Proliferating Cell Nuclear Antigen Expression in Childhood Acute Leukemia

By David Keim, Nabil Hailat, David Hodge, and Samir M. Hanash

The primary source of leukemic cells was a group of 171 patients with acute leukemia studied at the time of initial diagnosis. All patients were advised of procedures and attendant risks in accordance with the institutional guidelines, and gave informed consent. The specific diagnosis was made on the basis of morphologic features and histochemical staining of cells with peroxidase, esterase, and Sudan black; rosetting with sheep erythrocytes; and reactivity with a battery of monoclonal antibodies (MoAbs). Terminal deoxynucleotidyl transferase (TdT) was detected by indirect immunofluorescence. Leukemic cells were obtained by Ficoll-Hypaque gradient centrifugation of either heparinized peripheral blood or bone marrow. The leukemic cells constituted 85% or more of the total mononuclear cells in most preparations.

Surface immunophenotyping. Cell samples were analyzed for reactivity with a battery of MoAbs against the following antigens: CD1 (T6), CD2 (T11), CD3 (T4), CD4 (T9), CD7 (Leu 9), CD8 (T8), CD10 (CALLA), CD11 (Mol), CD13 (My7), CD14 (Mo2), CD19 (B4), CD20 (B1), CD33 (My9), CD38 (T10), and I2 (HLA D/DR). The diagnosis of T-ALL was excluded on the basis of lack of reactivity with specific T-cell markers and lack of rosetting with sheep erythrocytes. The percentage of the cell population stained with each MoAb and the mean channel of fluorescent intensity of staining (a measure of antigen density) were quantitated on a flow cytometer (Epics V, Coulter Electronics, Inc, Hialeah, FL). Nonspecific binding of the primary antibodies was assessed with an unrelated isotype-specific mouse MoAb.

Samples included leukemic cells from 21 patients with T-ALL; 113 with CALLA-positive, non-T ALL; 22 with CALLA-negative, non-T ALL; and 15 with ANLL. Peripheral blood T lymphocytes and phagocytizing (PHA)-stimulated peripheral blood T lymphocytes from six normal, healthy individuals were also used. Lymphocytes were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 0.8% L-glutamine (29.2 µg/mL), and 1% of penicillin/streptomycin (5,000 IU/mL and 5,000 µg/mL, respectively). PHA was used to stimulate lymphocytes at a concentration of 1 µg/mL. Harvesting occurred at 72 hours after the addition of PHA.

2-D PAGE. Cell pellets were solubilized by addition of lysis buffer consisting of (per liter) 8 mol/L urea, 20 mL of Nonidet P-40 surfactant (Hoefer Scientific Instruments, San Francisco, CA), 20 mL of amphoties (pH 3.5 to 10), 20 mL of 2-mercaptoethanol, and 0.2 mmol/L of phenylmethylsulfonyl fluoride in deionized water. 2-D PAGE was performed as previously described. In most cases 25-µL aliquots containing solubilized cells (2.5 × 10⁶) were immediately applied into isofocusing gels. First-dimension gels contained 50 mL of amphoties per liter (pH 3.5 to 10). Isofocusing was done at 1,200 V for 16 hours and 1,500 V for the last 2 hours. Twenty gels were run simultaneously. For the second-dimension separation, an acrylamide gradient of 11.4 to 14.0 g/dL was used. Protein spots in gels were visualized by the silver-staining technique of Merril et al. Unsolubilized aliquots were frozen as pellets at -80°C.

Amino acid sequence analysis of PCNA. For NH₂-terminal sequence analysis, six 2-D gels were electroblotted onto siliconized glass fiber filters. Pieces containing putative PCNA were applied directly to a gas phase sequencer (model 470A, Applied Biosystems, Foster City, CA) as described. As a control to ascertain that PCNA was above the detection level of staining (a measure of antigen density) were quantitated on a flow cytometer (Epics V, Coulter Electronics, Inc, Hialeah, FL). Nonspecific binding of the primary antibodies was assessed with an unrelated isotype-specific mouse MoAb.

Supported in part by a grant from the Public Health Service no. CA 32146.

Address reprint requests to David Keim, MD, University of Michigan Medical School, R4451 Kresge I, Box 0510, Ann Arbor, MI 48109.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.
NH₂ terminus was excised from the same filter from which PCNA was cut. Microsequencing yielded the expected NH₂-terminal sequence for HuCha 60. The Coomassie blue staining intensity of HuCha 60, relative to PCNA, indicated that there was sufficient PCNA for sequencing.

Quantification of PCNA. Each gel was scanned in a 1,024 × 1,024 pixel format, giving 160 µm as the pixel width. Spot detection and quantitation were performed as previously described. For the group of 171 patients with acute leukemia, the digitized gel images were assigned coded numbers and analyzed for the presence and quantity of PCNA without knowledge of the patient to whom a gel image corresponded, or of the immunologic or other phenotypic traits of the leukemic cells. The integrated intensity of PCNA and 20 other reference proteins in the gel were measured in units of optical density times square millimeter. Data on these spots was transferred to an IBM 4361 at the University of Michigan running the Michigan Terminal System (MTS) operating system to analyze data using the Michigan Interactive Data Analysis System (MIDAS), a statistical software package. The 20 reference protein spots were used to adjust the PCNA spot-integrated intensity for gel-to-gel variation due to any differences in gel staining or amount of protein loaded. The usual ANOVA was used for all comparisons.

S-phase determination. DNA content determination and estimation of the percent of cells in the S phase was by a standard method as described by Braylan et al.

RESULTS

PCNA identification. PCNA was identified by 2-D PAGE on the basis of molecular weight, pI, and up-regulation in normal peripheral blood lymphocytes after activation with PHA (mean PCNA intensity for N = 6 before stimulation was 0.592, and at 72 hours following stimulation was 1.41; P = .001). Figure 1 displays PCNA’s location and relative intensity for both populations. To confirm that the protein being analyzed is indeed PCNA, N-terminal sequencing was undertaken. The N-terminal sequence K, F, E, A, R, L, V, Q, G, S, I, L, K was generated that uniquely corresponded to PCNA, thus confirming the identity of the polypeptide being analyzed.

Differential expression of PCNA in acute leukemia subtypes. Quantitative analysis of PCNA in T-ALL, CALLA-positive, and CALLA-negative non-T ALL, and ANLL, showed significant differences in PCNA levels between the groups. Figure 2 displays PCNA in leukemic cells for a patient from each group and for unstimulated lymphocytes from a healthy donor. The quantitative analysis is summarized in Fig 3, which graphically displays the mean and range (1 SD) of PCNA for each subgroup. The groups of ANLL, non-T ALL (combining CALLA-positive and -negative), and T-ALL exhibited statistically significantly different levels of PCNA. All groups of acute leukemia studied had significantly greater mean levels of PCNA relative to unstimulated lymphocytes from healthy donors (P < .01). The group of non-T ALL exhibited a PCNA level that was intermediate between ANLL and T-ALL, and statistically significantly different from either group (P < .03). The difference in PCNA level between CALLA-positive and CALLA-negative non-T ALL was not statistically significant.

Relationship between PCNA levels and peripheral white blood cell (WBC) count. We analyzed PCNA levels in relation to the initial peripheral WBC count for the total leukemia group and for each of the four subtypes. Patients were divided into four WBC groups, based on peripheral WBC at presentation: less than 5,000; 5,000 to 50,000; 50,000 to 100,000; and greater than 100,000. The mean levels for PCNA, for all patients with acute leukemia, were 2.10, 1.96, 2.00, and 2.36, for the respective WBC groups (respectively N = 37, 79, 24, 28). These values were not significantly different from each other. Furthermore, patients within each subtype of acute leukemia were subdivided into the four WBC groups to determine if a significant relationship existed between PCNA and WBC for any of the four subtypes. No significant differences in PCNA relative to WBC levels were observed for any subtype. A comparison between patients with different subtypes of acute leukemia but within the same WBC group showed that T-ALL exhibited the highest mean levels of PCNA in each of the WBC categories, non-T ALL (CALLA-positive and -negative) intermediate levels, while ANLL exhibited the lowest levels. The differences between the T-ALL group, the non-T ALL group (CALLA-positive and -negative), and ANLL were significant. This concurred with the initial analysis of patients not categorized by WBC (Fig 3).

Analysis of PCNA with regard to S phase of the cell cycle. PCNA levels were analyzed in relation to percentage of cells in the S phase. Patients were first divided into two groups: Those with up to 8% of cells in the S phase were in one group and the remainder with greater than 8% were in the other. Eighty-five percent of cell samples were obtained from bone marrow. When all patients with acute leukemia were analyzed, a higher level of PCNA was seen in the group with greater than 8% of cells in the S phase (2.55 v 2.15, N = 45 and 30), although the difference was not statistically significant (P = .17). When each subtype of acute leukemia was examined individually, T-ALL displayed a statistically significant higher level of PCNA in the S phase > 8% group, relative to T-ALL < 8% group (4.54 v 2.54, N = 6 and 5; P = .01). Non-T ALL (CALLA-negative and -positive) patients did not display a statistically significant difference in PCNA above and below the S-phase boundary of 8%, although PCNA was higher in those patients with greater than 8% of cells in the S phase (2.45 v 2.00, N = 21 and 40, P = .12). The number of patients with ANLL and S-phase analysis was too small to allow statistically meaningful analysis.

Analysis of PCNA with regard to patient’s age or sex. No significant variation was found in PCNA levels for the entire group or the four subtypes of acute leukemia in relation to age or sex.

DISCUSSION

Cell kinetic studies have progressed from a predominantly descriptive analysis of cell cycle events to a molecular approach aimed at identifying genes or gene products that regulate progression of cells through different phases of the cell cycle. While it is generally agreed that there are no fundamental differences in the kinetics of normal hematopoietic progenitor cells and their leukemic counterparts, there is evidence for substantial heterogeneity between and within subgroups of leukemia with respect to protooncogenes and other factors involved in cell proliferation. PCNA has been
identified as a major nuclear protein involved in cell proliferation. After activation of T lymphocytes, PCNA reaches maximum levels during the S phase of the cell cycle. PCNA levels have been found to reflect cell proliferative activity. Takasaki et al. observed a highly significant correlation between numbers of blast cells and cells staining for PCNA in chronic myelogenous leukemia in blast crisis. Thus, we have examined the extent of variability in PCNA levels between and within the major childhood acute leukemia subtypes.

The mean levels of PCNA observed for all leukemia subtypes analyzed were significantly higher than for normal peripheral blood lymphocytes. However, in a study of clones derived from a nonleukemic, Epstein-Barr virus-transformed human lymphoid cell line, in a logarithmic growth phase with a generation time of 18 hours, we observed a mean PCNA
level that was significantly higher than for any of the acute leukemia subtypes (4.44 v 3.35 for T-ALL). Thus, PCNA levels observed in acute leukemia were intermediate between resting lymphocytes and maximally proliferating lymphoid cells in culture. Interestingly, ANLL exhibited the lowest mean level of PCNA. This finding is consistent with a relatively long average cell cycle time observed in ANLL, based on in vivo labeling with tritiated \(^3\)H-thymidine. Furthermore, the observation of a higher mean level of PCNA in T-ALL relative to non-T ALL is consistent with the observation of a higher cell labeling index and mitotic index in T-ALL reported by Murphy et al. In this subtype of acute leukemia we also observed a marked difference in PCNA levels between the subgroups with high or low percentage of cells in the S phase. Thus, it appears that PCNA levels likely reflect differences in proliferative activity between leukemia subtypes.

The two subtypes of non-T ALL recognized as representing pre-B cells exhibited very similar mean levels of PCNA. Furthermore, leukemia cells from two patients with B-cell ALL that were not included in the statistical analysis, exhibited levels of PCNA of 1.7 and 2.2 that were close to the mean for pre-B ALL, suggesting differences in PCNA levels between acute leukemia of B- and T-cell lineage. Alternatively, the differences observed could have reflected differences in tumor load between subtypes. However, the absence of a significant relationship between PCNA levels and WBC counts at the time of diagnosis would indicate that this is not the case. Previous studies have also shown lack of correlation between WBC counts and cell proliferation in acute leukemia, as determined on the basis of the fractional birth rate that represents the percentage of leukemic cells newly formed per unit of time. In light of these results, it has been postulated that the tumor load observed in a leukemia does not accurately reflect the rate of proliferation of leukemic cells.

Because ANLL, a subtype of acute leukemia associated with a poor prognosis, displayed low levels of PCNA, the potential prognostic significance of PCNA in acute leukemia is of interest. Preliminary analysis in which patients with
ANLL were divided into subgroups based on PCNA levels indicated that patients with PCNA levels below the mean for their respective group had a statistically significantly greater incidence of relapse than the subgroups with higher PCNA levels than the mean. Three of four ANLL patients with initial mean PCNA levels above the mean have not relapsed, while 6 of 7 patients with initial mean PCNA levels below the mean have relapsed. When data was analyzed in an alternative way, patients who relapsed were found to have a lower mean initial PCNA level (0.81, N = 7) than those patients without relapse (1.71, N = 4, P = .02). If this finding is validated in studies involving a larger number of patients and additional follow-up, then determination of PCNA levels at the time of prognosis could have prognostic as well as therapeutic significance.

The factors responsible for regulating PCNA level in leukemia remain to be determined. Coordinate synthesis of PCNA and DNA has been demonstrated in serum- or growth factor-induced quiescent cells. DNA synthesis is not required for synthesis of PCNA. PCNA messenger RNA (mRNA) level is very low or undetectable in quiescent cells, but increases 8 to 10 hours after serum stimulation, reaching peak levels at about 16 hours. PCNA level decreases dramatically at the end of S phase as determined by immunofluorescence or 2-D PAGE. Cells without appreciable PCNA staining by immunofluorescence or labeling by 3H-thymidine are no longer engaged in DNA synthesis. Studies involving cell stimulation, followed by treatment with hydroxyurea to block progression of the cell cycle from G1 phase to S-phase, showed that PCNA remains elevated despite the lack of elevation of DNA polymerase δ, thymidine kinase (TK), and H1 mRNA. Similar results were obtained using aphidicolin, a drug that interacts with DNAase I and holds dividing cells at the G1,S boundary. Conversely, cyclohexamide inhibits formation of both PCNA and TK mRNA, although other nuclear proteins are produced, suggesting PCNA synthesis requires expression of other growth regulatory genes. Therefore, it is likely that PCNA levels in leukemia reflect the activity of other growth regulatory genes that are associated with cell proliferation and as such could be an important marker for proliferative activity in acute leukemia.

REFERENCES

2. Celis JE, Madsen P, Celis A, Nielsen HV, Gesser B: Cyclin (PCNA, auxiliary protein of DNA polymerase δ) is a central component of the pathway(s) leading to DNA replication and cell division. FEBS Lett 220:1, 1987


Proliferating cell nuclear antigen expression in childhood acute leukemia

D Keim, N Hailat, D Hodge and SM Hanash