Premature Chromosome Condensation Studies in Human Leukemia. I. Pretreatment Characteristics

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The phenomenon of premature chromosome condensation (PCC) was used to compare the bone marrow proliferation characteristics of 163 patients with various forms of leukemia prior to the initiation of new therapy. The proliferative potential index (PPI, or fraction of G1 cells in late G1 phase) and the fraction of cells in S phase was determined and compared to the type of disease and the bone marrow blast infiltrate for each patient. Previously untreated patients with acute leukemia exhibited an average PPI value three times that of normal bone marrow (37.5% for acute myeloblastic leukemia [AML], acute monocytic leukemia [AMML], or acute promyelocytic leukemia [APML] and 42% for acute lymphocytic leukemia [ALL] or acute undifferentiated leukemia [AUL]). Untreated chronic myelogenous leukemia (CML) patients showed intermediate PPI values (25.2%), whereas CML patients with controlled disease exhibited nearly normal PPI values (14.8%). On the other hand, blastic-phase CML patients exhibited PPI values closer to that observed in patients with acute leukemia (35.4%). Seven patients with chronic lymphocytic leukemia (CLL) exhibited even higher PPI values. No correlations were observed between PPI values, fraction of cells in S phase, and marrow blast infiltrate. For untreated acute disease patients, PPI values were prognostic for response only at low and high PPI values. These results suggest that the PCC-determined proliferative potential is a biologic reflection of the degree of malignancy within the bone marrow.

NEW ADVANCES in the management of human leukemia require a better understanding of certain biologic aspects of growth and regulation at the cellular level. Toward this end, we have applied the phenomenon of premature chromosome condensation (PCC) to the study of bone marrow cells obtained from leukemic patients at various points of their disease.

Chromosomes of cells are normally only visible at mitosis. During the rest of the cell cycle, the chromosomes are diffuse within the interphase nucleus. However, if a mitotic cell and an interphase cell are fused together, the diffuse chromatin of the interphase cell immediately condenses into discrete units. This phenomenon has been termed "chromosome pulverization," "prophasing," and "premature chromosome condensation." The morphology of the prematurely condensed chromosomes reflects the phase of the cell growth cycle of the interphase cell at the time of fusion. G1-PCC exhibit a single chromatid per chromosome. Moreover, early G1 cells give rise to condensed G1-PCC, while late G1 cells result in extended PCC. Cells fused from S phase exhibit a pulverized appearance, with both single and double elements, while G2-PCC exhibit two chromatids per chromosome. The PCC...
technique, therefore, allows a rapid characterization of the cell cycle distribution of populations of cells such as those obtained from the bone marrow.9

Early studies showed that normal cells come to rest in early G1.8,10 Furthermore, in initial studies11 we showed that bone marrow cell populations obtained from patients with leukemia exhibited higher fractions of cells in late G1 than did bone marrow cells obtained from clinically normal marrow. In addition, the early studies suggested that these measurements might be useful in the prediction of progression of disease in patients with morphologically normal bone marrows.

This article is the first in a series describing a systematic study of the usefulness of the PCC technique in determining clinical prognosis at various stages of leukemic disease. The first step in such a study is the determination of baseline characteristics prior to initiation of chemotherapy. In this article, we compare the pretreatment PCC characteristics of bone marrow cell populations obtained from myelogenous and lymphocytic leukemia patients and relate these results to clinical outcome.

MATERIALS AND METHODS

Patient Groupings

For this study, patients with the following classes of disease were studied: acute myelogenous leukemia, including myeloblastic (AML), monomyeloblastic (AMML), and promyelocytic (APML) leukemia; acute lymphocytic leukemia (ALL); acute undifferentiated leukemia (AUL); chronic myelogenous leukemia (CML); and chronic lymphocytic leukemia (CLL). All patients in the acute disease category were previously untreated with chemotherapy, and the PCC values reflect the results with bone marrow specimens obtained just prior to the initiation of therapy. The CML patients are divided into the following three general groups for the sake of this paper: (1) Newly presenting and untreated CML patients who are subsequently entered on a combination chemotherapy program of cyclophosphamide, cytosine arabinoside, vincristine, and prednisone, with splenectomy and Bacillus Calmette-Guerin (BCG) immunotherapy; (2) previously treated CML patients adequately maintained with either no treatment or periodic treatment with hydroxyurea or myleran; and (3) CML patients clinically in blast crisis who were subsequently entered on a chemotherapy program of rubidizone alone or in combination with cytosine arabinoside, vincristine, and prednisone. Many of the CML blast crisis patients had previously been treated with less aggressive therapy. The majority of patients with chronic lymphocytic leukemia had been previously treated with a variety of therapy regimens; however, their disease was clinically determined to require more intensive therapy (cyclophosphamide, adriamycin, and prednisone).

Bone Marrow Cell Preparation

Bone marrow cells were drawn by aspiration from the iliac crest of patients, with their informed consent. The cells were placed in phosphate-buffered saline, including 50 μg/ml preservative-free heparin (Fisher, Fair Lawn, N.J.) and 1% calf serum (Gibco, Grand Island, N.Y.), and the buffy coat was obtained by centrifugation. Mononuclear cells were obtained by Ficoll-Hypaque gradient centrifugation and were washed twice in Hanks' balanced salt solution (Hanks' BSS) without serum.

Mitotic Cell Preparation

Chinese hamster ovary (CHO) cells were used in these studies as the mitotic inducer cells. CHO cells were routinely grown as monolayer cultures on plastic petri dishes (Falcon Plastics, Oxnard, Calif.) at 37°C in a humidified CO2 incubator in modified McCoy's medium supplemented with 16% heat-inactivated fetal calf serum (Gibco) and 1% antibiotic mixture (penicillin, 10,000 U/ml, and streptomycin, 10,000 μg/mg). Mitotic CHO cells were obtained by a 3-4 hr Colcemid treatment, followed by selective detachment of the mitotic cells. The mitotic index of such populations is routinely greater than 95%. These cells were then washed in Hanks' BSS.
Pretreatment PCC Values in Leukemia

Cell Fusion

The procedure to induce PCC in the bone marrow has been previously described. Briefly, bone marrow mononuclear cells and mitotic CHO cells were mixed together and washed twice with Hanks' BSS without serum. The mixture was then centrifuged, and the cell pellet was resuspended in 0.5 ml of Hanks' BSS without glucose, containing about 2000 hemagglutinating units of uv-inactivated Sendai virus. The fusion mixture was placed at 4°C for 15 min (to promote cell agglutination) and then transferred to a 37°C water bath for a 45-min incubation. By this time, cell fusion and the induction of PCC in fused bone marrow cells were completed.

Slide Preparation

At the end of fusion, two drops of fetal calf serum were added (to minimize cell lysis), and the fusion mixture was resuspended in 12 ml of hypotonic 0.075 M KCl for 10 min. The cells were then centrifuged, fixed twice in methanol:glacial acetic acid (3:1), and dropped onto wet slides. After drying, the slides were stained with Giemsa (Fisher).

Scoring of Slides

With the use of the light microscope, 100 PCC spreads were first located under low power and then examined under high power. The PCC were scored for their positions in the cell cycle according to PCC morphology (G1, S, or G2) (Fig. 1). As previously reported, the G1-PCC were arbitrarily graded on a scale...
Fig. 2. G₀-PCC of human bone marrow cells exhibiting varying degrees of chromosome condensation. The G₀-PCC are classified on the basis of degree of condensation and are rated on a scale of 1–6, with a value of 6 representing the most decondensed state.
scale of 1–6 on the basis of their degree of chromosome condensation. As shown in Fig. 2, class 1 represents the most compact G1-PCC. Classes 2 and 3 G1-PCC are somewhat more extended, yet the chromosomes can easily be delineated from telomere to telomere. Classes 4, 5, and 6 represent G1-PCC where the chromosomes have uncoiled and are increasingly extended with increasing number.

To quantitate the distribution of cells within G1 phase, we have defined the term proliferative potential index (PPI) as the ratio of the number of highly extended G1-PCC (classes 4, 5, and 6) to the total number of G1-PCC scored.

CHO-to-CHO fusions did not present a problem in these studies, since the mitotic cell populations were routinely better than 95% pure. Furthermore, any contaminating G1 and G2 CHO PCC could be distinguished on a cytogenetic basis. To avoid any bias, bone marrow PCC slide preparations were coded and scored without knowledge of the patient's case history or type of disease.

RESULTS

It was shown in a previous article that the proliferative potential index (or the fraction of G1 cells in late G1 phase), as determined using the PCC technique, was useful in distinguishing normal bone marrow populations from leukemic bone marrow populations. In that study, the average PPI value for bone marrows from 25 untreated leukemia patients was 27.5% as compared to average PPI value of 11.7% for bone marrow populations obtained from solid tumor patients with no evidence of disease in their bone marrow (normal bone marrow populations give similar low PPI values). The leukemic patients, however, were diverse in their type of disease, and the PPI values ranged from 6.0% to 76.0%. It was of interest, therefore, to determine if there was a relationship between the type and clinical extent of disease and the proliferation characteristics as determined by the PCC technique. The results are reported here according to leukemic disease type.

Acute Myelogenous Leukemia

In this group, the bone marrow characteristics of 75 previously untreated patients with either AML, AMML, or APML were determined by the PCC technique and compared to morphologically determined bone marrow characteristics such as blast percentage and blast infiltrate in the marrow (blast percentage multiplied by the cellularity of bone marrow particles). Included within this group were 62, 10, and 3 patients determined to have AML, AMML, and APML, respectively.

Figure 3 shows the relationship between the PPI and the fraction of S-PCC for all of the patients studied with acute myelogenous leukemia. For the group as a whole, the average PPI was found to be 37.5% (median 36.2%, ranging from a low of 10% to a high of 74.5%). The average fraction of cells in S phase was 7.3% (median 7.9% with a low of 0% to a high of 20%). However, only 2 of the 75 patients (2.7%) showed greater than 16% of the cells in S phase. In the previous study, bone marrow populations from solid tumor in the patients exhibited an average S-PCC fraction of 10%. From the data in Fig. 3, it is apparent that no correlation exists between the PPI value and fraction of cells in S phase. Furthermore, all three subgroupings of patients show no obvious differences in the proliferation characteristics.

Figure 4 illustrates the relationship between the blast infiltrate and the PPI values of those patients where adequate morphological determinations could be made. In some cases, marrows were too packed for adequate aspiration or the material obtained was inadequate for proper analysis. The average blast count for this group as a whole was 74.3%, with a range from 20% to 96.4%. The average
Fig. 3. Scatter diagram illustrating the relationship between PPI values and the fraction of cells in S phase for patients with AML, AMML, or APML. The open symbols represent patients not achieving complete remission, while the closed symbols represent patients achieving complete remission with induction therapy.

Fig. 4. Scatter diagram comparing the PPI values and the degree of blast infiltrate in the bone marrow at the time of sampling. Symbols are the same as in Fig. 3.
blast infiltrate was 67.1%, with a range of 18%-94.3%. There appeared to be little correlation between the PPI values and the bone marrow infiltrate. Although not shown here, there also appeared to be no correlation between blast percentage and the value of the PPI. The possible biologic relevance of noncorrelation will be discussed later in the article.

The relationship between the fraction of cells in S phase and the blast infiltrate is shown in Fig. 5. While there appears to be no direct correlation between the fraction of cells in S phase and the blast infiltrate, there is a suggested trend toward lowered S phase values as the blast infiltrate is increased. Each of these values, however, represents only one patient. A more meaningful relationship might be determined if one could follow a patient as the blast infiltrate increases. This can be observed in some patients with slowly progressing disease and will be reported elsewhere.

Most of the patients described in this group were subsequently treated with combination chemotherapy, including either adriamycin or rubidizone, cytosine arabinoside, vincristine, and prednisone. Of the patients followed sufficiently to date, 13 of 52 (25%) did not achieve a complete remission. Those patients not obtaining complete remission either obtained a partial remission, remained stable, or died during remission-induction therapy. It was of interest to determine whether the proliferation characteristics determined by the PCC technique were of prognostic significance for response. The open symbols in Figs. 3, 4, and 5 represent those patients not obtaining a complete remission. The data of Fig. 1 indicate that a high PPI value is suggestive of poor prognosis, while a low PPI suggests a better prognosis. So far in this analysis, 36% (9 of 25) of the patients above the average
PPI did not achieve complete remission, while only 14.8% (4 of 27) of the patients below the PPI mean did not obtain complete remission. All of the 11 patients with PPI values below 20 achieved complete remission and only 10% (2 of 20) of those patients with PPI values below 30% did not achieve complete remission. On the other hand, 50% (8 of 16) of those patients with PPI values above 50% (with sufficient follow-up) did not achieve a complete remission. The data shown in Figs. 4 and 5 do not suggest further prognostic value from the fraction of cells in S phase or the blast infiltrate.

**Acute Lymphocytic Leukemia**

The proliferation characteristics of bone marrow taken from 27 patients with a diagnosis of ALL or AUL were studied using the PCC technique. None of these patients had received chemotherapy prior to these measurements. The average PPI value for this group was 42.0% (median 36.3%), with a range from 11.3% to 92.9%. The average fraction of cells in S phase was 12.2% (median 9.0%, with a range of 1%–30.7%). A portion of the patients exhibited raised fractions of cells in S phase. Nine of the 27 patients (33.3%) exhibited S phase fractions of 16% or greater (in contrast to the situation with AML patients in which only 2 of 75 patients exhibited S phase fractions greater than 16%). As is shown in Fig. 6, there was little correlation between the PPI values and the fraction of cells in S phase.

Figure 7 illustrates the relationship between the PPI values and the blast infiltrate determinations for those ALL or AUL patients for whom adequate
morphological analyses were obtained. The average blast count for these patients was 92.4%, and the average blast infiltrate was 87.8%. As shown in Figs. 7 and 8, there was little correlation observed between either the PPI values or fraction of cells in S phase and the bone marrow blast infiltrate.

Most of the patients in this group were subsequently treated with combination chemotherapy, including adriamycin, cytosine arabinoside, vincristine, and prednisone, or cyclophosphamide, adriamycin, vincristine, prednisone, and bleomycin. Of 21 patients who have received therapy and have been analyzed for response, 7 patients did not achieve complete remission. These patients are represented by open symbols in Fig. 6. There appears to be no clear prognostic pattern of response for these patients using the proliferation characteristics defined by the PCC technique.

Chronic Myelogenous Leukemia

The PCC characteristics of bone marrow cells from 54 CML patients were determined, and the results are shown in Table 1. Twenty-eight of these patients were previously untreated and not clinically in the blastic phase. These patients exhibited an average PPI of 25.2%, which is significantly higher than the values previously observed for normal bone marrow. For those 14 patients who were adequately maintained with hydroxyurea or Myleran or no therapy, the PPI values were observed to return to the normal range (average PPI, 14.6%). On the other hand, the 12 patients studied in the blastic phase of their disease exhibited high PPI values (average, 35.4%), comparable to those values observed for patients with
Fig. 8. Scatter diagram comparing the fraction of cells in S phase with the degree of blast infiltrate in the bone marrow at the time of sampling. Symbols are the same as in Fig. 6.

acute leukemia. Despite these differences in PPI values for the three classes of CML patients, the fraction of cells in S phase remained somewhat similar (Table 1).

Two of the patients with CML were studied at different times during the course of their disease. The bone marrow PCC characteristics of one patient was determined once during the benign phase (when the PPI was found to be 14.3%) and once a year later during the blastic phase (when the PPI was 38.8%). Thus, a rise in the PPI accompanied the progression of disease. Another patient presented

<table>
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<tr>
<th>Stage of Disease</th>
<th>Number of Patients</th>
<th>PPI Values (%)</th>
<th>S-PCC (%)</th>
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<tbody>
<tr>
<td>Previously untreated</td>
<td>28</td>
<td>25.2</td>
<td>14.9</td>
</tr>
<tr>
<td>Controlled</td>
<td>14</td>
<td>14.6</td>
<td>17.1</td>
</tr>
<tr>
<td>Blastic phase</td>
<td>12</td>
<td>35.4</td>
<td>13.5</td>
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initially with a PPI value of 33.8%, and 1 mo later, showed a PPI value of 35.7%. Five months later, the patient exhibited progression to the blastic phase of disease. Thus, in this case, higher PPI values appeared to precede progression of disease.

**Chronic Lymphocytic Leukemia**

The bone marrows of seven patients with CLL were studied with the PCC technique (Table 2). These subjects represented patients whose disease was clinically active enough to warrant combination chemotherapy with cyclophosphamide, adriamycin, and prednisone. These patients generally exhibited high PPI values (average 59.4%). The fusion indices obtained for these cells were found to be significantly lower than those obtained with bone marrow cells from patients in the other disease categories. This observation suggests the surface receptor for the Sendai virus particle was either reduced or hidden from the surface in these cells.

**DISCUSSION**

Early studies have indicated that the PCC technique is useful in distinguishing normal and malignant populations of cells in various states of growth. By prematurely condensing the chromosomes of interphase cells, one can not only determine the phase distribution of the cell population (G₁, S, G₂), but one can also determine the distribution of cells within a phase, such as G₁. The distribution of cells within G₁ phase appears to differ between normal and malignant cell populations regardless of the fraction of cells actually entering S phase. In previously reported studies using the PCC technique, we have shown that under growth-limiting condition normal cell populations accumulate in early G₁ phase, while transformed cell populations accumulate in late G₁ phase with highly extended G₁-PCC. A similar situation is observed in vivo, where normal resting tissue cells tend to rest in early G₁ (low PPI); whereas in a solid tumor, most of the cells give rise to an extended G₁ morphology (high PPI).

In a previously reported study, it was observed that the same biologic phenomenon occurs for human bone marrow. Cells obtained from normal bone marrow and marrow of solid tumor patients with no evidence of disease exhibit low PPI values (averaging 12%). Bone marrow populations from leukemic patients, on the other hand, exhibited significantly higher PPI values. In the present study, we observed that the average PPI value for previously untreated patients with AML and ALL was at least three times that of normal bone marrow. On the other hand, the fraction of cells in S phase was only slightly lower than that obtained from normal marrow. This observation and the data from Fig. 3 suggest that there is a disconcordance between the fraction of cells in late G₁ and the fraction of cells entering S phase. Thus, as in the case of other malignant cell populations, leukemic bone marrow cell populations appear to accumulate in late G₁ phase under certain growth conditions.
It is interesting to note that despite a general rise in PPI values for patients with acute disease, there exists a wide range of PPI values. Furthermore, no correlation was observed between the PPI values and morphologically derived data such as bone marrow blast count and blast infiltrate. These observations suggest it is not solely the bone marrow blast percentage that determines the PPI. Recently, bone marrow cells from both normal and leukemic individuals were subjected to separation by albumin density gradients, and the various fractions were analyzed by the PCC technique. In the gradients from normal subjects, the most dense and differentiated cells exhibited the lowest PPI values of the gradient. In contrast, the most dense fractions from gradients of leukemic marrow populations exhibited the highest PPI values of the gradient. These results support the notion that the apparently normal differentiated cells in the leukemic bone marrow can exhibit an accumulation in late G1 phase (Hittelman, unpublished results).

The biology behind our observation reported here (i.e., some patients exhibited a low bone marrow blast count yet had a high PPI) is not well understood; however, two possibilities exist. In light of recent observations that some mouse and human leukemic clones are capable of induced differentiation, these late G1 cells might represent malignant cells that have stopped dividing and have morphologically differentiated, to a certain degree, in vivo. On the other hand, in light of the reported observations that leukemic cells can have a growth inhibitory effect on normal hematopoiesis, these late G1 cells might represent normal cells accumulated in late G1 due to the influence of the leukemic cells. At this point we cannot distinguish which of these two possibilities best explains our results. Work in this regard is continuing. In any event, the wide range of PPI values for patients with morphologically similar disease emphasizes the biologic diversity of acute leukemia from patient to patient.

The results of measurements on the patients with chronic myelogenous leukemia shed some light on the clinical relevance and the biologic meaning of the PCC determinations. Bone marrows taken from previously untreated CML patients exhibited PPI values intermediate between those values observed for normal bone marrow and those observed for acute leukemia. With successful disease control, the PPI values were observed to drop to normal levels. However, transition to blastic phase despite therapy was accompanied by a rise in PPI values to levels comparable to those observed in patients with acute leukemia. Thus, the PPI values might reflect the degree of malignancy or the proliferative potential of the bone marrow. It will be important to serially follow CML patients to determine if a jump from a low PPI to a high PPI precedes or accompanies transition to blastic crisis.

It is also of interest that the three classes of CML patients generally showed similar fractions of cells in S phase, despite significantly different PPI values. This observation supports the in-vitro-derived results that malignant cells may accumulate in late G1 phase without a corresponding increase in the S phase fraction.

The accumulation phenomenon is even more apparent in the case of bone marrows obtained from patients with CLL. These patients with active disease showed extremely high PPI values with somewhat normal levels of cells in S phase. This result is interesting in light of the fact that many of the cells in the CLL bone marrow are more differentiated lymphocytes. This suggests that the leukemic lymphocyte comes to rest in late G1 phase, whereas we have shown previously that normal peripheral blood lymphocytes are resting in early G1.
The prognostic value of the initial pretreatment measurements are somewhat limited, yet a trend was observed in two leukemic situations. In acute myelogenous leukemia, patients with very low PPI (under 20%) values responded well to remission-induction therapy, whereas one-half of those patients with PPI values greater than 50% did not achieve complete remission. Similar correlations of PPI values with response were not observed in the ALL patients. Further follow-up on these patients will determine if the pretreatment values are related to the length of survival following therapy. As will be shown in subsequent reports, the PPI values drop to intermediate levels with complete remission and return to high values prior to clinical evidence of relapse.

In this article, therefore, we have shown the bone marrow mononuclear cell populations from leukemic patients exhibit increased fractions of cells in late G1 phase, as determined by the PCC technique. For patients with CML, the height of the PPI values reflected the activity of the disease. In subsequent papers we will report the changes in PCC characteristics subsequent to remission-induction therapy and describe the usefulness of the PCC technique in monitoring response to therapy and predicting progression of leukemic disease.

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