PARP, and PARP protein in normal PB and BM specimens and in seven CLL cases. HL-60, Jurkat, and K-562 cell lines were used as positive controls. Also, BM aspirates obtained from two hematologically normal individuals and two normal PB samples were used as controls.

are frequently found in the BM and PB of patients with CLL, and increased numbers of these cells are associated with accelerated disease, trisomy 12, and increased proliferative activity [1,6–9].

In the present study, we assessed CLL cells in BM specimens from patients with early and advanced stage disease and found both proliferating cells and cells undergoing apoptosis. The combined immunostaining for nuclear Ki-67 and membranous CD20 in BM biopsy specimens demonstrated that the Ki-67-positive proliferating cells were of B-cell lineage and derived from the CLL, and were not attributable to T-cells or erythroid or myeloid precursors. The changes that we observed in patients with CLL with atypical morphology were similar to those reported by Garcia-Marco *et al.* [6] However, our study extended the work of those authors by examining apoptosis with the use of immunoblotting, TUNEL, and immunostaining for cleaved caspase-3 in BM biopsy specimens. Other studies support our data, as a subpopulation of proliferating Ki-67$^+$, survivin$^+$, bcl-2$^+$, and p27Kip1$^{+/-}$ CLL cells has been identified in proliferation centers in lymph nodes and BM, which are the preferred sites of relapse [8,11,12,18].

The reasons for the Ki-67 expression in a subset of cells in atypical CLL cases are unknown. However, one likely factor is that genes regulating the cell cycle are involved [19,20]. For example, overexpression of cyclin D2 mRNA has been found to be an almost constant feature of CLL [21]. In addition, whereas cyclin D1 has been reported in patients having an aggressive form CLL [22], cyclin D3 has been shown to play a role in the exit of CLL lymphocytes from quiescence [23]. Nevertheless, Delmer *et al.* [21] reported that more than 96% of the CLL cells in their study were in the G0/G1 phase of the cell cycle. Although it is possible that overexpression of the cell cycle inhibitor p27 [24] and interaction of CLL cells with accessory cells and BM stroma [18,19] prevent cell cycle transition, these studies suggest that deregulation of the G1 restriction point is involved in CLL progression.

Our results are also consistent with those of previous studies demonstrating that CLL cells are heterogeneous with a hierarchy of progenitor and mature cells [25,26]. In fact, a recent study showed that the frequency of two or more clones is higher in CLL cases with atypical morphology (4/29 [13.8%]) compared with CLL cases with typical morphology (12/353 [3.4%]) [27]. A review of PB smears in this study revealed the presence of two or more morphologically different populations of lymphocytes in 89% of the cases.

In contrast with Oliveira *et al.* [28], who found a decrease in the susceptibility to apoptosis with CLL progression, as well as an increase in the tumor mass and proliferation rate, we found apoptotic cells in CLL cases at all disease stages. Similar to our previous findings in studies of acute leukemia [15–17], we detected procaspase-8, procaspase-9, procaspase-10, and procaspase-3 in CLL cells at higher levels than those observed in normal BM and PB cells. This finding is in agreement with a study by Krajewski *et al.* [29], who showed by immunoblotting that all of their CLL cases expressed procaspase-3. In that study, the authors evaluated 12 cases of CLL by immunocytochemistry using smears prepared from PB lymphocytes ($>$90% CD20$^+$). In all 12 cases the neoplastic cells were positive for caspase-3. Immunolocalization was cytoplasmic in all 12 patients, with 2 patients having distinct nuclear immunoreactivity.

It is unlikely that the increased procaspase levels result from accumulation due to a lack of cleavage, because we detected cleaved caspase-3, cleaved PARP, and TUNEL-positive cells in all BM specimens of the CLL cases assessed. Also, the apoptotic rate in BM biopsy specimens from normal donors, determined by TUNEL, was 0% [30]. By contrast, six of seven CLL patients (86%) had an apoptotic cell percentage $>$1% as determined via TUNEL.

Another interesting finding is that many of our patients can be classified as being at high risk, based on the presence of chromosomal abnormalities (+12, 11q-, and 17p-), the BM histologic pattern (diffuse versus other patterns), lymphocyte doubling time ($<$12 months), and elevated β2 microglobulin level [31–33]. As a result, all seven patients received therapy for some time after the completion of their diagnostic studies. Neither immunoglobulin gene mutation status nor the levels of CD38 and ZAP-70 expression in these CLL cases is available, because these tests were not routinely performed in our clinical laboratory when this study was undertaken. However, retrospective studies have established that patients whose CLL cells carry nonmutated IgV_H genes ($\leq$2% difference in nucleotide sequences from the nearest germline V_H gene) harbor high-risk chromosomal anomalies, express CD38 and ZAP-70, and have a shorter median survival duration (8–10 years) and are more in need of treatment when compared with patients whose CLL cells carry mutated IgV_H genes [34–37].

In conclusion, our data suggest that CLL with atypical morphology consists of a heterogeneous population of neoplastic cells. Some of these cells proliferate, whereas others undergo apoptosis. The mechanisms responsible for these events and their pathophysiologic significance and prognostic implications need to be addressed in future studies. Moreover, Bogner *et al.* [38] and Decker *et al.*[39] have found that cycling CLL cells are highly susceptible to inhibition of the proteasome with induction of apoptotic cell death. Thus, the use of agents that target the pool of proliferating cells in CLL with

atypical morphology may be of value in the treatment strategy in these patients.

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