Prognostic Value of Proliferating Cell Nuclear Antigen Expression in Chronic Lymphoid Leukemia

By Auro del Giglio, Susan O'Brien, Richard Ford, Hideyuki Saya, John Manning, Michael Keating, Dennis Johnston, Rainer Khetan, Adel El-Naggar, and Albert Deisseroth

Chronic lymphocytic leukemia (CLL) is a generally indolent disease that can assume a more aggressive behavior in some patients. Although several investigators have proposed CLL staging systems that correlate with survival, it is still difficult to prospectively identify CLL patients who will have a longer versus a shorter aggressive clinical course. Other prognostic factors have been studied, including bone marrow histology and lymphocyte doubling time (LDT). Montserrat et al showed that CLL patients with a LDT of less than 12 months had a worse prognosis than those patients with an LDT of more than 12 months. Julisson and Gahrton showed that, in CLL cells stimulated by mitogens, high-proliferative responses assessed by thymidine incorporation were associated with a prognosis worse than that associated with low-proliferative responses. Although kinetic parameters like the LDT and response to mitogens can help to estimate the clinical aggressiveness of the disease, they are complex to assess and cannot be applied to prospective analysis of prognosis at presentation.

Proliferating cell nuclear antigen (PCNA), which is a cofactor for DNA-dependent DNA polymerase, is a 36-Kd nuclear protein whose concentration reflects the proliferative state of normal and transformed cells. Kurki et al, using flow cytometry, showed that PCNA starts increasing during G1, peaks in S phase, and declines during the G2/M phase. Furthermore, PCNA expression by immunohistochemistry has been used in several types of tumors to assess the proliferative activity of malignant cells. PCNA expression has been shown to correlate with the proliferative activity of lymphoid cell lines, lymphoid tumors, including non-Hodgkin’s lymphomas, and acute lymphocytic leukemia in children. It was of interest, therefore, to determine if the level of PCNA in CLL lymphocytes would correlate with other known predictors of prognosis in this disease. The results of this analysis of PCNA levels in 40 patients with CLL show that PCNA correlates with other clinical characteristics of CLL patients that are presently used to predict the prognosis of this disease.

**Materials and Methods**

**Samples.** Peripheral blood or bone marrow aspiration samples were subjected to Ficoll-gradient separation. Cells were counted by a hemocytometer and adjusted to a final cell concentration of 1 x 10⁷ cells/mL. Protein was prepared by adding 2X electrophoresis sample buffer (Tris 0.25 mol/L, pH 6.8, 2% sodium dodecyl sulfate [SDS], 4% β-mercaptoethanol, 10% glycerol, and 0.5% Bromophenol blue) to an equal volume of cell suspension. Patients whose samples were analyzed participated in clinical protocols approved by The University of Texas M.D. Anderson Cancer Center Human Subjects Committee. The 40 patients studied had a mean age of 60 years; 55% were male and 45% were female, and 45% of them had received treatment in the past. The diagnosis of CLL was based on standard clinical and immunohistochemical criteria, and all bone marrow biopsies were reviewed by Dr J. Manning. The bone marrow sample submitted by two of the 40 patients showed no leukemic involvement. The immunophenotypic characteristics of the leukemic cells (CD56, CD25, CD19, CD10, FMC7, CD2, CD3, CD4, CD8, CD52, presence of surface immunoglobulin [Slg], κ or λ light chains, and HLA-DR) were measured by flow cytometry.

**Western blotting.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared as described previously. After boiling samples for 3 minutes, protein from 5 x 10⁶ cells was loaded and electrophoresed on 7.5% SDS-PAGE gels. The proteins were then transferred to an Immobilon PVDF membrane (Millipore Corporation, Bedford, MA) and probed with either anti-PCNA monoclonal antibody from the clone 2F4 (Boehringer Mannheim, Indianapolis, IN), or antiactin monoclonal antibody (Oncogene Science, Inc, Manhasset, NY). After hybridization with 125I-labeled secondary monoclonal antibody (Amersham Corp, Arlington Heights, IL), biots were exposed to Kodak XAR film (Eastman Kodak, Rochester, NY). Exposures that were still in the linear range of the films were selected for densitometric evaluation.

**Growth factor stimulation of leukemia cells.** To determine if...
PCNA expression could be associated with proliferation of CLL cells. CLL cells were incubated in RPMI medium (Irvine Scientific, Santa Anna, CA) with 20% fetal calf serum (HyClone Laboratories, Inc, Logan, UT) with or without 10% B-cell growth factors (BCGF; Cellular Products Inc, Buffalo, NY). Cells were harvested after 72 hours in culture, and protein was prepared as described above. Part of the samples was incubated for 16 hours with 100 μCi of tritiated thymidine (6 Ci/μmol/L) (ICN Biomedicals, Inc, Costa Mesa, CA), and the radioactivity incorporated into ethanol-precipitable DNA was measured using a Beckman liquid scintillation counter (Beckman Instruments, Irvine, CA).

Flow cytometric analysis of unstimulated CLL cells. Fresh samples from 10 patients were submitted for flow cytometric analysis concomitantly with protein sample preparation for the PCNA western blotting assay. We used the two-step acridine orange method for simultaneous DNA and RNA estimation as described previously. The analysis was performed on an EPICS-profile flow cytometer and the proliferative rate was defined as the number of cells in the S + G2/M phases of the cell cycle.

Densitometric scanning. Films were analyzed with a Beckman DU-70 spectrophotometer. The wavelength used was 510 nm and the slit width was 0.1 mm. Absorbance values were corrected by the background for each film, and the area under the curve was measured as recommended by the manufacturer.

Statistical methods. Patient samples were assayed in duplicate experiments. After excluding any significant difference between the two sets of measurement by the paired t-test, the values obtained by measuring the areas under the curves of absorbance for both actin and PCNA were averaged. To account for differences in protein loading, PCNA levels were normalized by the actin expression from the same sample. A logarithmic transformation of the data was used to stabilize the variance. Both one-way analysis of variance and simple regression analysis were used to study the correlations of the above variables and the clinical and pathologic characteristics of the patients.

The calculation of the doubling time was done using a linear regression analysis of the absolute lymphocyte counts and the interval in days between them. The absolute lymphocyte counts were derived from complete blood counts obtained before sample collection for PCNA Western assay. If the regression slope was negative, the doubling time was considered to be higher than 12 months. The LDT was calculated in 36 of 40 CLL patients as in those eight patients was the LDT slope negative.

RESULTS

To assess the reliability of the Western blotting assay used, we examined the correlation between the level of PCNA expression by Western blot with the level of proliferation stimulated in CLL B lymphocytes by B-cell growth factor (BCGF) (Table 1 and Fig 1). The expression level of PCNA decreased after 72 hours in culture to almost undetectable levels. Upon stimulation with BCGF, there was a striking increase in PCNA expression that was accompanied by a comparable increase in [3H]thymidine incorporation (Table 1).

Marked heterogeneity of expression of PCNA was observed among the 40 CLL patients examined (Fig 2). After normalization by actin expression, statistically significant higher levels of PCNA were noted in the more advanced disease stages when compared with earlier stages of disease presentation using both RAI and Binet classifications (Table 2). Significantly higher PCNA expression was also observed in patients with a shorter LDT (less than 12 months) as compared with patients with a more prolonged LDT (more than 12 months) (Table 2). We also observed a statistically significant correlation between the percentage of cells in S + G2/M phases and the PCNA expression in 10 unstimulated CLL samples (Table 2). However, no correlation was found between PCNA levels and the types of bone marrow involvement (nodular v diffuse) or with other clinical parameters like sex, age, peripheral white blood cell count, absolute lymphocyte count, percentage of marrow lymphocyte infiltration, hemoglobin levels, platelet count, or the presence of lymphadenopathy and hepatosplenomegaly. There was also no significant correlation noted with any of the immunophenotypic characteristics of the leukemic cells analyzed: CD56, CD25, CD19, CD10, FMC7, CD2,

Table 1. PCNA and Thymidine Incorporation of CLL Cells Stimulated With BCGF

<table>
<thead>
<tr>
<th></th>
<th>Control Before Stimulation</th>
<th>Control at 72 h Without BCGF</th>
<th>BCGF Treatment for 72 h</th>
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<tbody>
<tr>
<td>PCNA*</td>
<td>0.03</td>
<td>0.001</td>
<td>0.25</td>
</tr>
<tr>
<td>Thymidine incorporation (cpm)</td>
<td>ND</td>
<td>202.2</td>
<td>65,032.7</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

*PCNA values were normalized by actin as described in Materials and Methods. These results are from one patient with CLL and are representative of other similar stimulation experiments that were performed in our laboratory.
CD3, CD4, CD8, CD5, CD20, presence of Slg, κ or λ light chains, and HLA-DR.

DISCUSSION

Our results suggest that PCNA expression reflects the proliferative activity of CLL cells both before and after mitogen stimulation. We also observed a marked heterogeneity of PCNA levels among unstimulated samples. Furthermore, higher PCNA values correlated significantly with shorter doubling times and with the presence of more advanced disease. Therefore, as both clinical stage and the LDT have been shown to be important prognostic factors in CLL, it is conceivable that PCNA expression might also have prognostic relevance in this disease.

However, we did not observe a significant correlation between PCNA expression and white blood cell counts (WBCs), absolute lymphocyte counts, or diffuse versus nondiffuse patterns of bone marrow involvement. It is possible, therefore, that in some patients, the high WBCs or a diffuse pattern of bone marrow involvement might not reflect a higher leukemic cell proliferative activity, but rather the prolonged survival time of the cells. In those patients, the prognostic relevance of these parameters may reflect a measure of their tumor burden not necessarily related to the proliferative rate of the CLL lymphocytes.

The use of Western blotting to quantify PCNA expression is an alternative to the currently used immunohistochemistry methods, flow cytometry, and two-dimensional SDS-PAGE. The specificity of Western blotting for the detection of PCNA has been described previously, and our experiments suggest that Western blotting also reliably detects variations in PCNA-expression–associated differences in cell proliferative activity.

In this study, we also included previously treated patients. As the effects of cytotoxic chemotherapy on PCNA levels are unknown, we cannot exclude in the patients studied a treatment-related influence on PCNA expression.

Several investigators have shown that PCNA levels correlate with the cellular proliferative activity in several systems. However, there is recent experimental evidence to support the hypothesis that PCNA expression also increases independently of cell division. Cells in a study of human amnion cells after ultraviolet irradiation, observed a significant increase in PCNA levels in cells not in the S phase of the cell cycle. As δ polymerase may be involved in DNA synthesis associated with repair, it is possible that PCNA also participates in that process. Therefore, elevated PCNA levels may also indicate a higher activity of DNA repair synthesis that could facilitate the emergence of drug resistance and thus further compromise patient outcome.

In summary, PCNA expression can estimate CLL cell proliferative activity and correlates with known prognostic factors of this disease-like stage and LDT. Consequently, high levels of PCNA expression by unstimulated CLL lymphocytes at presentation may identify patients with a higher proliferative activity and, therefore, with a worse prognosis for whom intensive therapy should be considered. The validity of this hypothesis is currently being tested in a prospective study of PCNA levels and therapy outcome.

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